SPECTRAL AND REDOX PROPERTIES OF BACTERIOCHLOROPHYLL IN ITS NATURAL STATE

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SUMMARY

The behaviour of bacteriochlorophyll in its natural state with regard to oxidative bleaching, photobleaching and temperature bleaching was investigated. Reversible changes in absorption by the addition of oxidative and reductive agents, previously measured in organic solution, were also found to be present when bacteriochlorophyll is in its natural state in chromatophores of purple bacteria. Only the absorption band of longest wavelength in *Rhodospirillum rubrum*, *Rhodopseudomonas spheroides* and *Chromatium* could be bleached reversibly. Also a reversible shift in position of the 800-mμ absorption band in *Rh. rubrum* occurs while there are indications of a similar shift in the other species. At a potential of approximately 515 mV the difference spectrum obtained by addition of ferri-ferrocyanide mixtures to chromatophores of *Rh. rubrum* was similar to the difference spectrum obtained by DUYSENS by illumination of living bacteria. Additional bleaching experiments were done by heating and illumination of the chromatophore extracts.

The present experiments may yield a link between the *in vitro* and *in vivo* phenomena obtained with chlorophyllous pigments.

INTRODUCTION

The study of the chemistry and photochemistry of the chlorophylls has been confined predominantly to pigments extracted from the cells and dissolved in organic solvents. In the photosynthetic apparatus these pigments occur under conditions quite different from those in organic solution. For example, the pigment concentration in the photosynthetic structure usually is more than a thousand times higher than the concentration used in most experiments with chlorophylls in organic solvents. Also there are many indications that the pigments cannot move freely in the structure as they do in organic solvents, but are concentrated in thin layers and probably attached to carriers such as protein macromolecules[1]. These differences are reflected, among other ways, in differences in both absorption spectra and fluorescence yields.

It seems promising to investigate which of the *in vitro* properties of the chloro-

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phylls are retained when the pigments are present in their natural state and how these properties are modified under these conditions.

For this type of investigation bacteriochlorophyll proved to be the most useful pigment. There is a considerable difference in absorption spectra of bacteriochlorophyll dissolved in organic solvents and bacteriochlorophyll located in photosynthetic bacteria. Moreover, rather stable extracts of chromatophores can be prepared from these bacteria. These extracts usually have the same absorption spectrum as living bacteria.

Earlier experiments made with bacteriochlorophyll in organic solution[1,2] showed that this pigment could be bleached by addition of different oxidants, such as ferric chloride, iodine or potassium permanganate. The original spectrum could be restored completely by immediate addition of ferrous salts, ascorbic acid or other reductants. In contrast to the behaviour of chlorophyll *a*[2], no slow restoration of the spectrum occurred upon standing in the dark or addition of non-reducing salts. With bacteriochlorophyll—as well as with chlorophylls *a* and *b* and bacterioviridin—dissolved in methanol it was possible to measure a fairly reproducible oxidation-reduction potential[3].

The "oxy" chlorophyll forms are unstable in air saturated solution. They lead to different secondary products in the case of bacteriochlorophyll and chlorophyll *a*.

We investigated the capacity for reversible oxidation-reduction of bacteriochlorophyll in its natural state in the different modifications occurring in various photosynthetic purple bacteria, and also the question was considered whether this redox phenomenon can be related to absorption changes in the bacteriochlorophyll spectrum measured by DUYSENS[4] with living bacteria. In connection with these phenomena the absorption changes brought about by heating and intense illumination were also studied.

METHODS

Chromatophores of the photosynthetic purple bacteria *Rhodospirillum rubrum* strain 1-1-1, *Rhodopseudomonas spheroides* and *Chromatium* strain D were obtained by the use of a FRENCH-MILNER homogeniser[5]. Intact bacteria and cell debris were removed by 10-min centrifugation at 5000 $\times$ *g*. The chromatophores then were precipitated by 1-h centrifugation at 10,000 $\times$ *g* and the supernatant discarded. The pellet was taken up in distilled water or a buffer solution. As a rule phosphate buffer pH 7.5 was used.

Absorption spectra and difference spectra were measured with a Beckmann DK-2 recording spectrophotometer. Difference spectra were obtained by addition of the required amount of a ferri-ferrocyanide mixture to one of the cuvettes with bacterial extract, while an equal amount of distilled water was added to the other cuvette. A correction was applied for the absorption of the ferri-ferrocyanide mixture. Experiments on chromatophore bleaching were carried out by illumination with a 500 or 1000 W incandescent lamp with light absorbed only by bacteriochlorophyll which was isolated by a Corning 3480 filter and a 5-cm water filter.

For the determination of the shift in position of the 800-mμ absorption band, occurring when living bacteria and chromatophores are illuminated, the derivative spectrophotometer of FRENCH[6] was used.

RESULTS

The oxidation-reduction potential of bacteriochlorophyll in methanol (the potential of the solution at which the infrared absorption band with a maximum at 770 mμ is bleached halfway) was previously found to be approximately 550 mV *versus* a standard hydrogen electrode. This value suggested that reversible oxidation-reductions of aqueous chromatophore suspensions might be measured by the use of ferri-ferrocyanide systems. These systems also have the advantage that it is possible to work with solutions buffered at pH 7.5. Thus absorption changes and coagulation due to low pH as well as the influence of low and probably non-physiological pH on potential values are eliminated (*cf.* THOMAS *et al.*[7]).

The results of absorption and potential measurements obtained with chromatophores of three different bacterial species, each having a different near infrared absorption spectrum due to bacteriochlorophyll, will be given. Two of these species belong to the group of non-sulfur bacteria, the third to that of the sulfur bacteria.

Rhodospirillum rubrum *strain 1-1-1*

Fig. 1a shows the near infrared absorption spectrum of a chromatophore suspension from *Rhodospirillum rubrum* and Fig. 1b the absorption spectrum of a methanolic bacteriochlorophyll solution. In this bacterium both the main infrared absorption band at 880 mμ and the weak absorption band at about 800 mμ in the natural state correspond to the 770-mμ absorption band of bacteriochlorophyll in methanol[2]. Thus the main fraction of the pigment molecules shows a greater shift in absorption maximum—from the *in vitro* position—than a minor fraction of the pigment molecules. It can be estimated that at 800 mμ the absorption due to the

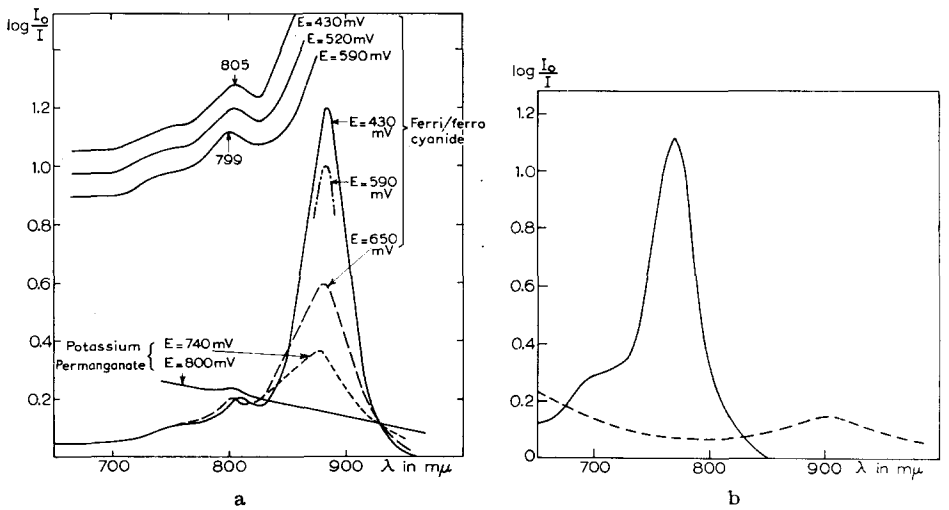

Fig. 1a. Absorption spectrum of a chromatophore extract of *Rhodospirillum rubrum*. The dashed lines show the absorption spectrum after ferri-ferrocyanide mixtures (or potassium permanganate) were added. The potentials of the solution are given in mV *vs.* normal hydrogen electrode. The values below 700 mV are obtained with ferri-ferrocyanide, the higher ones with permanganate. In the insert the position of the 800-mμ band is given at three different potentials. 1b. Near infrared part of the absorption spectrum of a methanolic extract of *Rhodospirillum rubrum*. The dashed lines give the spectrum after addition of ferric chloride, iodine or potassiumpermanganate.

molecules with the small shift is approximately one third of the total absorption at this wavelength. Then the number of molecules showing a shift to 800 mμ is of the order of 1 % of those showing a shift to 880 mμ.

The results of addition of ferri-ferrocyanide mixtures with different potential and of potassium permanganate are also presented in Fig. 1a. It appears from this figure that with increasing potential two different processes occur: the 880-mμ band is bleached while the 800-mμ band is shifted in position from 805 to 799 mμ but not bleached. Even after a total bleaching of the 880-mμ band the weak band is still visible at 799 mμ. The potential at which the 800-mμ band is halfway shifted is approximately 520 mV, while the potential at which the 880-mμ band is halfway bleached is approximately 650 mV. The bleaching of the 880-mμ band is not fully reversible: the sooner the addition of reductant the higher the percentage of reversibility. This is similar to the behaviour in organic solvents, where complete reversibility is obtained only after immediate addition of reductant. Fig. 1b shows the

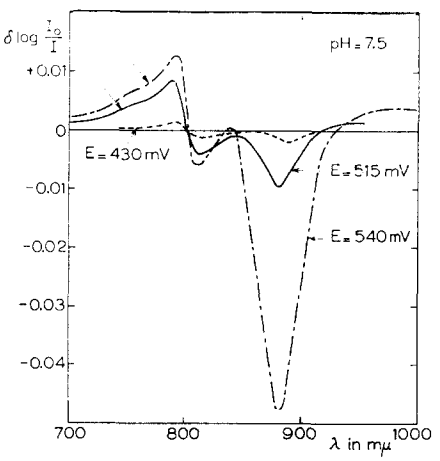

Fig. 2. Difference spectra of *Rhodospirillum rubrum* chromatophores obtained by the addition of ferri-ferrocyanide mixtures: ----- potential 430 mV, ——— 515 mV, —·— 540 mV.

—reversible—bleaching of bacteriochlorophyll in methanol. A reversible shift in position was not encountered here. If present, such a shift may have been masked by the bleaching. An irreversible shift towards shorter wavelength after addition of dilute iodine to an ether solution of bacteriochlorophyll could be noticed sometimes.

The reversible bleaching induced by the addition of ferri-ferrocyanide can be compared with the difference spectra of bacteriochlorophyll absorption observed when living bacteria are illuminated (*cf.* DUYSENS[4]). Therefore absorption difference spectra measured as the difference between the untreated suspension and that adjusted to the redox potentials 430, 515 and 540 mV are presented in Fig. 2. This figure shows that the difference spectrum obtained at a potential of 515 mV is similar to the difference spectrum measured by DUYSENS. Not only are the shapes of the difference spectra similar but also the absolute values of the changes are of the same order of magnitude. This was measured with the derivative spectrophotometer. With this instrument the determination of the exact location of the maximum of the 800-mμ band is possible. Side illumination with blue light (isolated by a Corning 5031 filter and a 5-cm 6 % copper sulfate solution) of $1.1 \cdot 10^4$ ergs/cm²/sec produced a band

shift from 805 to 800 mμ with bacteria suspended in distilled water. This shift was reversed after a few seconds storage in the dark. When the intensity was reduced to a tenth, the shift still was 3 mμ, suggesting that the higher intensity was sufficient to cause the maximum possible shift. In chromatophore suspensions the reversible shift upon illumination was only 1 or 2 mμ, while addition of ferricyanide in the dark to the same extract caused a shift of 5 mμ.

A chromatophore suspension is bleached irreversible if illuminated with strong light (approximately 150,000 f.c. with filters removed). The stability against bleaching of bacteriochlorophyll in chromatophores exceeds the stability for bacteriochlorophyll dissolved in methanol more than a thousand times. Irreversible photobleaching was found to proceed in freshly prepared extracts at a rate approximately 4 times higher than in extracts which had been stored in the dark for a considerable time. Usually these freshly prepared extracts also showed photophosphorylation. Another chemical activity exhibited by these freshly prepared extracts is their capacity to reduce ferricyanide in the dark after addition of succinate or growth medium (peptone). The reduction of ferricyanide in the dark could be used to demonstrate the reversibility of the oxidative bleaching with chromatophore suspensions: addition of an equal amount of succinate to the cuvettes in the spectrophotometer, both containing chromatophores, one of them with ferricyanide, resulted in a gradual disappearence of the difference spectra presented in Fig. 2.

Photobleaching at these high light intensities usually was not totally irreversible. Up to 10 %, and in some cases up to 30 %, of the bleaching proved to be reversible. As shown in Fig. 3, the difference spectrum in the near-infrared also consists of a decrease of the 880-mμ band and a shift of the 800-mμ band. This spectrum differs

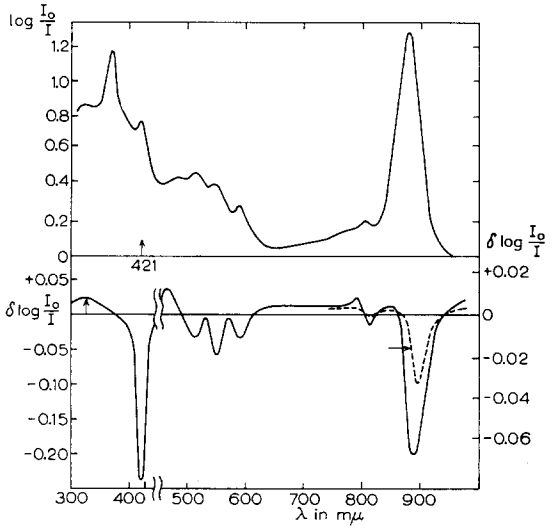

Fig. 3. Absorption spectrum and difference spectrum of a chromatophore extract of *Rh. rubrum* bleached 15 min by light absorbed by bacteriochlorophyll (150,000 f.c. with filters removed). The dashed line shows the near infrared part of the spectrum after 15 min storage in the dark, in the rest of the spectrum no measurable change in absorption occurred during this dark period. The marked decrease in absorption at 421 mμ is due to the bleaching of un unknown ether soluble pigment, present to an unusually large extent in this special extract. Whether the increase around 340 mμ (marked by left arrow) is due to this same pigment or to a different one, could not be established.

from the completely reversible difference spectrum, measured by DUYSENS at much lower intensities of illumination, in the ratio between the values of decrease and shift. Bacteriochlorophyll bleaching in organic solvents also is slightly reversible. After a nearly complete bleaching of the 770-mμ band by light this band regenerated about 10 %. The percentage of reversibility could be enhanced to 70 % by addition of ascorbic acid, but only in alcoholic solvents. With chromatophores no increase in the percentage of reversibility of photobleaching of bacteriochlorophyll was found after addition of ascorbic acid.

Fig. 3 also shows irreversible decrease in absorption (occurring after a few minutes of illumination) in the short wavelength part of the spectrum (421 mμ). This decrease was due to an ether soluble pigment different from bacteriochlorophyll, carotenoids and most probably also from cytochromes[8].

The temperature of the chromatophore extract rose from 3 to 5° during 15 min illumination with strong light. Therefore it was investigated whether heating of the extract exhibits a marked influence on the absorption spectrum. Heating the extract from room temperature (20°) to 30° or cooling it 10° resulted in the appearance of a difference spectrum of approximately the same shape as measured with high intensity photobleaching. The magnitudes of these differences were smaller than those encountered in the bleaching experiments. It thus seems unlikely that the high intensity reversible photobleaching is due to the influence of temperature only. After heating a freshly prepared extract for less than 1 min at 80°, the absorption band at 880 mμ disappears while a new band arises st about 780 mμ, in a position close to that of bacteriochlorophyll in organic solvents. In order to investigate whether or not bacteriochlorophyll in this state is separated from the chromatophores and present in an aqueous colloidal phase, heated and non-heated chromatophores were centrifuged 1 h at 20,000 $\times$ g. The particles were precipitated, no pigment was present in either one of the supernatants. Colloidal bacteriochlorophyll, prepared by dilution of a concentrated methanolic solution with water, was not precipitated under the same conditions. It thus appears that no separation of the pigment from the chromatophores occurs as a result of heating to 80°.

Rhodopseudomonas spheroides

The absorption spectrum of a chromatophore extract of *Rhodopseudomonas spheroides* is given in Fig. 4. In the near infrared the bacteriochlorophyll spectrum is more complicated than that of *Rhodospirillum rubrum*. Extraction with methanol results in a bacteriochlorophyll spectrum indentical to that of Fig. 1a. In the chromatophore two maxima are present at 800 and 850 mμ, while a shoulder appears at about 890 mμ.

The results of addition of ferri-ferrocyanide mixtures are different for freshly prepared extracts and for extracts which have been stored for some time. The results with the latter ("old") extracts, measured as difference spectra, are given in Fig. 5a. A reversible decrease in absorption occurs at 885 mμ and around 805 mμ, a reversible increase around 790 mμ. This difference spectrum looks similar to that measured with *Rhodospirillum rubrum*. Also at potentials between 600 and 650 mV only the long wave length shoulder in the absorption spectrum appears to be bleached reversibly. At potentials higher than 700 mV a marked irreversible decrease occurs with the other bands.

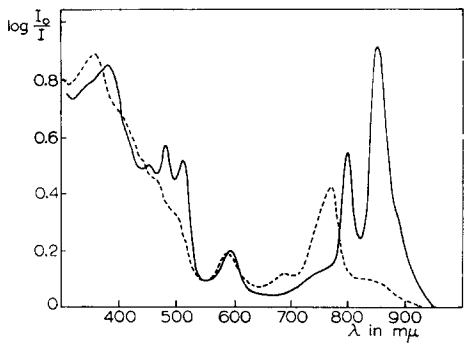

Fig. 4. Absorption spectrum of a chromatophore extract of *Rhodopseudomonas spheroides* before (———) and after (– – – –) a 1-min heating to 83°.

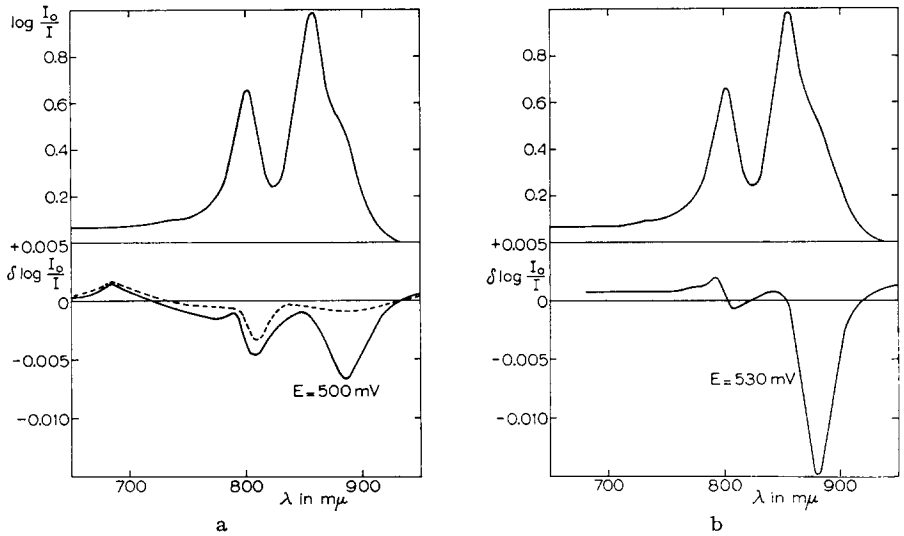

Fig. 5a. Difference spectrum of an "old" extract of *Rhodopseudomonas spheroides*, obtained by the addition of ferricyanide. 5b. Difference spectrum of a "fresh" extract of *Rh. spheroides* buffered at pH 7.5. The dashed line shows the spectrum after addition of succinate to the cuvettes. Such an addition results in a reduction of ferri- to ferrocyanide and consequently in a disappearence of the reversible fraction of the spectrum.

With fresh extracts, besides a reversible change in absorption, also an irreversible decrease at 800 mμ occurs already at potentials around 500 mV. This decrease, which is strongly enhanced at lower pH (around 5), coincides with an irreversible increase in absorption at 690 mμ. Most probably this is due to an oxidation of "800 mμ bacteriochlorophyll" to a green oxidation product encountered in experiments with bacteriochlorophyll in organic solution[2,9]. In Fig. 5b the difference spectrum with a ferri-ferrocyanide mixture at a potential of 500 mV is given for a fresh extract buffered at pH 7.5. In this figure the reversible and irreversible fractions are given by the curve obtained after addition of succinate to the oxidised chromatophore extract, as such an addition results in a reduction of ferricyanide and a disappearence of the reversible bleaching. The graphs indicate that the reversible change in absorption in this spectral region most probably is similar to that measured with "old" extracts.

Bleaching at high light intensities also proceeded differently with "fresh" and "old" extracts. In fresh extracts the 800-mμ band was bleached at an appreciably higher rate than the other bands while an increase in absorption occurs with a maximum around 690 mμ. With old extracts bleaching proceeded much slower and at an approximately equal rate for the different infrared bands. As shown in Fig. 6, this high intensity bleaching also was slightly reversible. Heating and cooling produced a difference spectrum of an analogous type (all three bands are present in this spectrum) and of the same order of magnitude. It thus is possible that this reversible bleaching is, at least partly, a temperature effect.

As a change in temperature causes a change in refractive index, the differences measured in the recording spectrophotometer may be caused not only by a change in absorption, but also by a change in selective scattering of the extract. The existence of such a selective scattering was observed by LATIMER[10] in chloroplast suspensions and was found to influence the location of the red absorption maximum of chlorophyll *a*. An experiment was made in which the refractive index of the solvent was increased

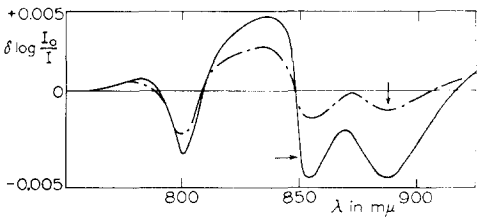

Fig. 6. Difference spectrum between an unilluminated extract of *Rh. spheroides* and one illuminated for 15 min at high intensity. This difference spectrum is similar in shape to the spectrum obtained after raising the temperature of the extract from 20 to 30°. —·— the same spectrum after 10 min storage in the dark. In contrast to the ones presented in Fig. 5 these difference spectra show bands both at 850 mμ and 890 mμ as indicated by arrows.

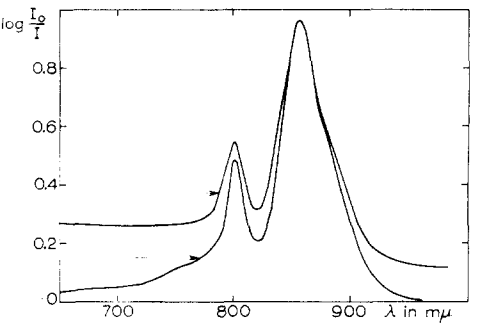

Fig. 7. Absorption spectrum of *Rh. spheroides* before (lower arrow) and after (upper arrow) addition of enough albumin to increase the refractive index of the extract to 1.42.

to 1.42 by addition of albumin. As shown in Fig. 7, the scattering is increased, but the location of the absorption maxima is not influenced by this treatment. Also the difference spectrum upon heating was not influenced as should be expected if this difference spectrum were due to selective scattering. It thus seems doubtful that the temperature effects are due to scattering and not to absorption differences. The results of a 1-min heating at 80° of a chromatophore extract of *Rhodopseudomonas spheroides* is also presented in Fig. 4. The near infrared absorption spectrum of the extract after heating looks similar to the spectrum of bacteriochlorophyll dissolved in organic solvents, shown in Fig. 1b. Centrifugation experiments showed that, analogous to the results with *Rhodospirillum rubrum* extracts, the pigment had not been released from the chromatophores, but it may be released from its normal position in the chromatophores. The remnants of the carotenoid bands and the bacteriochlorophyll band in the near u.v. also are shifted towards shorter wavelengths. No fluorescence could be measured with these heated extracts. The new band at 780 mμ, however, is broader than the bands of the original spectrum and the corresponding band in organic solvents. The area covered by this band is approximately

one third of the area of the original infrared spectrum. The height of the most pronounced band at 850 mμ is decreased 80 % by a 1-min heating to 76° and 20 % by a 1-min heating at 60°.

Chromatium *strain D*

The near infrared absorption spectrum of a chromatophore extract of *Chromatium* strain D as given in Fig. 8, shows marked variability. In Fig. 8a the main absorption band is located at 850 mμ, while a lower band occurs at 800 mμ and an absorption shoulder at about 890 mμ. This picture is similar to that of *Rhodopseudomonas*

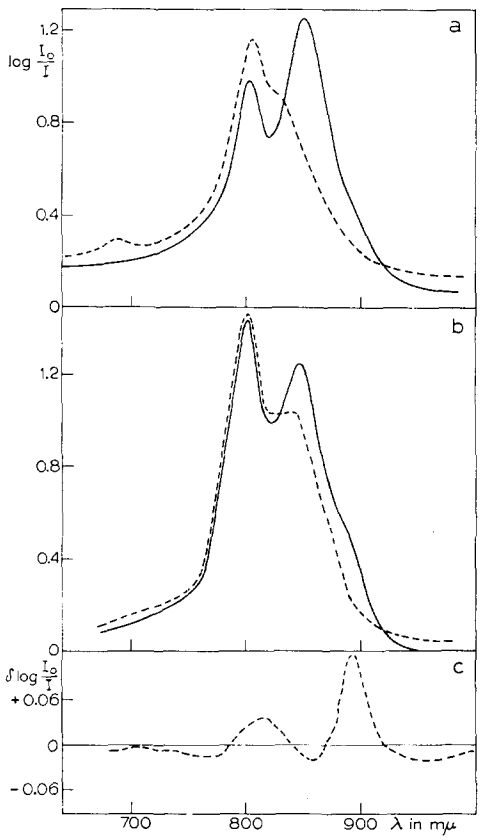

Fig. 8a and 8b. Near infrared absorption spectra of extracts of *Chromatium* strain D. The effect of addition of ferri-ferrocyanide mixtures is indicated by a dashed line. 8c. Difference spectrum drawn from the values obtained when reducing agent (ascorbic acid) was added to the extract of Fig. 8b.

spheroides, although the half widths of the bands of the latter species are markedly smaller. In the absorption spectrum presented in Fig. 8b the main band is located at 800 mμ. In some cases the band at 850 mμ is even much less pronounced. Although it is difficult to measure the total absorption due to a band responsible for the absorption shoulder at 890 mμ, it is estimated that the absorption due to this band is of the same order of magnitude in both types of spectra. The remark of WASSINK

et al.[11] that both types are interconvertable by a change of light intensity during the period of growing could not be confirmed as yet. A decrease of the light intensity from 1000 to 200 lux had no effect on the absorption spectrum.

Addition of ferri-ferrocyanide resulted in an irreversible decrease in absorption of the 850-mμ band, more so in freshly prepared extracts. In some cases an irreversible increase of the 800-mμ band was observed. Reversible bleaching was found to occur only with the 890-mμ absorption shoulder. Fig. 8c shows the difference spectrum obtained after addition of ascorbic acid as reductant of the oxidised chromatophore extract. This addition resulted in an increase in absorption at 890 mμ and 815 mμ, while the absorption at 780 and 820 mμ decreased slightly. This difference spectrum is similar in shape to the spectrum of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*.

Heating of a chromatophore extract of *Chromatium* for one minute at 83° resulted in the disappearance of only the 890-mμ absorption shoulder, the other bands seemed to be unaffected.

DISCUSSION

There is a similarity between the reversible oxidative bleaching of bacteriochlorophyll in organic solvents and bacteriochlorophyll in its natural state. This similarity holds only for the absorption band with longest wavelength in all three bacterial species investigated. To this band, according to DUYSENS[12], all light energy is transferred which is absorbed by the other bacteriochlorophyll bands. With these other near infrared absorption no reversible oxidative bleaching was evident. However, irreversible bleaching processes which are related to the age and probably to the photochemical activity of the chromatophore extract do occur with these bands.

A second reversible proces is a shift in position of the weak absorption band at 800 mμ in *Rhodospirillum rubrum* extracts. No counterpart of such a reversible shift in position was found with bacteriochlorophyll in organic solvents. It is, however, not necessary that such a shift be produced by a change in pigment composition. If we assume that the positions of the absorption bands of bacteriochlorophyll *in vivo* are mainly determined by a pigment-protein (or lipoprotein) combination, a small shift in position of an absorption band may be explained by a change in the protein part as well.

The difference spectra suggest that a similar reversible shift occurs with *Rhodopseudomonas spheroides* and *Chromatium* strain D, although here it concerns only a small fraction of the total absorption at 800 mμ. Consequently in these latter species an absorption spectrum similar to that of *Rhodospirillum rubrum*—showing reversible oxidative changes—seems to underlie the total absorption spectrum. It may be a plausible hypothesis that the spectrum as presented by *Rhodospirillum rubrum* is a basic spectrum for the functioning of photosynthetic purple bacteria. In this respect it may also be mentioned here that this latter spectrum appears, according to SISTROM *et al.*[13] in carotenoid free photosynthetic mutants of *Rhodopseudomonas spheroides*.

The difference spectrum obtained by illumination of *Chromatium* (*cf.* DUYSENS[4]) showing a reversible decrease at 890 and 800 mμ and a reversible increase at about 790 mμ, may also be explained in terms of reversible bleaching of the 890-mμ shoulder and a shift of a small fraction of the 800-mμ band.

The two phenomena of bleaching and shift may be caused either by a single process or by two different processes. The finding that the shift of the 800-mμ absorption band upon illumination is smaller with chromatophores than with living bacteria, while DUYSENS mentions that the bleaching of the 890-mμ band with chromatophores exceeds the bleaching with bacteria, might point to the latter of the two possibilities. In this respect also the relatively low value of light saturation of the shift may be mentioned.

The changes of the near infrared absorption spectrum upon heating to 80°, especially with *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*, is in accordance with the hypothesis that the position of the bacteriochlorophyll absorption bands is mainly determined by a pigment–protein linkage. Denaturation of the protein by heating to 80° apparently results in a change to the *in vitro* type of spectrum combined with absorption changes due to denaturation of the pigment. The different behaviour and also the difference in shape of the 800- and 850-mμ bands of the sulfur bacterium *Chromatium* with respect to the bands of the non-sulfur bacteria *Rhodopseudomonas spheroides* may point to a difference in molecular pigment structure between those species.

The experiments with the three different bacterial species, indicating that only the longest wavelength absorption band is bleached reversibly, show that the molecular structure of the chromatophore has a marked influence on the chemical properties of bacteriochlorophyll. The possibility of obtaining with chromatophores at a given potential a similarity of the spectrum of reversible bleaching by light with living bacteria indicates that reversible oxidative bleaching can play a role *in vivo*. Whether this property represents the primary light reaction of bacterial photosynthesis or merely a sideline cannot yet be concluded. Being specific to the chlorophyllous pigments (reversible oxidative bleaching could not be observed with pheophytins, the green oxidation product of bacteriochlorophyll or with carotenoids) it gives one of the first bridges between *in vitro* chlorophyll chemistry and processes occurring in the living cell.

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