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LIGHT-INDUCED RAPID ABSORPTION CHANGES DURING PHOTOSYNTHESIS

VIII. CYTOCHROME AND BACTERIOCHLOROPHYLL REACTIONS IN *RHODOSPIRILLUM RUBRUM* CELLS*

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SUMMARY

By means of flash spectrophotometry, the oxidation reactions of cytochromes c_2 and b can be differentiated. Cytochrome c_2 oxidation had a risetime of 1–2 msec; that of cytochrome b was several times longer. Both risetimes were several orders of magnitude longer than the duration of the excitation flash, suggesting that both cytochromes were oxidized in the dark.

Unique for the cytochrome-b transients was the appearance of sharp spikes occurring always in the direction opposite of those of the cytochrome-b oxidation. These transient spikes occurred at an excitation intensity level beyond the saturation level for both cytochromes c_2 and b. The risetime for the sharp spike transients was approx. 50 μsec.

In the wild-type *Rhodospirillum rubrum* cells, reactions due to the carotenoids caused spectral changes in the 470–560-mμ region. This resulted in an unproportionately large negative α- and β-bands in the light-*minus*-dark difference spectrum. There was no noticeable difference in the decay kinetics between the cytochromes and the carotenoids.

The oxidation of both cytochromes c_2 and b was stopped near 0°. At −196°, positive bands appeared at 430 and 790 mμ, and negative bands appeared at 380, 810 and 870 mμ. All reactions taking place at the liquid-nitrogen temperature had a risetime of approx. 50 μsec. The low-temperature difference spectrum probably represents changes originated from the bacteriochlorophyll molecules.

The net effect of 4 inhibitors (*p*-chloromercuribenzoate (PCMB), phenylmercuriacetate, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), and antimycin A) on *R. rubrum* cells was similar, *i.e.*, the light-*minus*-dark difference spectrum resembled closely that obtained with whole cells at −196° or from chromatophores at room temperature.

The effect of PCMB on the transients at all wavelengths was immediate. In

Abbreviations: PCMB, *p*-chloromercuribenzoate; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

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the presence of phenylmercuriacetate, HOQNO or antimycin A, however, the final transient profile at 420 mμ took more than 2 h to become established. Prior to that time, the transient underwent a series of intermediate stages. More unusally, the 420-mμ transient in the presence of HOQNO or antimycin A appeared to completely revert to the initial profile at approx. 120 min after the inhibitor was added.

INTRODUCTION

The presence of a *c*-type cytochrome in *Rhodospirillum rubrum* was first reported by VERNON AND KAMEN[1,2]. Subsequently, DUYSENS[3] and CHANCE AND SMITH[4] observed light-induced absorption changes in intact *R. rubrum* cells and attributed them to a photooxidation of endogenous cytochromes.

The bacterial cytochromes are photooxidized with high quantum efficiency[5,6]. In *Chromatium* cells, photooxidation of the cytochromes absorbing at 423.5 mμ was found by CHANCE and co-workers to proceed with very fast risetimes[7] and at liquid-nitrogen temperature[8], indicating that the electron-transfer reaction involving the cytochrome is a temperature-independent primary process. However, VREDENBERG AND DUYSENS[9] later reported that some cytochrome reactions may be stopped at sufficiently low temperatures.

Light-induced absorption changes in the bacteriochlorophyll absorption regions were also observed by DUYSENS[10] in *R. rubrum* cells and in *Chromatium* extracts. Subsequently, GOEDHEER[11] showed that chemical oxidation with ferricyanide could bring about a similar absorption change in bacterial chromatophores. This led to the suggestion that the light-induced changes are associated with the photooxidation of a small fraction of the bacteriochlorophyll molecules called P870 or P890 at the photochemical reaction center[12].

Many spectrophotometric studies of photosynthetic bacteria have been reported in the literature, but most of them were limited to the spectral region pertaining to either the cytochromes or bacteriochlorophyll. Short laser flashes (30-nsecond duration) have been used recently for the examination of cytochrome absorption changes in the Soret region[7]. The present work is an attempt to explore a possible relationship between bacteriochlorophyll absorption changes and those of the other redox components in *R. rubrum* in the wavelength region from 350 to 900 mμ using 20 μsec excitation flashes.

EXPERIMENTAL

R. rubrum cells, strain S-1, and the carotenoidless mutant cells were grown in a medium previously described by NEWTON[13]. Anaerobic conditions were created by dark respiration when the cells were kept in a stoppered cuvette.

The construction of the flash spectrophotometer and its operation in conjunction with a CAT computer were described earlier[14]. The spectrophotometer was used in the single-beam mode[15]. For measuring wavelengths below about 700 mμ the excitation flash was isolated by a Baird-Atomic interference filter with a half-bandwidth of 50 mμ and centered at 875 mμ. When measuring above 700 mμ, a broad 590 mμ interference filter was used for isolating the excitation flash. Individual narrow-band

interference filters were used to shield the photomultiplier from the excitation flash. The measuring beam was isolated by a Bausch and Lomb 500-mm grating monochromator, and a uniform bandwidth of 1.5 mμ was used throughout the entire measuring-wavelength range. An EMI 9558 photomultiplier was used for the wavelength below 700 mμ, and an RCA 7102 photomultiplier for the near-infrared region.

For low-temperature measurements, a thin layer of the cell suspension was placed in a demountable cuvette formed by 2 microscope slides separated by a spacer 0.7 to 1 mm thick. The cuvette assembly was then mounted in a cold-finger frame which formed an integral part of a liquid-nitrogen container. An exploded view of this assembly is shown in Fig. 1. The entire assembly was placed inside a dewar which was pre-flushed with dry nitrogen. The front surface of the cuvette was oriented 45° with respect to both the measuring and the excitation beams. A 32-gauge copper–constantan thermocouple was inserted into the suspension for monitoring the temperature.

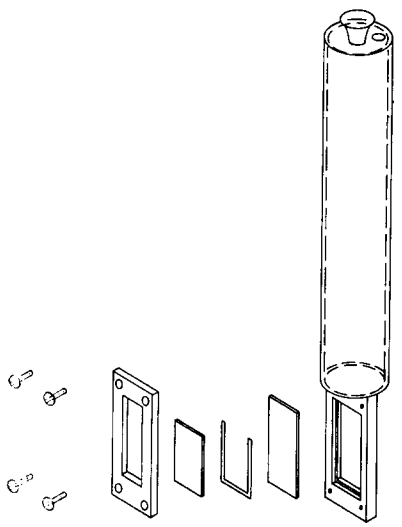


Fig. 1. An exploded view of the cuvette assembly used for low-temperature measurements (see text for details).

RESULTS

Light-induced absorption changes in anaerobic cells

Light-induced absorption changes in anaerobic *R. rubrum* cells were measured for the region from 350 to 900 mμ, which covers the major absorption bands of the cytochromes, carotenoids and bacteriochlorophyll. In different wavelength regions, the rates of both the onset and decay of the absorption changes were different. However, the absorption changes that may be ascribed to one particular pigment were usually very similar or identical in kinetics. For instance, the absorption-change transients that are ascribable to the photooxidation of cytochromes c_2 and b at 405, 420, 520 and 550 mμ and at 415, 428, 522.5 and 558 mμ, respectively, as shown in Fig. 2, had practically identical kinetics. The risetime of cytochrome-c_2 photooxidation was estimated to be 1 to 2 msec, and the decay halftime 40 msec. The

absorption-change transients associated with cytochrome *b* always started with a sharp spike (downward or upward) which was followed by a slow rise in the opposite direction and then a slow decay. The sharp spike of the 428.5-mμ transient had a risetime of approx. 50 μsec and an apparent decay time of approx. 2 msec. The rise-time of the opposite signal was of the order of 10–20 msec.

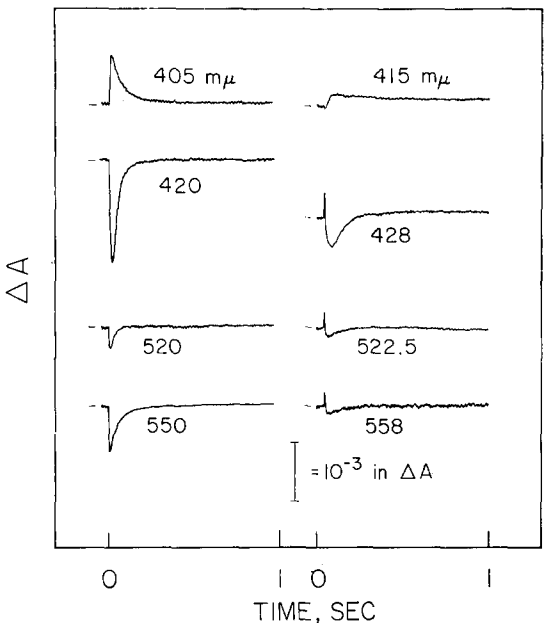


Fig. 2. Absorption-change transients in the absorption regions of cytochromes c_2 (left column) and *b* (right column) of anaerobic *R. rubrum* cells.

No transients due to the carotenoids can be separately identified by their kinetics in the 470–560-mμ region. However, the light-*minus*-dark difference spectrum presented below (Fig. 3) indicates that carotenoid reactions were present. When the carotenoidless mutant cells of *R. rubrum* were used in examining the light-induced absorption changes, transients with practically the same kinetics as those of the wild-type cells were obtained. Because of the absence of carotenoid reactions, changes in the carotenoid absorption regions of the mutant cells were smaller.

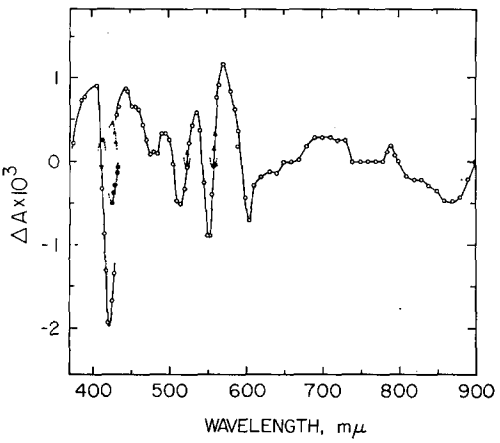
In anaerobic *R. rubrum* cells, absorption-change transients in the bacteriochlorophyll absorption regions were relatively small. These transients had a risetime of 50 μsec or less and a decay time of 25 msec (*cf.* Fig. 8).

The light-minus-dark difference spectra

Since the absorption-change transients in the wavelength region examined differ in kinetics, the difference spectrum represents reactions caused by more than one species. Figs. 3 shows such a composite difference spectrum, in which some transients of similar kinetics were separately indicated.

In the Soret region, one may conclude that cytochrome c_2 and cytochrome *b* are both oxidized. Presumably as a result of interference by carotenoid changes, the

α- and β-difference bands of cytochrome c_2 are large relative to its Soret band. On the other hand, the absorption changes in the α- and β-bands of cytochrome *b* were less influenced by the carotenoid changes.



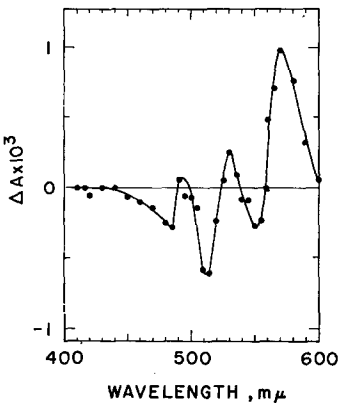


Fig. 3. The light-*minus*-dark difference spectrum of anaerobic *R. rubrum* cells. (The solid dot and triangle represent, respectively, the slow changes and rapid spike in the cytochrome *b* transients).

Fig. 4. Difference between the difference spectra of the wild-type and the carotenoidless *R. rubrum* cells.

Similar to that found previously by SMITH AND RAMIREZ[16] for *Rhodopseudomonas spheroides*, the carotenoid absorption decreased and new absorption bands appeared at longer wavelengths in *R. rubrum* cells, as reflected in the large positive bands at 535 and 570 mμ in the difference spectrum shown in Fig. 3. The carotenoid changes can be more clearly shown in Fig. 4 by plotting the difference between the light-*minus*-dark difference spectra of the wild-type and carotenoidless mutant cells.

Unique for the absorption-change transients in the cytochrome-*b* absorption regions is their complex kinetics, as shown earlier in Fig. 2. Thus far, the sharp spikes were observed only in the cytochrome *b* absorption regions. Beyond 430 mμ, a positive signal persisted, but it was apparently different from the sharp positive spike because it decayed in 10 to 15 msec.

The general profile of the difference spectrum in the bacteriochlorophyll absorption regions is similar to that observed by DUYSENS *et al.*[17] in washed aerobic cells of *R. rubrum*, except the magnitude obtained here in anaerobic whole cells was much smaller.

Dependence of the absorption changes in the Soret region on the excitation intensity

The transients at 420 and 428.5 mμ, which were ascribed to cytochromes c_2 and *b*, respectively, were examined over a range of excitation intensities. A plot of the magnitude of the absorption change *vs.* relative intensity is shown in Fig. 5. For the 428.5-mμ transient, the positive spike and the negative slow change are plotted separately.

As seen in Fig. 5, the magnitude of cytochrome-c_2 photooxidation (420(—)) increased linearly at low intensities, and became saturated at 45 % of the maximum

intensity. On the other hand, cytochrome *b* photooxidation (428(—)) became saturated at a much lower intensity of 20–25 %, and stayed level at higher intensities. The sharp spike (428(+)) started to appear at about 35–40 %, slightly beyond the saturating intensity of the negative absorption change at 428 mμ. It remained constant between 50 and 70 %. Above 70 % it increased to a second plateau. The magnitude of the relatively rapidly decaying positive absorption change at 438 mμ (438(+)) appeared to increase linearly with the intensity up to the maximum value used. Separate experiments showed that at an intensity 50 % higher than the maximum value used in the figure it also started to level off.

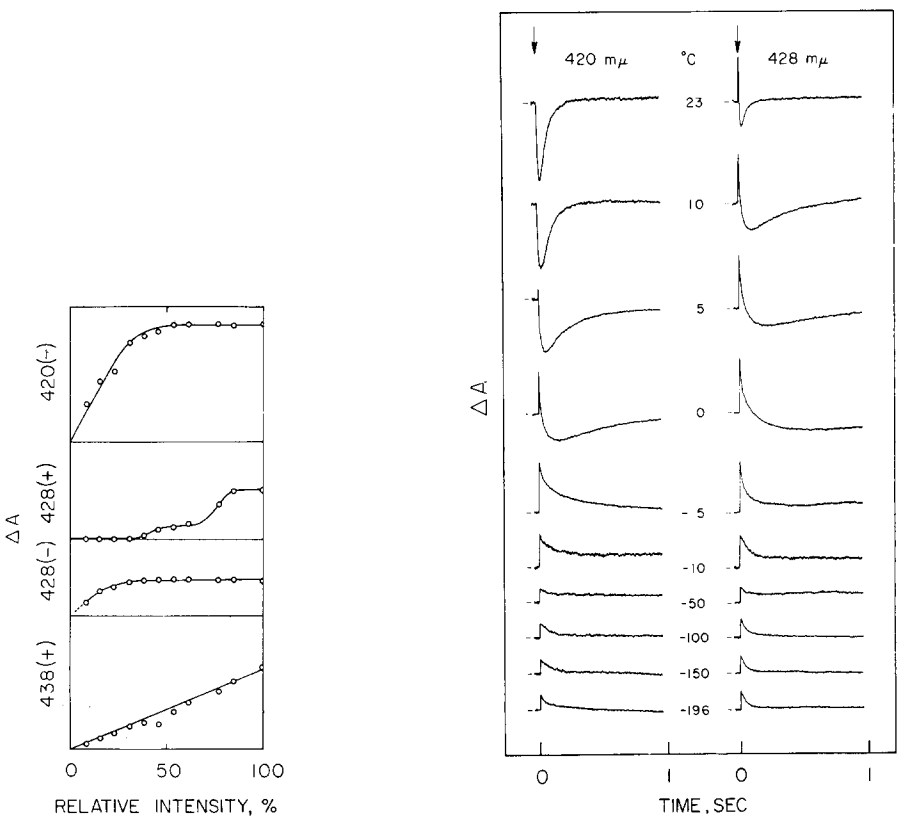


Fig. 5. Dependence of the transient absorption changes at several wavelengths on the excitation intensity. The intensity of the near infrared flash usually used in these experiments (instantaneous peak intensity = $1.29 \cdot 10^4$ ergs/cm^2·sec) was set as 100 %.

Fig. 6. Absorption-change transients at 420 and 428 mμ at different temperatures.

Light-induced absorption changes at —196°

The light-induced absorption changes in anaerobic *R. rubrum* cells in the 350–900-mμ region were examined at low temperatures. Fig. 6 shows the effect of decreasing temperature on the light-induced absorption changes of cytochromes c_2 and *b* at 420 and 428 mμ. When the temperature was lowered from 25° to 10°, the decay of the 420-mμ transient became slower. At 5°, a small positive spike appeared,

which was followed by a slow decrease and a much slower decay. The effects were further enhanced at 0°. At −5° or below, only a rapid positive change followed by a decay occurred upon flash illuminaton. This pattern persisted up to −196°, and the decay showed little temperature dependence.

The 428.5-mμ transient at 10° was similar to the 420-mμ transient at 0°. Below −10°, the two transients were nearly the same.

The light-*minus*-dark difference spectrum at −196° for the spectral region from 350 to 900 mμ is shown in Fig. 7. The spectrum shows a large decrease at 380 mμ and a broad positive band between 410 and 465 mμ. In the bacteriochlorophyll-band region, a positive shift at 790 mμ and a decrease in absorption at 810 and 870 mμ occurred. The temperature appeared to have no significant effect on the kinetics of the bacteriochlorophyll changes, except the magnitude of these changes was much greater at liquid-nitrogen temperature. Note that the low-temperature difference spectrum developed more structure in the form of extra shoulders at 380 and 765 mμ.

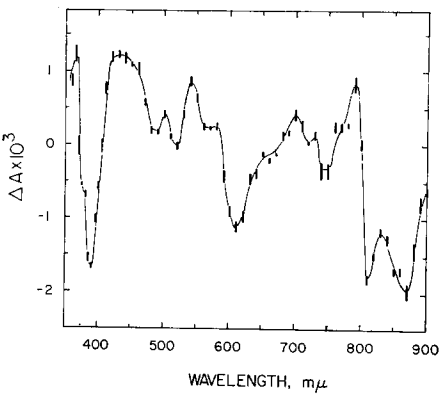


Fig. 7. The light-*minus*-dark difference spectrum of *R. rubrum* whole cells at −196°.

Effect of several inhibitors

The profiles of the absorption-change transients in anaerobic *R. rubrum* cells were drastically altered immediately after *p*-chloromercuribenzoate (PCMB) was added to the cell suspension to a concentration of 2 mM. Light-induced absorption-change transients at several representative wavelengths in untreated cells and in cells containing PCMB are shown in the first two columns of Fig. 8. The negative absorption changes in the Soret region due to cytochrome oxidations were replaced with positive changes forming a broad positive difference band. In the region where carotenoids absorb, only absorption increases were found. The absorption changes in the bacteriochlorophyll absorption regions were greatly enhanced and had much longer decay times.

The effect of PCMB on the profiles of the absorption-change transients was immediate. With longer incubation time, only the decay time became longer, the magnitude of the absorption change remained practically constant. For instance, the half-decay times of the 430-mμ signal at 5, 60 and 120 min after PCMB addition were respectively 0.4, 0.5 and 0.6 sec. It is worth noting that the peaks of transients at wavelengths corresponding to those of cytochrome *b*, namely 430 and 560 mμ,

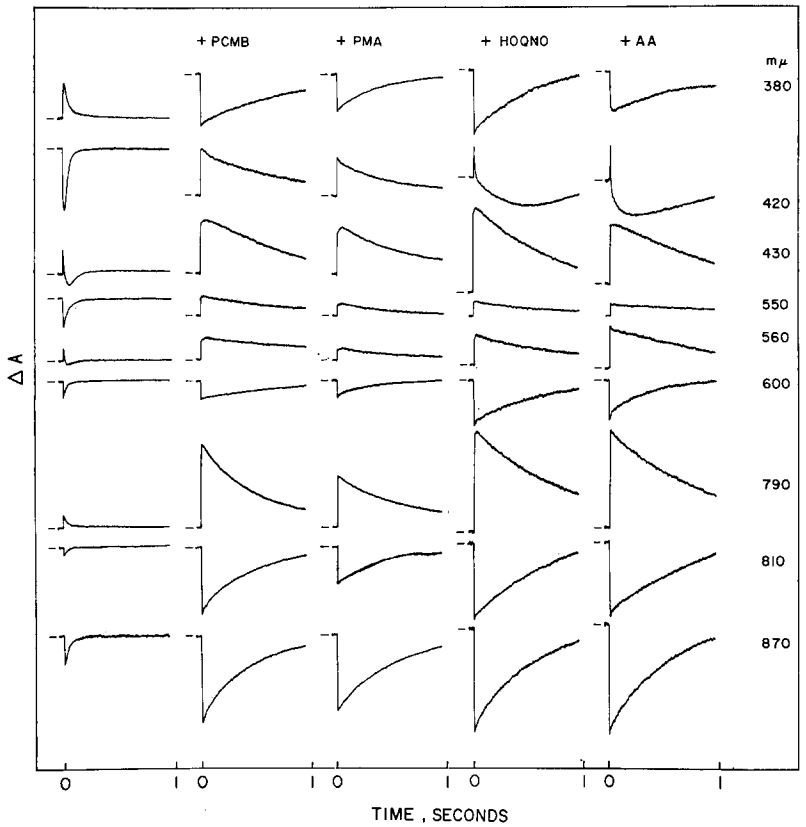


Fig. 8. Absorption-change transients of *R. rubrum* cells at several representative wavelengths. First column, anaerobic cells; second column, cell suspension containing 2 mM PCMB; third column, 0.1 mM phenylmercuriacetate (PMA); fourth column: 0.1 mM HOQNO; fifth column: 0.05 mM antimycin A (AA).

were slightly truncated. Most of the transient absorption changes in the presence of PCMB had rapid risetimes, namely, $\leqq 50$ μsec.

The net effect of phenylmercuriacetate on the light-induced absorption changes in *R. rubrum* cells is shown by the affected transients in the third column of Fig. 8 and by the difference spectrum in Fig. 9. Unlike PCMB, however, the establishment of the final transient profiles in the Soret region took approx. 3 h. Prior to that time, the transient underwent a series of intermediate stages. A typical set of transients at 420 mμ at various times after phenylmercuriacetate addition are shown in the first column of Fig. 10. At wavelengths outside the Soret region, phenylmercuriacetate produced immediate changes; only the dark decay times increased slightly with incubation time. As seen from Figs. 8 and 9, 3 h after phenylmercuriacetate addition, the transients and the difference spectrum were almost identical to those caused by PCMB. Similar to the low-temperature difference spectrum, those in the presence of PCMB or phenylmercuriacetate also developed shoulders at 405 and 760 mμ and a band split at 430 mμ.

The inhibitors 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) and anti-

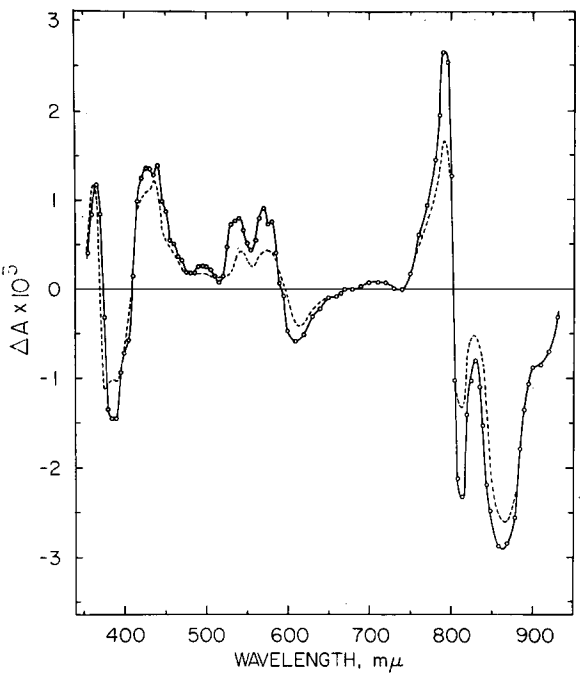


Fig. 9. Light-*minus*-dark difference spectra of *R. rubrum* cells in the presence of PCMB (solid curve) and phenylmercuriacetate (PMA) (dashed curve).

mycin A also drastically altered the profile of the light-induced absorption-change transients in anaerobic *R. rubrum* cells, as shown in the last two columns of Fig. 8. Most of these transients required about 20 min to reach the final profile with a maximum constant decay time. However, the transient at 420 mμ underwent a series of intermediate stages. More unusually, as can be seen in the second and third columns of Fig. 10, the 420 mμ absorption-change transient appeared to completely revert to the initial profile at approx. 120 min after the inhibitor was added. The value of 120 min was an average of 4 separate measurements, with a deviation of less than 10 min. In one particular *R. rubrum* sample, the reversion occurred at 90 min for both HOQNO and antimycin A. After the reversion, the transient profile changed to those shown in the bottom row of Fig. 10, and did not vary any further with time.

From the individual transients shown in Fig. 8, it can be seen that the light-*minus*-dark difference spectrum of *R. rubrum* cells in the presence of HOQNO or antimycin A is also very similar to those with PCMB or phenylmercuriacetate shown in Fig. 9, except for the discontinuity near 420 mμ.

DISCUSSION

The use of short flashes in inducing reactions with overlapping absorptions allowed a differentiation of the two cytochrome reactions by their onset and decay kinetics. Cytochrome-c_2 oxidation has a risetime of 1–2 msec; that of cytochrome *b* is several times longer. Both risetimes are several orders of magnitude longer than the duration of the excitation flash.

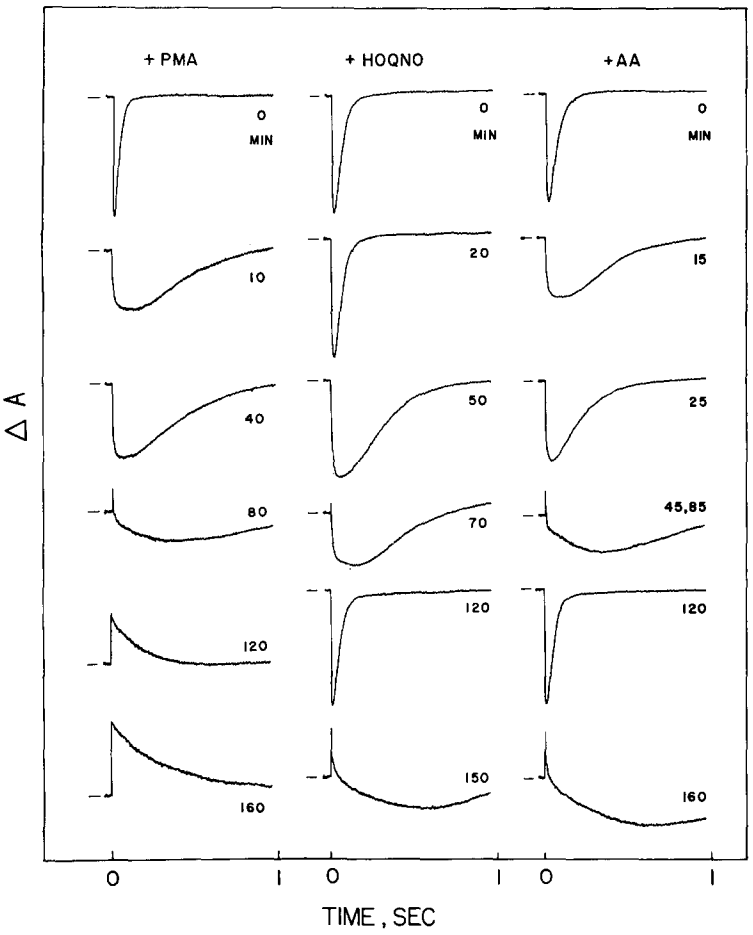


Fig. 10. Light-induced absorption-change transients of *R. rubrum* cells at 420 mμ at different times after addition of phenylmercuriacetate (PMA) (first column); HOQNO (second column); and antimycin A (AA) (third column).

The involvement of both cytochromes in photooxidation was previously reported by DUYSENS[3,18] and by CHANCE[4,19] and their co-workers. The different response of the 420 and 430 mμ absorption changes to light intensity[18] and the biphasic dark decay of the transients in the 420–430 mμ region[19] were cited as evidence for the involvement of both cytochromes. Results from this study are in agreement with these earlier observations. The maximum wavelength in the Soret region of the light-*minus*-dark difference spectrum obtained under steady illumination[3,4,18,19] was previously reported to be near 430 mμ, with only a shoulder at 420 mμ. The apparent difference between the literature values and those observed here may be due partly to the fact that the cytochrome-*b* reaction saturates at a much lower excitation intensity than cytochrome c_2 and partly to the fact of a slower back reaction of cytochrome *b*.

Unique for the cytochrome-*b* transients was the appearance of a sharp spike

in the opposite direction when the excitation intensity exceeded the saturation level for cytochrome-*b* oxidation. Since these spikes were observed mostly in the cytochrome-*b* absorption region, it is possible that they represent cytochrome-*b* photoreduction at these high light intensities. A more plausible alternative is that the sharp positive spike at 428 mμ and the rapidly decaying transient at 438 mμ actually belong to the same reaction. At 428.5 mμ where the absorption decrease due to cytochrome-*b* oxidation was maximum, cancellation by the cytochrome-*b* rise would result in a sharp positive spike. However, such an explanation is not applicable to the spikes observed at other cytochrome-*b* wavelengths (*cf.* Fig. 2).

Contrary to the report that photooxidation of cytochrome c_2 in *R. rubrum* stops at −30° (ref. 9), photooxidation of both cytochromes c_2 and *b* appear to stop near 0° (see Fig. 6). The temperature sensitivity of both the onset and decay of the oxidation reactions of cytochromes further indicates that these are not primary reactions.

The difference spectra at room temperature (Fig. 3) and at liquid-nitrogen temperature (Fig. 7) both show the presence of a broad positive difference band near 430 mμ. This is presumably the same broad positive band, reported previously by many other investigators, which occur whenever photosynthesis is inhibited[18,20].

The broad 430-mμ positive band has been attributed at various times to a cytochrome reaction[4] or to bacteriochlorophyll oxidation[12,17]. OLSON AND KOK[21], and later VREDENBERG AND DUYSENS[9] found that the 430-mμ change was not correlated with those in the infrared region, and the authors concluded that the change was probably due to an unknown pigment and not due to the cytochromes or bacteriochlorophyll. More recently, RUBY, KUNTZ AND CALVIN[22] reported that the 433- and the 865-mμ changes in *R. rubrum* chromatophores have different decay kinetics. Furthermore, they found the 433-mμ absorption-change signal and the ESR signal to have identical kinetics, and concluded that they are produced by the same molecular species.

Although it is not yet possible to determine the nature of the 430-mμ positive changes, several features observed here which the 430 mμ and the infrared changes have in common may be worth mentioning. For instance, both transients have rapid risetimes, namely, $\leqq 50$ μsec, which is the limit of time resolution of the instrument used here. More significantly, both transients can occur at −196° without great change in decay kinetics. At room temperature, the decay times of the 440 and 870 mμ transients are both fast but not identical. At −196°, the decay kinetics of the 870-mμ change were practically unaltered, those of the 430- and 440-mμ changes were slightly slower. Also, the decay times of the 430- and 870-mμ transients are both lengthened in the presence of inhibitors. The similarity between the difference spectrum taken in the presence of inhibitors and at −196° further suggests that these spectral changes most probably originated from the bacteriochlorophyll molecules.

Although cytochrome oxidation was caused by light absorbed by bacteriochlorophyll, evidence for any direct coupling and transfer between it and the cytochromes is still lacking. The results from this study suggest that bacteriochlorophyll photooxidation could be the primary reaction because of (a) its rapid risetime and (b) its independence of temperature. On the other hand, the magnitude of the bacteriochlorophyll-absorption change observed here in fresh anaerobic cells was small and could not account for the amount of cytochrome oxidized if, as often suggested[7], cytochrome and bacteriochlorophyll are tightly bound into a charge-transfer complex

and cytochrome oxidation occurs as a result of an electron transfer to the bacteriochlorophyll. However, the small change in bacteriochlorophyll absorption is consistent with a recent interpretation put forward by CHANCE. By means of laser-flash excitation, CHANCE AND DEVAULT[7] found the risetime for cytochrome c_2 photooxidation in *Chromatium* to be less than 20 μsec, and they concluded that if bacteriochlorophyll is the electron-transfer intermediate, its lifetime must be shorter than 20 μsec and it may not be detectable by the presently available methods. By the same token, the 1–2 msec risetime observed here for cytochrome c_2 oxidation could suggest that an equivalent bacteriochlorophyll absorption change which decays in about 1 msec occurred but was experimentally difficult to detect because of instrument time response and the interference by bacteriochlorophyll fluorescence.

With sufficient incubation, the net effect of the inhibitors appears to be the abolition of the cytochrome and carotenoid reactions and the enhancement of the bacteriochlorophyll changes. In the phenylmercuriacetate case (*cf.* Fig. 10), the intermediate stages may be a combination of the slow disappearance of cytochrome changes and the onset of bacteriochlorophyll changes. The temporal phenomenon and signal reversion observed for HOQNO and antimycin A are far more complicated and puzzling. The intermediate stages and the 120-min reversion occurred only over a very narrow wavelength region centered at 420 mμ. No corresponding changes were observed at other wavelengths such as the α- or β-band region of the cytochromes.

The effects of phenylmercuriacetate on the light-induced absorption-spectrum change reported in the literature have been variable. Among the effects reported are: (a) the appearance of a narrow positive peak at 431 mμ (ref. 4); (b) the appearance of a broad positive peak[20]; and (c) the appearance of a narrow positive peak attributed to the reduction of a *b*-type cytochrome[23]. The temporal dependence of the absorption changes observed here may partly account for the variations reported in the earlier observations.

NISHIMURA[23] reported earlier that HOQNO and antimycin A shift the steady state of cytochromes in *R. rubrum* cells, namely, to an increased oxidation of cytochrome c_2 and an increased reduction of cytochrome *b*. It was also observed that infrared illumination caused a further oxidation of cytochrome c_2 and a further reduction of cytochrome *b* (ref. 23). From the transients shown in Fig. 8 for *R. rubrum* cells in the presence of HOQNO and antimycin A, one could expect a steady-state light-*minus*-dark difference spectrum to mimic an oxidation of cytochrome c_2 and a reduction of cytochrome *b*. But the positive changes at both 550 and 560 mμ rule this out. On the other hand, the truncation of the peaks in the 430- and 560-mμ transients in Fig. 8 in the presence of phenylmercuriacetate, HOQNO, antimycin A, and even PCMB does represent an additional positive absorption change, however small it may be, superimposed on the large absorption changes. These additional small changes are consistent with a reduction of cytochrome *b*. At the presently available resolution, small negative changes at 420 and 550 mμ corresponding to cytochrome c_2 oxidation would not be as easy to decipher from the transients.

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