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Multiple Light-Induced Reactions of Cytochromes *b* and *c* in *Rhodopseudomonas spheroides*

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Illumination of chromatophore preparations from *Rhodopseudomonas spheroides* causes the oxidation of a cytochrome *c* and a slight oxidation of a cytochrome *b* with a maximum at 560nm. When illuminated in the presence of antimycin A the oxidation of cytochrome *c* was more pronounced and cytochrome b_{560} was reduced; the dark oxidation of cytochrome b_{560} was biphasic in the presence of succinate, but not in the presence of NADH, a less effective reductant. Split-beam spectroscopy showed that, in addition to the reduction of cytochrome b_{560}, another pigment with maxima at 565 and 537nm. was reduced and was more rapidly oxidized in the dark than cytochrome b_{560}. This pigment, tentatively identified as cytochrome b_{565}, was also detected in spectra at 77°K, after brief illumination at room temperature; the maxima at 77°K were at 562 and 536nm. In the absence of antimycin A, light caused a transient reduction of cytochrome b_{565} and an oxidation of cytochrome b_{560}. Dark oxidation of b_{565} was rapid, even in the presence of antimycin A and succinate. Difference spectra, at 77°K, of ascorbate-reduced minus succinate-reduced chromatophores or of anaerobic succinate-reduced minus aerobic succinate-reduced chromatophores suggested that two cytochromes *c* were present, with maxima at 547 and 549nm. When chromatophores frozen at 77°K were illuminated both these cytochromes *c* were oxidized, indicating a close association with the photochemical reaction centre. A scheme involving two reaction centres is proposed to explain these results.

The photosynthetic bacteria contain a cytochrome *c* that is photo-oxidized with a high quantum efficiency and that is intimately associated with the photoreaction centre (cf. Chance, De Vault, Hildreth, Parson & Nishimura, 1967). In *Rhodopseudomonas spheroides* the photo-oxidation of cytochrome c_2 is accompanied by the reduction of a *b*-type cytochrome, which is presumably near to the photochemical reducing site (Nishimura, 1963). These two cytochromes may be linked in a cyclic electron flow that can be blocked by antimycin A in a way analogous to its inhibition of mitochondrial electron flow. There is evidence that in the purple photosynthetic bacterium *Rhodospirillum rubrum* two different cytochromes may be photo-oxidized by the activation of two different centres (Sybesma, 1969), and action spectra for light-induced cytochrome oxidation in *Chromatium* (Morita, 1968) indicate that in this purple sulphur bacterium more than one reaction centre is involved in the oxidation of *c*-type cytochromes. The present paper gives evidence suggesting that two *c*-type cytochromes are present in chromatophores of *R. spheroides* and that they may be closely coupled to the reaction-centre chlorophyll.

In the presence of antimycin A a pigment spectroscopically resembling a cytochrome *b* is rapidly reduced in *R. spheroides* chromatophores; by adjusting the experimental conditions a second pigment, provisionally called cytochrome b_{565}, is also reduced. The spectroscopic properties and the kinetics of the light-induced reduction and dark oxidation of these pigments are described in this paper.

MATERIALS AND METHODS

Organisms. The carotenoid-deficient blue–green mutant of *R. spheroides*, strain R22, was a gift from Dr W. R. Sistrom. Cells were grown anaerobically with continuous illumination in the medium of Sistrom (1960) at 30°. Harvested cells were washed twice with 100mM-potassium phosphate buffer, pH 6·8, and were either used immediately or stored as a pellet in the deep-freeze.

Preparation of chromatophores. Cells were suspended in 100mM-tris chloride buffer, pH 7·5, and disrupted by ultrasonic vibration at 0° for 90sec. (Dawes Soniprobe, large probe, 100w output). The extract was centrifuged at 20000*g* for 15min. and the chromatophores were collected from the supernatant by centrifuging at 100000*g* for 60min. The chromatophores were washed once in 100mM-tris chloride buffer, pH 7·5. The bacteriochlorophyll content of the chromatophores was determined from the extinction

at 870nm. by using the extinction coefficient *in vivo* ϵ_{mM} 127 at 870nm. (Clayton, 1963).

Absorption spectra. Difference spectra of chromatophore pigments were obtained at room temperature and at 77°K with a split-beam spectrophotometer (Yang & Legallais, 1954) constructed by Dr P. B. Garland of this Department. The monochromator used (Higler D.400) had a reciprocal dispersion of 1·3nm./mm. slit-width; for room-temperature spectra slit-widths were 1·0mm.; for spectra in liquid N_2 slit-widths were 0·5mm. For light-minus-dark spectra illumination was by means of a 100w quartz–iodine lamp fitted with a Kodak–Wratten 88A filter. The photomultiplier was protected by a filter of saturated $CuSO_4$ solution 1cm. deep.

Dual-wavelength spectrophotometry. A dual-wavelength spectrophotometer (Chance, 1951), adapted for actinic illumination, was used. The 100w quartz–iodine actinic lamp was screened with a Kodak–Wratten 88A filter and the photomultiplier was protected with a 1cm. filter of saturated $CuSO_4$ solution. Changes in transmission were recorded on a Rikadenki potentiometric recorder with a full-scale response time of 1sec.

RESULTS

When succinate is added to a chromatophore suspension there is an immediate change in the absorption spectrum corresponding apparently to the reduction of some cytochromes *b* and *c*. After an interval of 1–2min. a further increase in absorption occurs (Fig. 1) that coincides with the onset of anaerobiosis. Stirring causes the spectrum to revert to its early reduced form, but it rapidly changes back to the anaerobic state. The addition of 2mM-potassium cyanide inhibits this oxidative change, but does not increase the state of reduction of the cytochromes. Illumination of the anaerobic chromatophores causes a slight change in the absorption at 560nm. (attributed to a cytochrome *b*) and a fall at 552nm. (attributed to the oxidation of cytochrome c_2) (Fig. 2*a*). A similar picture was obtained with either succinate or NADH as the electron donor, although the extent of dark reduction of both cytochromes *b* and *c* was significantly higher with succinate. Addition of antimycin A to the anaerobic chromatophores induced more pronounced changes in the cytochromes on illumination (Fig. 2*b*), with reduction of cytochrome *b* and oxidation of cytochrome *c*. There was an increase in absorption, not only at 560nm., but also on the long-wavelength side of the maximum, which suggested that more than one cytochrome was being reduced, the longer-wavelength pigment

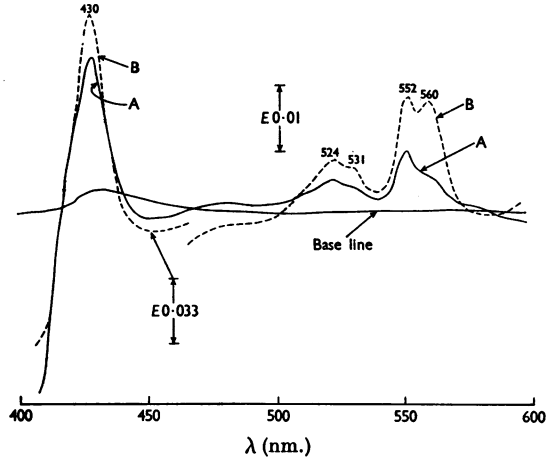


Fig. 1. Effect of the addition of succinate on the absorption spectrum of chromatophores of the blue–green mutant of *R. spheroides*. Chromatophores (12·6μM-bacteriochlorophyll), suspended in 100mM-tris chloride buffer, pH7·5, were placed in both cuvettes of the split-beam spectrophotometer. Succinate (2mM) was added to the test cuvette and the difference spectrum A (—) immediately recorded. Spectrum B (---) was recorded after an interval of 4min., which allowed the oxidative metabolism of succinate by chromatophores to remove the available dissolved oxygen from the test cuvette.

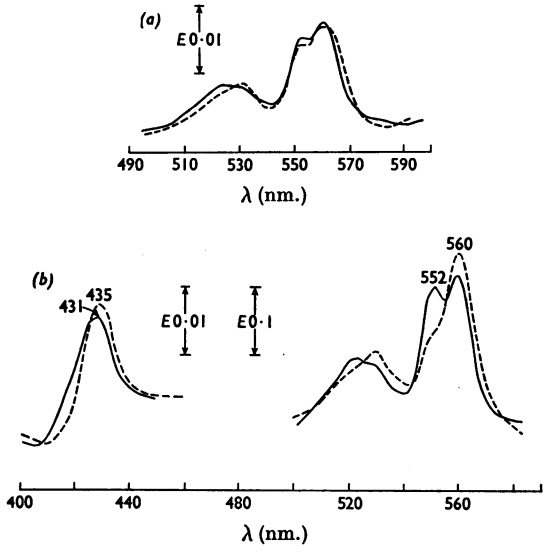


Fig. 2. Effect of illumination on *R. spheroides* chromatophores. (*a*) Chromatophores (12·6μM-bacteriochlorophyll) in 100mM-tris chloride buffer, pH7·5, were placed in the split-beam spectrophotometer and the test sample was allowed to become anaerobic in the presence of succinate, as described for spectrum B in Fig. 1. The test sample was then illuminated with far-red light and the steady-state spectrum recorded. (*b*) Conditions were as described in (*a*) except that 10μl. of antimycin A solution (200μg./ml.) in ethanol was added to the test cuvette. —, Difference spectrum in dark; ---, difference spectrum in light.

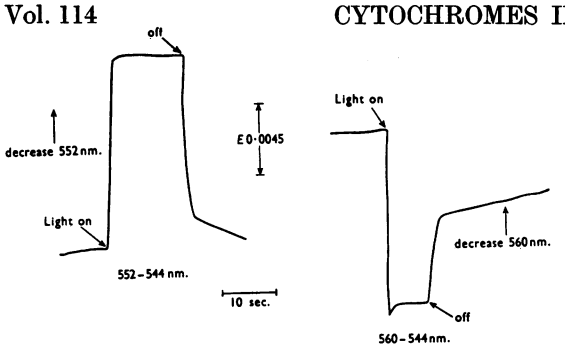


Fig. 3. Effect of illumination of *R. spheroides* chromatophores in the presence of succinate and antimycin A on cytochromes *c* and b_{560}. Measurements were made in a dual-wavelength spectrophotometer. The cuvette contained chromatophores (13 μM-bacteriochlorophyll) in 2·3 ml. of 100 mM-tris chloride buffer, pH 7·5, containing succinate (2 mM) and antimycin A (2 μg.). Cytochrome *c* was measured at 552 minus 554 nm. and cytochrome b_{560} at 560 minus 544 nm. An upward deflexion on the chart indicates a decrease in absorption at 552 or 560 nm. relative to 544 nm., i.e. cytochrome oxidation. Far-red light was switched on and off at the points shown.

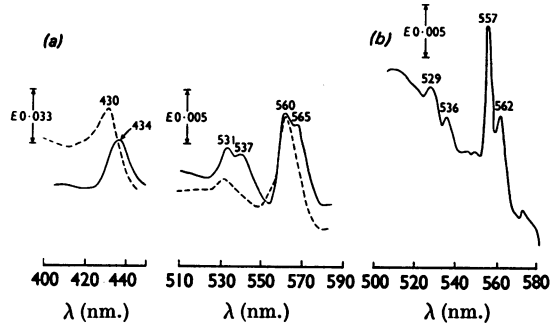


Fig. 4. Demonstration of cytochrome b_{565} in chromatophores of *R. spheroides*. The two cuvettes of the split-beam spectrophotometer contained chromatophores (12 μM-bacteriochlorophyll), succinate (2 mM), antimycin A (2 μg.), ascorbate (2 mM) and 2,6-dichlorophenol-indophenol (10 μM). (*a*) The test sample was illuminated in the split-beam spectrophotometer and the steady-state difference spectrum recorded (—); the light was then switched off and the spectrum immediately recorded (– – –). (*b*) The test sample was illuminated and then both cuvettes, in their special holder, were plunged into liquid nitrogen. The difference spectrum between illuminated and non-illuminated samples was recorded at 77°K.

possibly being cytochromoid *c*. The possibility of the formation of a second reduced carrier was supported by the kinetic experiments shown in Fig. 3. The photo-oxidation of cytochrome c_2 at 552 nm. is very rapid, as is its dark reduction. Although the photoreduction at 560 nm. is rapid and monophasic, the off reaction was biphasic, a rapid phase followed by a much slower oxidation.

Evidence for the participation of two cytochromes b *in electron transport in* R. spheroides. To make the changes in the *b* cytochromes clearer the light-induced oxidation of cytochrome c_2 was diminished by the addition of ascorbate + 2,6-dichlorophenol-indophenol to the cuvettes; this maintains the cytochrome *c* almost fully reduced, even in light. Succinate and antimycin were also added to the cuvette to maintain reduced any succinate dehydrogenase-linked cytochrome *b*. When these chromatophores were illuminated a very striking difference spectrum was obtained (Fig. 4*a*) with twin α peaks at 560 and 565 nm. and with β peaks at 531 and 537 nm. respectively. The trough at 552 nm. suggests that some cytochrome *c* was oxidized. When the light was switched off the 565 nm. peak rapidly disappeared, leaving a normal cytochrome *b* spectrum with a Soret band at 430 nm. (435 nm. in the illuminated state). These same pigments were seen at 77°K. Samples were illuminated at room temperature and then immediately plunged into liquid nitrogen (Fig. 4*b*). It was not essential to have ascorbate + 2,6-dichlorophenol-indophenol in this assay system; the longer-wavelength cytochrome was also observed in its absence. The presence of a β-band indicates that the pigment could not be cytochromoid *c*; the positions of its wavelength maxima suggested that it is probably a cytochrome *b* and it is referred to here as cytochrome b_{565}.

The kinetics of the reduction and oxidation of these cytochromes were followed at the α- and β-peaks of each pigment (Fig. 5); this figure illustrates the rapid oxidation of cytochrome b_{565} and slow oxidation of cytochrome b_{560}. The kinetics of α- and β-peaks changes coincided well and it also appeared that the rapid component of 560 nm. oxidation could be due to some interference from absorption of cytochrome b_{565}, and the slow component of cytochrome b_{565} to interference from cytochrome b_{560}. The difference in reactivity of cytochromes b_{560} and b_{565} could also be shown in uninhibited anaerobic preparations (Fig. 6). Illumination caused an oxidation of cytochrome b_{560}; cytochrome b_{565} was reduced on illumination, but it became more oxidized when the light was switched off.

NADH was less efficient in reducing cytochromes *b* and *c* than was succinate, as judged by an increased reduction obtained when succinate was added to cuvettes after NADH (Fig. 7). When NADH was the electron donor the dark oxidation of cytochrome b_{560} is more nearly monophasic (Fig. 8), suggesting that its slow oxidation, in the presence of succinate when the light is switched off, results from the continuing flow of electrons from succinate tending to reduce cytochrome b_{560} again.

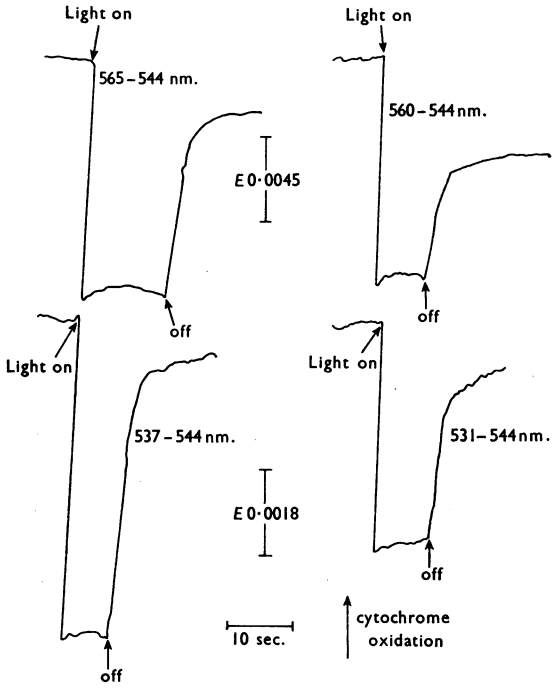


Fig. 5. Comparison of light-induced changes in cytochromes b_{560} and b_{565} measured at the maxima of the α-peaks and the β-peaks. Experimental conditions for the *R. spheroides* chromatophores were as described in Fig. 3.

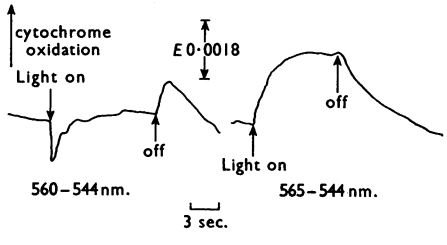


Fig. 6. Light-induced changes of cytochromes b_{560} and b_{565} in illuminated anaerobic chromatophores of *R. spheroides*. The cuvette contained 13 μM-bacteriochlorophyll in 100 mM-tris chloride buffer, pH 7·5, and was allowed to become anaerobic after the addition of 2 mM-succinate. No antimycin was present.

Evidence for the participation of two cytochromes c *in electron transport in* R. spheroides. The difference spectrum of ascorbate-reduced minus succinate-reduced chromatophores (Fig. 9*a*) shows not only two cytochromes *b* but also twin peaks at 547 and 549 nm., probably due to two cytochromes of the

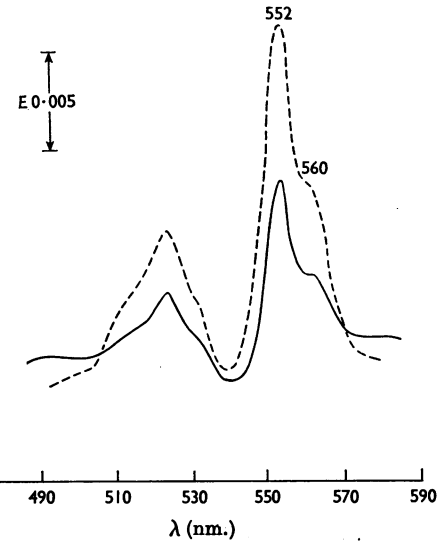
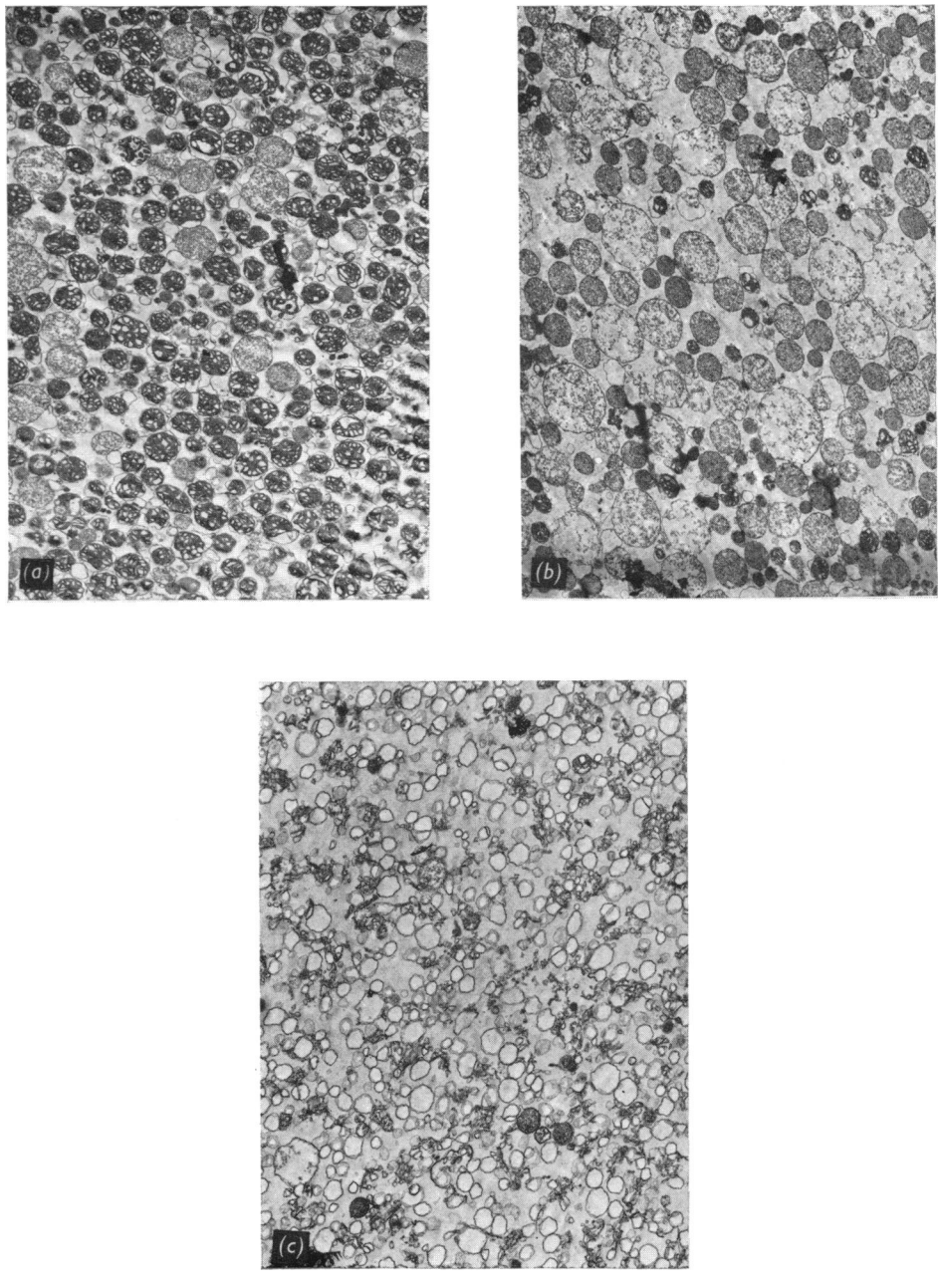


Fig. 7. Effect of NADH and succinate on the absorption spectrum of chromatophores of *R. spheroides*. Chromatophores (12 μM-bacteriochlorophyll) were suspended in 100 mM-tris chloride buffer, pH 7·5, and NADH (0·5 mM) was added to the test cuvette. The NADH caused a reduction of cytochromes that was complete after about 3 min., when the spectrum (—) was recorded. Then 2 mM-succinate was added to the test cuvette and another spectrum recorded (– – –).

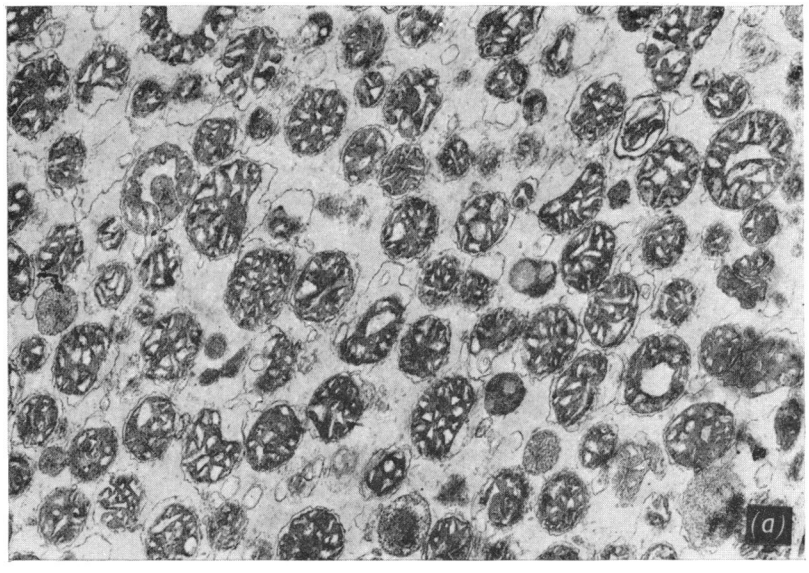
c type, although the major cytochrome *c* absorbs at 549 nm. at 77°K (Fig. 10). The minor component is also readily made apparent in difference spectra between 'aerobic succinate' and 'anaerobic succinate' chromatophores (Fig. 9*b*). In both cases the small twin peaks at 547 and 549 nm. stand out. The relatively small concentrations of both cytochromes c_{547} and b_{562} is shown in $\pm$ dithionite difference spectrum (Fig. 10), where the major cytochrome peaks are at 549 and 558 nm. with only slight shoulders in the regions at 547 and 562 nm.

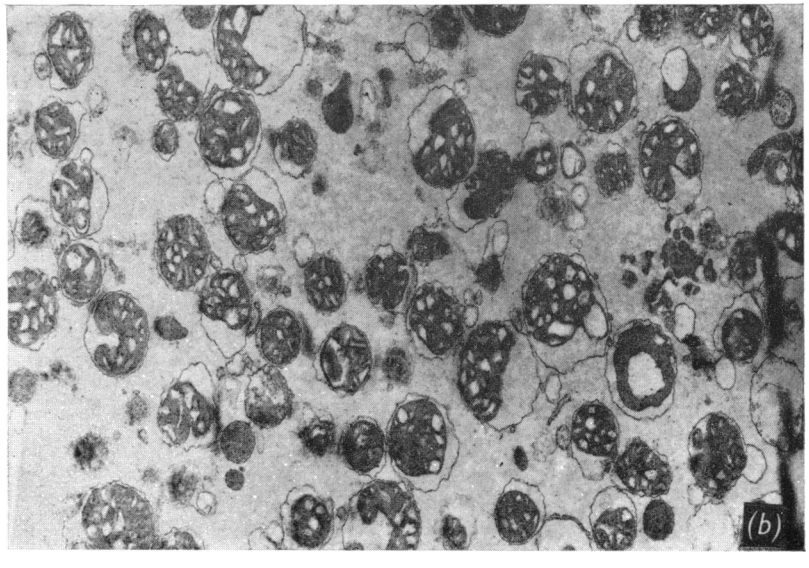
Difference spectra after illumination at low temperatures. In many organisms the oxidation of *c*-type cytochromes is so closely coupled to the reduction of the photo-oxidized reaction-centre chlorophyll that the reaction takes place even at 77°K (Chance *et al.* 1967). Since enzymic re-reduction of the cytochrome c_2 and enzymic changes in other cytochromes *c* would not be possible at this temperature, illumination of *R. spheroides* chromatophores at very low temperatures might isolate the cytochrome *c* that was coupled to the reaction-centre chlorophyll. The results of such an experiment are shown in Fig. 11. The cuvette holder was withdrawn from the Dewar flask containing liquid nitrogen, and one cuvette was illuminated for 5 sec.



EXPLANATION OF PLATE 1

Electron micrographs of thin sections of rat liver mitochondria, after treatment *in vitro* with (*a*) propylene glycol, (*b*) NFCH and (*c*) OFCH. Experimental details are given in the text. Magnification × 12000.





EXPLANATION OF PLATE 2

Electron micrographs of thin sections of rat liver mitochondria, after treatment *in vivo* with (*a*) NFCH and (*b*) OFCH. Experimental details are given in the text. Magnification × 20000.

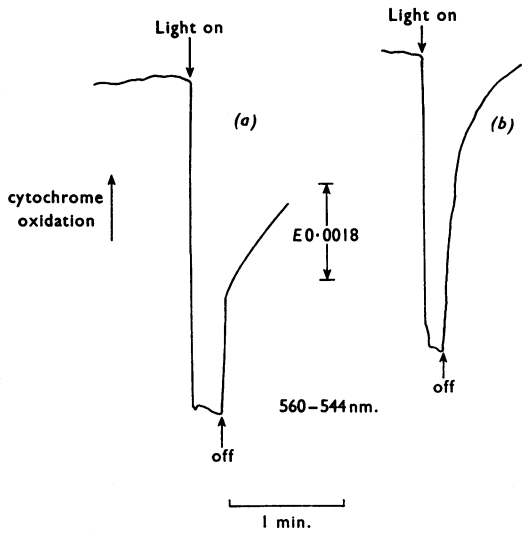


Fig. 8. Comparison of the kinetics of light-induced reduction and dark oxidation of cytochrome b_{560} of *R. spheroides* chromatophores when succinate or NADH was present in the cuvette. The cuvette contained chromatophores (13 μM-bacteriochlorophyll) in 2·3 ml. of 100 mM-tris chloride buffer, pH 7·5, containing antimycin A (2 μg.). (*a*) 2 mM-Succinate present; (*b*) 0·5 mM-NADH present.

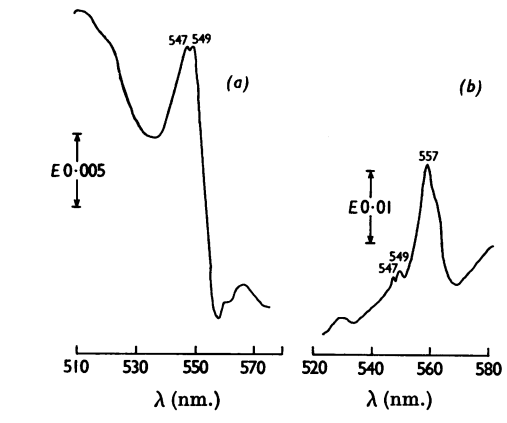


Fig. 9. Detection of two cytochrome *c* peaks in *R. spheroides* difference spectra measured at 77°K. Both cuvettes contained chromatophores (12 μM-bacteriochlorophyll) in 50 mM-tris chloride buffer, pH 7·5, containing glycerol (50%, v/v). (*a*) To the test cuvette ascorbate (1 mM) was added and to the other cuvette succinate (2 mM) was added. After sufficient time had been allowed for the chromatophores to become anaerobic, the cuvettes were plunged into liquid nitrogen and the spectrum was recorded at 77°K. (*b*) The test cuvette was allowed to become anaerobic after the addition of 2 mM-succinate and then cuvettes were frozen immediately after the addition of 2 mM-succinate to the blank cuvette. The difference spectrum was recorded at 77°K.

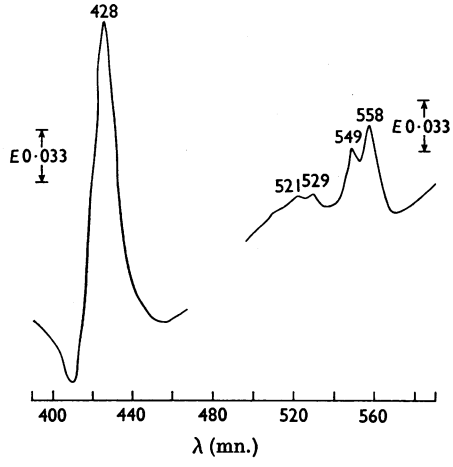


Fig. 10. Difference spectrum of *R. spheroides* chromatophores at 77°K after the addition of dithionite. Chromatophores (13 μM-bacteriochlorophyll) were suspended in the two cuvettes of the split-beam spectrophotometer in 50 mM-tris chloride buffer, pH 7·5, containing glycerol (50%, v/v). A few crystals of dithionite were added to the test cuvette and the samples frozen in liquid nitrogen. Spectra were recorded at 77°K.

with red light and replaced in the Dewar flask for spectroscopy. Some temperature rise would obviously take place during this time, but there was little or no change in the physical appearance of the frozen samples. The major spectroscopic shift caused by illumination was an absorption decrease in the region of cytochrome *c* absorption, with double maxima at 547 and 549 nm.

DISCUSSION

The purple non-sulphur photosynthetic bacterium *Rsp. rubrum* can be grown aerobically non-photosynthetically, and the terminal oxidase has many of the spectroscopic properties of a *b*-type cytochrome (Taniguchi & Kamen, 1965). The oxidase system of *R. spheroides* studied by Chance, Horio, Kamen & Taniguchi (1966) was shown by kinetic studies not to be cytochromoid *c*, and the position of its absorption maximum at 428 nm. suggested the possibility that it might be a cytochrome *b*. The difference spectra (Fig. 4) suggest that the pigment absorbing at 565 nm. is a cytochrome and not a cytochromoid. It has a well-defined β-peak at 537 nm. and a Soret band at 435 nm. These positions would fit most closely with a cytochrome of the *b* type. The position of the Soret band makes it unlikely that this is the oxidase described by Chance *et al.* (1966).

When the oxidation of the cytochromes *b* was

inhibited by the addition of antimycin, the oxidation of cytochrome b_{560} was slower than that of cytochrome b_{565} when succinate was present (Fig. 5), but not as markedly when the less-efficient electron donor NADH was present. This suggests that cytochrome b_{560} may be more directly linked to succinate dehydrogenase. The time-response of the equipment used did not permit any decision to be made about the relative rates of reduction of the two cytochromes *b* on illumination.

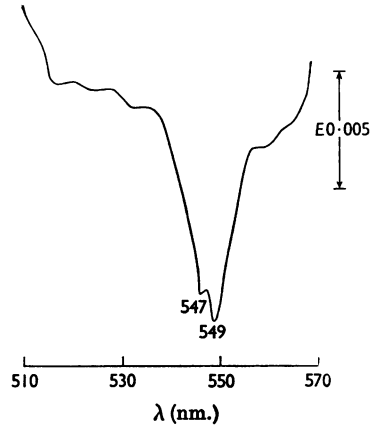
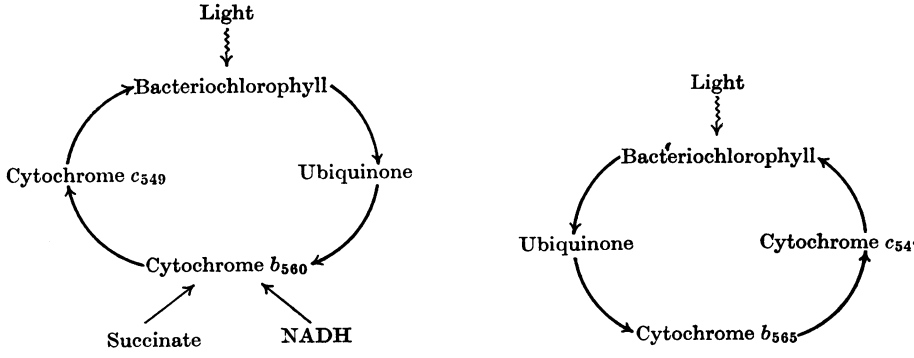


Fig. 11. Effect of illumination of chromatophores of *R. spheroides* at the temperature of liquid nitrogen. The two low-temperature cuvettes of the split-beam spectrophotometer were filled with a suspension of chromatophores (12 μM-bacteriochlorophyll) in 50 mM-tris chloride buffer, pH 7·5, containing glycerol (50%, v/v). Ascorbate (1·0 mM) and 2,6-dichlorophenol-indophenol (20 μM) were added to both cuvettes, which were then frozen in liquid nitrogen. The cuvettes were withdrawn and the test cuvette was illuminated for 5 sec. with red light. The samples were returned to liquid nitrogen and the difference spectrum was recorded.

Difference spectra at liquid-nitrogen temperature (e.g. Fig. 4*b*) showed two peaks, at 547 and 549 nm., both of which may be due to cytochromes of the *c* type. Both peaks could be seen at 77°K in difference spectra between anaerobic succinate-reduced and aerobic succinate-reduced cells. The α-peak of cytochrome *c* in dithionite-reduced chromatophores is at 549 nm., with a very slight shoulder at shorter wavelength (Fig. 10). Since these two pigments, both tentatively identified as cytochromes *c*, react at temperatures approaching 77°K they must both be associated with the primary reaction centres, and since two different cytochromes *c* appear to be involved there may be two types of reaction centre. Sybesma (1969) has evidence for two such centres in the related organism *Rsp. rubrum*. One reaction centre contains the special bacteriochlorophyll P800 molecules in association with bacteriochlorophyll P890; the other centre contains bacteriochlorophyll P890 molecules unrelated to bacteriochlorophyll P800. Each reaction centre has its own electron-transport chain. Similar schemes have been proposed for the purple sulphur bacterium *Chromatium* (Cusanovich, Bartsch & Kamen, 1968), and in each case it has been suggested that one reaction centre activates cyclic photophosphorylation and the other drives electron flow to reduced nicotinamide nucleotide. In *R. spheroides* NADH is formed on illumination of whole cells, after a slight lag, and its formation is abolished by the addition of uncouplers or 2-heptyl-4-hydroxyquinoline *N*-oxide (O. T. G. Jones, unpublished work). This means that in this organism NADH is formed from succinate by reversed electron flow driven by ATP produced from cyclic photophosphorylation, as has already been shown in *Rsp. rubrum* (Keister & Yike, 1967; Jackson & Crofts, 1968). Such a mechanism requires that, if only one ATP is formed in cyclic electron flow, then, in the extreme case, when NADH is being produced there



Scheme 1. Scheme for photosynthetic electron flow in chromatophores of *R. spheroides*.

would be no ATP available for reactions of carbon dioxide fixation or for other synthetic processes. A second reaction centre, perhaps concerned only with cyclic photophosphorylation, would increase the efficiency of the process. A scheme that would fit the experimental results described in this paper is given in Scheme 1. Cytochrome c_{547} is placed in the cyclic scheme because it tends to be less readily reduced by succinate than is cytochrome c_{549} (it is readily observed in anaerobic succinate-reduced minus aerobic succinate-reduced spectra). Cytochromoid *c* is not included in this scheme only because no spectroscopic shifts in the appropriate spectral regions were detected (*R. spheroides* chromatophores differ markedly from *Rsp. rubrum* chromatophores in this respect). Ubiquinone is placed on the scheme as an early electron acceptor as a consequence of the kinetic measurements of Vernon and his associates that have been reviewed by Vernon (1968). The potential drop from ubiquinone (+0·098v; Moret, Pinamonti & Fornasari, 1961) to cytochrome *c* (about +0·3v in the photosynthetic bacteria examined by Kamen & Vernon, 1955) seems sufficient only for one phosphorylation step and makes the availability of two reaction centres reasonable. Clayton has described two major changes in bacteriochlorophyll of *R. spheroides* on illumination: a bleaching at 870nm. (bacteriochlorophyll P870) and a blue shift in a component absorbing at 805nm. (bacteriochlorophyll P800), and has discussed the possibility that these are due to two different chlorophyll centres (cf. Clayton, 1967) that are closely associated. The absorption changes in the two similar centres in *Rsp. rubrum* were found to have different decay rates (Kuntz, Loach & Calvin, 1964).

In summary it can be stated that the results described in this paper would be consistent with a scheme involving two reaction centres. Some association between these centres, possibly at the level of ubiquinone or cytochrome *b*, occurs, since all pigments are reduced to a greater or smaller extent on the addition of succinate in aerobic conditions.

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