Plant & Cell Physiol., **11**, 519–530 (1970)

Light-induced absorbance change of carotenoid in chromatophores of photosynthetic bacterium, *Rhodopseudomonas spheroides*

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(Received November 20, 1969)

The nature of the light-induced absorbance change of carotenoid, spheroidene, was investigated with the chromatophores of *Rhodopseudomonas spheroides*. The experimental results indicate that the change does not represent an oxidation-reduction reaction of the carotenoid, but is caused by a change in the state of the chromatophores closely related to the high energy state of the photophosphorylation. Since the change almost vanishes at liquid nitrogen temperature, it probably does not represent a primary photochemical reaction in the chromatophores. The values of the quantum yield for the change of carotenoid were above unity; 2.5 on an average.

A reversible change in the absorption spectrum of carotenoid induced by illumination of photosynthetic bacteria was first observed by SMITH and RAMIREZ (*1*) in intact cells of *Rhodospirillum rubrum*. Such change in the absorption spectrum of carotenoid seems to be one of the common features of photosynthetic bacteria since similar changes have been detected in intact cells of *Rhodopseudomonas spheroides* (*2–4*), *Rhodospirillum fulvum* (*5*) and *Rhodomicrobium vannielii* (*6*), as well as in the chromatophores of *Rhodospirillum rubrum* (*7, 17*) and *Rhodopseudomonas spheroides* (*8, 9, 16*). The absorbance change was explained as a shift of the absorption spectrum of the carotenoid caused by a light-induced conversion of carotenoid from the original short wavelength-absorbing form to a long wavelength-absorbing form (*3*).

AMESZ and VREDENBERG (*5*) calculated the efficiency of the light-induced absorbance change of carotenoid in intact cells of *Rhodopseudomonas spheroides*, assuming a shift of the absorption spectrum of the carotenoid, and obtained a value of 3 for the quantum yield of the carotenoid conversion. They also suggested that the absorbance change was not due to any chemical reaction of the carotenoid.

Recently, FLEISHMAN and CLAYTON (*16*), and BALTSCHEFFSKY (*17*) reported evidence for the involvement of a high energy state, or phosphorylation intermediate, in the mechanism of the light-induced absorbance change of carotenoid in the chromatophores.

The present work deals with the light-induced absorbance change of carotenoid in the chromatophores of *Rhodopseudomonas spheroides*, with the aim of elucidating the mechanism of the change under various experimental conditions.

Abbreviation: CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone.

Material and methods

Rhodopseudomonas spheroides was grown photoheterotrophically in a liquid organic medium after the method of ORMEROD *et al.* (*10*). The culturing was carried out at 30°C semiaerobically under the illumination of incandescent light (about 8,000 lux). After 2 days' growth, the cells were harvested by centrifugation and washed once with a large volume of 0.05 M phosphate buffer, pH 7.4, containing 0.5 M sucrose. The washed cells were suspended in the phosphate-sucrose medium and subjected to rupturing on a French press operated at 400 kg cm^{-2}. The disrupted cell suspension was centrifuged and the fraction precipitating between $15,000 \times g$ for 20 min and $59,000 \times g$ for 60 min was collected. This fraction (chromatophores) was washed once with a large volume of fresh phosphate-sucrose medium and suspended in a small volume of the same medium. For measurement, the chromatophore suspension was diluted with the same medium to make the absorbance at 855 nm about 1.0.

For the measurement of light-induced absorbance changes, a single beam-spectrophotometer constructed in this laboratory was used, the apparatus was designed so that the measuring light and the actinic light were incident on the cuvette in the same direction. The measuring light (approximately 0.1 erg cm^{-2} sec^{-1}) was obtained by use of a prism-monochromator; an incandescent lamp operated with a stabilized direct current was used for the light source. The 855 nm actinic light (approximately 1,000 erg cm^{-2} sec^{-1}) was obtained from a BAUSCH and LOMB grating monochromator equipped with a filter (Kodak-Wratten 88 A). Cuvettes of either a 10 mm or 3.5 mm light path were used. Changes in intensity of the transmittent light at desired wavelengths were measured with a photomultiplier (Hamamatsu TV, 7696) placed closely behind the cuvette containing the chromatophore suspension. Blue filters, V-B46 and IRO-IA (Toshiba), were placed between the cuvette and the photomultiplier to cut off the actinic light. The signal from the photomultiplier was amplified, using an electric circuit with a time constant shorter than one m sec. For recording, a strip chart servo-recorder (Riken Densi Co.; SP-J2) or a synchroscope (Toshiba Electric Co., 1554) was used.

For measurement of the light-induced absorbance change at −196°C, the chromatophore suspension was placed in a transparent plastic cuvette (2.0 mm light path) with an alminium fin, and chilled by immersing in liquid nitrogen placed in a transparent Dewar flask.

The absorption spectra of the chromatophores and their changes on addition of oxidation-reduction reagents were measured with a Shimadzu MPS spectrophotometer.

The intensity of the actinic light was altered by the use of glass filters, N-70, N-50 and N-10 (Hoya Glass Co.). Absolute intensity of the actinic light was measured with a calibrated thermopile (Kipp and Zonen, E l-type) combined with a microvoltmeter (Toa Dempa Co. PM-18).

The effects of molecular oxygen were tested by introducing the air to an anaerobic suspension of the chromatophores. The anaerobic condition was furnished by repeated evacuation and flashing with pure nitrogen gas, followed by further exhaustion of any trace of molecular oxygen by the use of a reaction mixture containing glucose, glucose-oxydase and catalase (*13*).

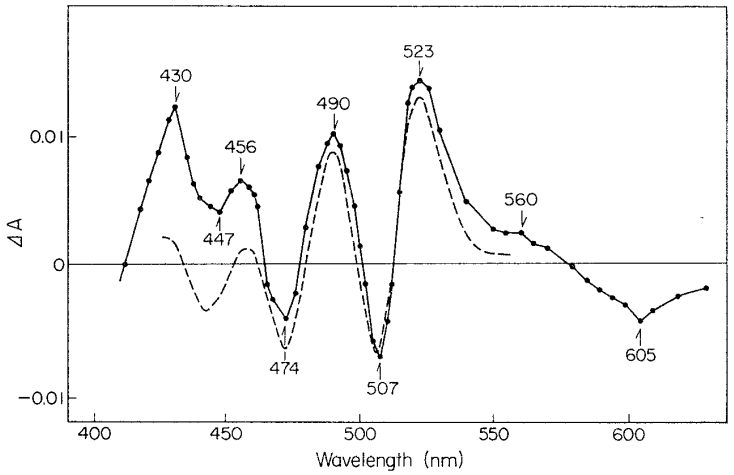

Fig. 2. Illuminated-*minus*-dark difference spectrum of the chromatophores. Dotted line represents the difference spectrum calculated assuming a uniform shift of the absorption spectrum of the chromatophores towards longer wavelength side. (400 cm^{-1} decrease in wavenumber; see text).

independent of the intensities of the actinic light used. As will be fully discussed in a later section of this article (**Discussion**), the light-induced absorbance changes occurring at 440-530 nm are to be ascribed to a change in the carotenoid in the chromatophore. On the other hand, the absorbance changes at 605 and 430 nm are probably due to changes of bacteriochlorophyll (*11, 18*). The reduction of the b-type cytochrome indicated by the absorbance increase at 560 nm might also have contributed to the absorbance increase at 430 nm (*12*).

In the following experiments, the light-induced absorbance changes ascribed to the change of carotenoid were investigated mainly at the above-mentioned peaks or troughs of the difference spectrum, mostly at 490 nm. For comparison, the changes at other wavelengths were also examined.

Effects of uncouplers and inhibitors on the light-induced absorbance changes

The light-induced absorbance change at 490 nm was suppressed by addition of CCCP, an uncoupler of photophosphorylation in chromatophores of photosynthetic bacteria (*19*) (Fig. 3). A marked suppression of the steady state level change was observed at low concentrations of CCCP; 50%-suppression was obtained at 3×10^{-7} M CCCP. It can be seen from the figure that CCCP accelerated the rate of light-off change (recovery) whereas it was without effect on the light-induced change at the onset of the light.

On the other hand, the light-induced absorbance change at 430 nm was hardly affected by 10^{-6} M CCCP both with respect to the magnitude of the steady state level change and to the kinetics of the on- and off-responses.

The effects of CCCP on the light intensity dependence of the light-induced absorbance change at 490 nm were examined (Fig. 4). The absorbance change at the steady state level was saturated at a light intensity of about 1,000 erg cm^{-2} sec^{-1} in the absence of CCCP. The presence of CCCP modified the light intensity

Results

Light-induced absorbance changes in chromatophores of Rhodopseudomonas spheroides

Light-induced absorbance changes in the chromatophores of *Rhodopseudomonas spheroides* were followed at various wavelengths in the visible region. Fig. 1 shows the time courses of the changes at 523, 507, 490, 474, 430 and 605 nm. On illumination of the chromatophores, there occurred increases at 523, 490 and 430 nm, and concomitant decreases at 507, 474 and 605 nm. Steady state levels of these changes were reached within 30 sec after onset of actinic illumination, and the original levels of absorbance were recovered within 60 sec after cessation of the light. As will be seen from the figures, the time courses of the absorbance changes at 523, 507, 490 and 474 nm were all similar in shape.

The light-*minus*-dark difference spectrum was obtained by measuring the steady state level of the light-induced absorbance change at respective wavelengths (Fig. 2). Repeated illumination with one sample did not alter the results of the measurements when sufficiently long dark periods (*e.g.*, 3 min) were inserted between successive light periods. As can be seen in Fig. 2, there are peaks at 430, 456, 490, 523 and 560 nm, troughs at 447, 474, 507 and 605 nm and isosbestic points at about 464, 477, 501 and 512 nm. These characteristics of the difference spectrum were

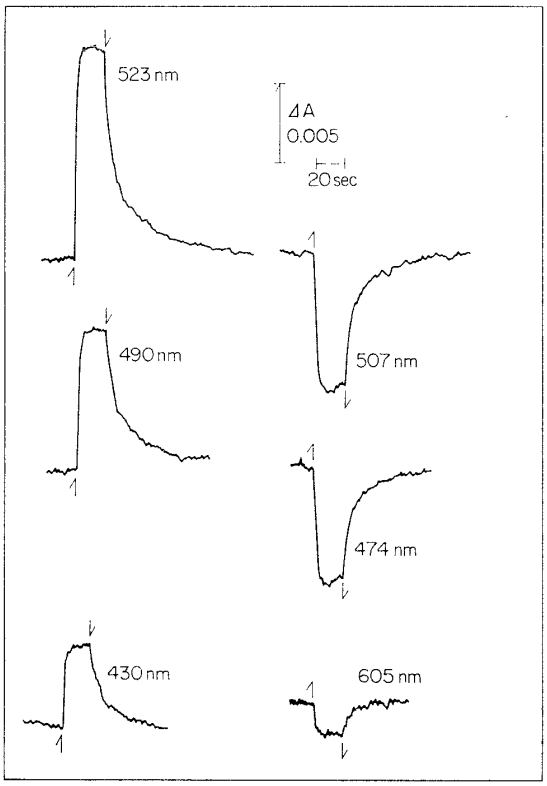

Fig. 1. Time courses of light-induced absorbance changes at 523, 507, 490, 474, 430 and 605 nm in chromatophores of *Rhodopseudomonas spheroides*. Upward and downward arrows represent, respectively, onset and cessation of actinic illumination.

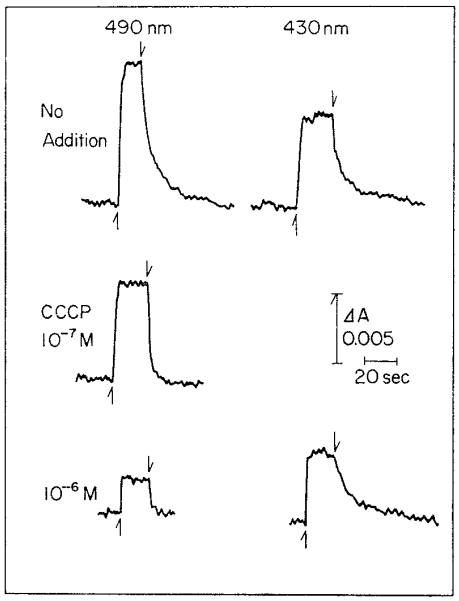

Fig. 3. Effects of CCCP on the light-induced absorbance changes at 490 nm (carotenoid) and at 430 nm.

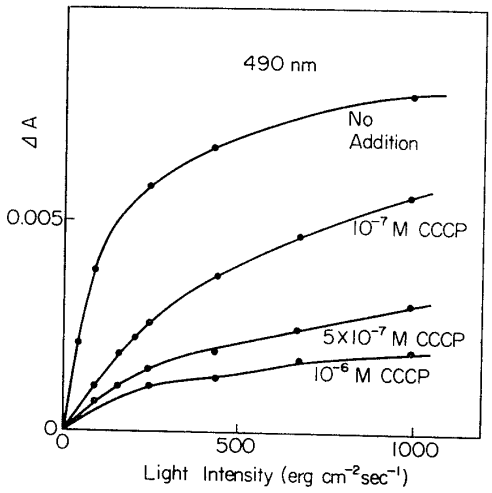

Fig. 4. Relationship between intensity of actinic light and the light-induced absorbance increase (steady state) at 490 nm in the presence and absence of CCCP.

curve so that the suppressing effect of CCCP became more marked at lower intensities of light. Similar effects of inhibitors, including CCCP, HOQNO and desaspidine, have been reported by Fleishman and Clayton (*16*) in the case of the chromatophores of *Rhodopseudomonas spheroides*. In contrast, the inhibitory actions of gramicidin D and antimycin A were not affected by varying the intensity of actinic light (*16*).

It was found that other uncouplers of photophosphorylation, gramicidin J and Triton X-100 (*20*), also suppressed the light-induced absorbance change at

490 nm. The concentrations for 50%-suppression of the steady state level change were 3×10^{-6} M for gramicidin J and 2×10^{-2} % for Triton X-100. Inhibitors of electron transport in photosynthetic bacteria, including antimycin A, *o*-phenanthroline and piericidin A (*21*), also suppressed the light-induced absorbance change at 490 nm; with 50%-suppression concentrations at 4×10^{-5}, 3×10^{-3} and 5×10^{-5} M, respectively. The effect of antimycin A was not removed by addition of phenazine methosulfate (10^{-4} M) either in the presence or absence of ascorbate.

Effects of molecular oxygen and oxidation-reduction reagents on absorption spectrum of chromatophores

SMITH and RAMIREZ (*2*), using intact cells of *Rhodopseudomonas spheroides,* found that introduction of molecular oxygen to the reaction medium caused a change in the absorption spectrum of carotenoid similar in pattern to the light-induced absorbance change under investigation. It was found in the present study, however, that introduction of molecular oxygen did not cause any change in the absorption spectrum of the chromatophores at the region of 460–540 nm, nor was the light-induced absorbance change at these wavelengths affected by its presence or absence.

The effects of the addition of oxidation-reduction reagents on the absorption spectrum of the chromatophores were investigated (Fig. 5). The addition of dithionite (grains) caused increases in absorbance at 430 nm and at 550–560 nm and a decrease at 406 nm, which can be ascribed to the reduction of the cytochromes. The addition of ferricyanide decreased the absorbance at 590 nm, which is probably due to the bleaching of the bacteriochlorophyll. It should be noted that these reagents did not induce any significant absorbance change at wavelengths, at which carotenoid showed absorption. It seems, therefore, unlikely that the light-induced

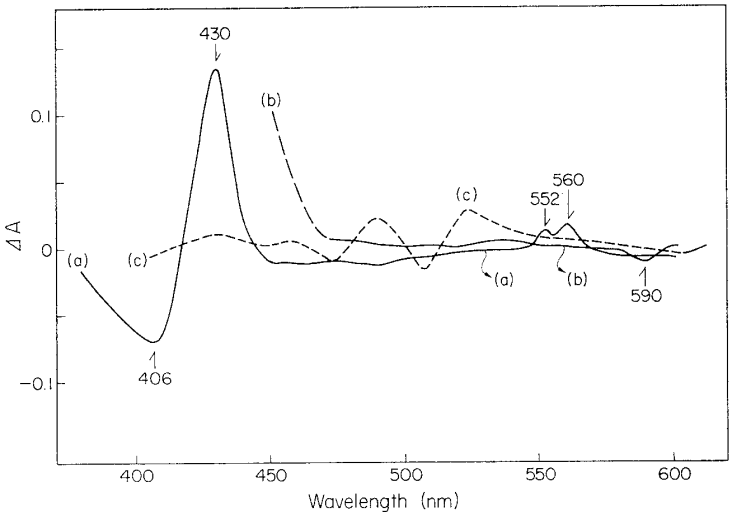

Fig. 5. Difference spectra of changes induced by addition of oxidation-reduction reagents to the chromatophores. (a) Dithionite-*minus*-no addition. (b) Ferricyanide-*minus*-no addition. Dithionite, few grains. Ferricyanide, 5×10^{-5} M. Spectral changes were measured after sufficient incubation with these reagents (10 min). (c) Light-*minus*-dark difference spectrum.

absorbance changes at 450–540 nm (Fig. 2 and 5) represent an oxidation-reduction reaction of carotenoid in the chromatophore.

Kinetics of light-induced absorbance changes of chromatophores

The time course of the light induced absorbance change on onset of actinic illumination was measured at 508 nm with the aid of a synchroscope (Fig. 6). Under the experimental condition of illumination (*i.e.*, 1,000 erg cm^{-2} sec^{-1} ; 855 nm) the initial rate of change was 0.039 (ΔA/sec). The whole time course of this change followed a formula for a unimolecular reaction, having $k_{uni}=5.8$ sec^{-1}.

A similar time-course pattern was also obtained when the absorbance changes were measured at 490 or 523 nm under the same experimental conditions.

The initial rapid phase of absorbance change (recovery) on cessation of light was also examined. In this case also, the initial rapid part of the time course was expressed by a formula for a unimolecular reaction. The initial rate was 0.018 (ΔA/sec) corresponding to a unimolecular reaction constant of 4.3 sec^{-1}. Similar results were obtained at other wavelengths mentioned above.

The relationship between the intensity of actinic light and the initial rate of absorbance change at 508 nm is illustrated in Fig. 7. At the range of light intensities studied, *i.e.*, below 1,000 erg cm^{-2} sec^{-1} (855 nm), the initial rate was proportional to the light intensity. On the other hand, the magnitude of the change in steady state level of absorbance was represented by a saturation curve showing a gradual

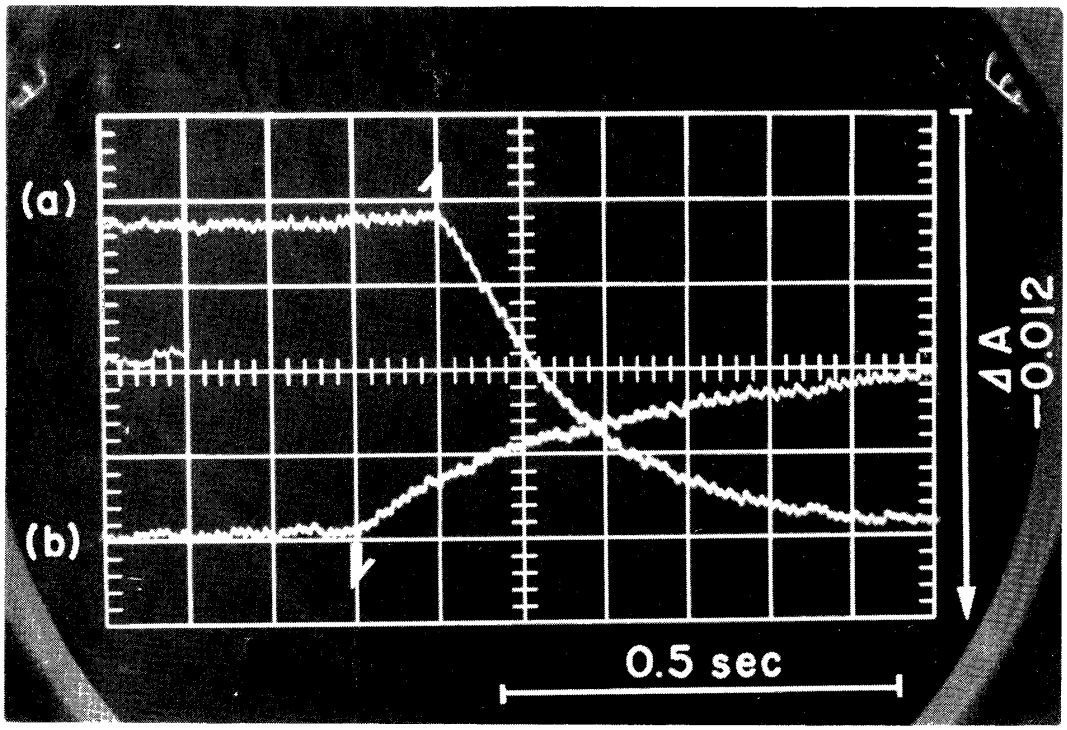

Fig. 6. Time courses of light-induced absorbance change at 508 nm traced on a synchroscope. Actinic light; 855 nm, 1,000 erg cm^{-2} sec^{-1}. (a) Absorbance decrease on onset of light (↑ arrow). (b) Absorbance increase on cessation of light (↓ arrow).

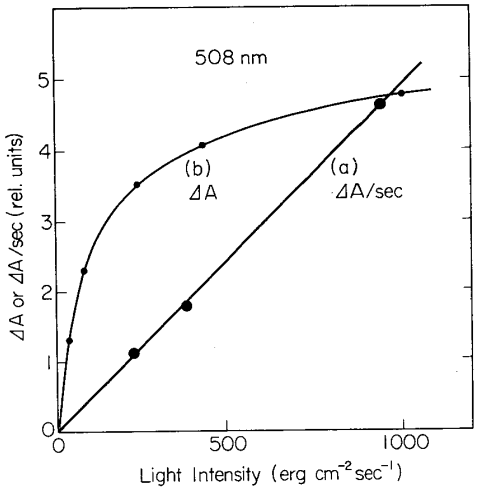

Fig. 7. Light-intensity dependence of the initial rate (a), and the steady state level (b) of light-induced absorbance change measured at 508 nm.

decrease of the slope with increasing intensity of the actinic light. A 50%-saturation was attained at about 100 erg cm^{-2} sec^{-1} (855 nm). A similar pattern of light-intensity dependence was also obtained for the changes at 490 nm and 523 nm. This will be self-evident when we consider the above-mentioned fact (*cf.* Fig. 2) that the shape of the light-*minus*-dark difference spectrum is independent of the intensity of actinic light.

Light-induced absorbance changes at liquid nitrogen temperature

The chromatophores were tested for their capacity for light-induced absorbance

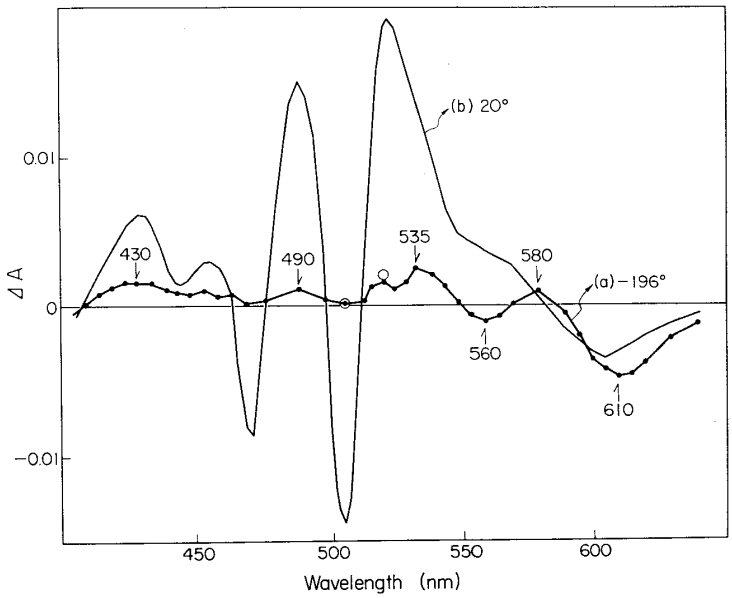

Fig. 8. Light-induced difference spectra at room and liquid nitrogen temperatures. (a) −196°C. ○; The chromatophore sample was kept in the dark during and after cooling to −196°C and illuminated only once for the measurement except for a short exposure to the weak measuring light, which was unavoidable in preparation for the measurement, (b) Room temperature (20°C).

changes at liquid nitrogen temperature (−196°C) (Fig. 8). It can be seen from the figure that the absorbance changes at 450–550 nm almost vanished with the lowering of the temperature whereas the change at 610 nm ascribed to the change of bacteriochlorophyll was similar in extent as that observed at room temperature.

At this low-temperature, however, there is a possibility that the effect of the preceding exposure to the light might have survived during the dark interval and thus gave rise to a suppression of further response of the chromatophore to subsequent illumination. To test this possibility, some experiments were carried out in a way that the chromatophore sample was frozen in the dark so as to be illuminated only once for that particular measurement. The results of such experiments are indicated with open circles in the figure. There was no difference as compared with the results of the repeatedly illuminated experiments.

Discussion

The assignment of the absorbance changes observed at 450–540 nm to some change of carotenoid in the chromatophores is quite admissible if one considers the characteristic three-peaked pattern of the light-*minus*-dark difference spectrum localized at the spectral region at which carotenoid showed absorption (*1–9*). Suggestions have also been made by various investigators (*2*, *3*, *5*) that the observed absorbance changes are a result of a shift of the absorption spectrum of carotenoid in the bacterial cells or chromatophores. In the present study, detailed examination of this point was made by drawing a theoretical curve for the difference spectrum on a basis of the assumed shift of the absorption spectrum of the chromatophore towards the longer wavelength side. Actually, a uniform shift by 400 Kayser in wavenumber was adopted to fit the experimental results. The agreement of the theoretical curve (Fig. 2, dotted line) with the experimental results obtained was quite satisfactory. It should be noted that the same magnitude of shift in the absorption spectrum has been successfully used by Amesz and Vredenberg for explaining similar absorbance changes in intact cells of *Rhodopseudomonas spheroides*.

Rhodopseudomonas spheroides contains spheroidene and spheroidenone as its two major carotenoids (*14*). These carotenoids were extracted and purified on a silica-gel thin-layer chromatogram. Spheroidene showed an absorption maxima at 429, 456 and 486 nm; spheroidenone at 460 and 515 nm (acetone–methanol, 7:2, v/v), thus confirming the assignment of the absorption change under investigation to the change of the former carotenoid (*5*).

Amesz and Vredenberg (*5*) estimated a value of 3 for the quantum yield of the absorbance change of carotenoid in intact cells of *Rhodopseudomonas spheroides*. The calculation was based on the above-described assumption of a shift of the absorption spectrum of the carotenoid. However, no detailed accounts have been presented yet. In the present work the quantum yield of the change of the carotenoid in the chromatophores was calculated as follows: The molar absorption coefficient of spheroidene in the chromatophores was estimated by comparing the absorption of spheroidene in the chromatophores at the absorption maxima (*i.e.*, 476 nm, 508 nm) with that at the corresponding maxima in the acetone–methanol extract (*i.e.*, 456 nm, 487 nm); a value, $E^{1M}_{508nm}=9.3\times10^4$ was thus obtained, using a value of $E^{1\%}_{1cm,\,456nm}=2{,}700$ for the absorption coefficient of spheroidene in an acetone-

methanol solution as reported by SCHNEOUR (*15*). The molar difference absorption coefficient of the carotenoid *in vivo*, ΔE, was computed graphically by estimating the absorbance difference which may result from a shift of the absorption spectrum of the carotenoid *in vivo* by 400 cm^{-1} towards the lower wavenumber side (see above); a value, $\Delta E^{1M}_{508nm} = 1.6 \times 10^4$ was thus obtained for the absorbance change at 508 nm. The number of carotenoid molecules converted on illumination was calculated from the observed absorbance change, using the above-described value for the molar difference absorption coefficient.

The quantum yield of the light-induced change was calculated from the absorbed light intensity and the initial rate of the carotenoid from the absorbed light intensity and the initial rate for the change of carotenoid measured as shown in Fig. 6. The values thus obtained for the quantum yield were always larger than unity, (1.4 to 3.4; an average of about 2.5) which is in agreement with the results of AMESZ and VREDENBERG with intact cells of the same organism (*5*). Such values for the quantum yield that were greater than unity also indicate that the change in question is not a simple photochemical reaction, *e.g.*, photo-isomerization or photo-oxidation or -reduction, of the pigment. A similar instance of high quantum yield has also been reported concerning the light-induced H^+ uptake in the chloroplasts of higher plants (*23*). The question whether this is a mere coincidence or whether there is some connection between these two facts remains to be elucidated.

At liquid nitrogen temperature, the extent of the light-induced absorbance change of carotenoid, if any, was insignificant as compared with that observed at room temperature (Fig. 8). This fact indicates that the change does not represent a primary reaction. The above-described experimental results at the low temperature, however, differ from those reported by ARNOLD and CLAYTON (*8*, *9*) in that, in the air-dried film of the chromatophores of *Rhodopseudomonas spheroides*, the absorbance changes of carotenoid were observed even at 1°K. The cause of this difference has also to be elucidated.

SMITH and BALTSCHEFFSKY (*7*) reported that in intact cells of *Rhodopseudomonas spheroides*, the magnitude of light-induced absorbance change of carotenoid, as well as the time course of the change, depended on the concentration of molecular oxygen in the reaction medium. The introduction of air to an anaerobic reaction-mixture caused a spectral change of carotenoid which was similar to that observed upon illumination (*2*). These facts led them to the inference that the light-induced absorbance change of carotenoid may represent a light-induced oxidation-reduction of carotenoid. In the present study, working with the chromatophores, however, no influence of O_2 was detected concerning the absorption spectrum or the light-induced absorbance change of carotenoid. It is logical, therefore, to conclude that the light-induced absorbance change of carotenoid under investigation is not a result of an oxidation of the carotenoid, spheroidene. The difference in behaviors of the intact cells and chromatophores towards molecular oxygen may be explained as follows: In (the case of) intact cells, the introduction of O_2 to the reaction mixture will accelerate the electron transport which, in turn, may produce a change in the state of the carotenoid *in vivo*. In the case of chromatophores, molecular oxygen does not significantly enhance the electron transport, partly because of the lack of endogenous substrates; some factor(s), or structural element(s), operating in the above-described link of changes might also have been lost or injured. In fact, we

have previously shown in a study of the ubiquinone-mediated electron transport in *Chromatium* D, that the capacity for reacting with molecular oxygen is completely lost during the preparation of the chromatophores, whereas the activity of the light-induced cyclic transport of electrons remained uninjured (*24*).

The suppressing effects of uncouplers, such as CCCP, gramicidin J and Triton X-100, on the light-induced absorbance change of carotenoid suggest that the light-induced change is closely related to the formation of a high-energy state (or intermediate) of photophosphorylation in the chromatophores. Recent works of FLEISHMAN and CLAYTON (*16*) and BALTSCHEFFSKY (*17*) which appeared on conclusion of the present work, provide further support to this inference.

A most probable cause of the observed shift on the absorption spectrum is a structural change accompanying the light-induced high energy state of the chromatophores (*22*), which may result in a change in the environment of the carotenoid molecules, thus inducing an alteration of the electronic state of the pigment.

The present work was initiated by the suggestion of Prof. M. NISHIMURA (Kyushu University), a former member of this laboratory, to whom the authors express their cordial thanks. They acknowledge their gratitude to Dr. S. MORITA for valuable advice and discussion.

The work was supported by a grant from the Ministry of Education to which the authors' thanks are due. Financial aid afforded to one of the authors (M.O.) by the late Mr. SETSUYA YAMAMOTO is also acknowledged with deepest gratitude.

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