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STUDIES ON BACTERIAL CHROMATOPHORES

II. ENERGY TRANSFER AND PHOTOOXIDATIVE BLEACHING OF BACTERIOCHLOROPHYLL IN RELATION TO STRUCTURE IN NORMAL AND CAROTENOID-DEPLETED *CHROMATIUM*

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SUMMARY

Electronic energy transfer, fluorescence emission spectra and photooxidative bleaching of bacteriochlorophyll in *Chromatium* chromatophores were shown to be affected by detergent action and by inhibition of normal carotenoid synthesis in the parent cells. These phenomena are discussed in relation to the structure of the chromatophore and the state of bacteriochlorophyll *in vivo*. A structural role for carotenoids is suggested.

INTRODUCTION

In the pigment-carrying chromatophores of the photosynthetic purple bacterium ***Chromatium*** bacteriochlorophyll occurs in three different "types" which, according to the position of their absorption maxima in the near infrared, are called B_{800}, B_{850} and B_{890}, respectively.

As was shown by DUYSENS[1], light energy absorbed by B_{800} and B_{850} is transferred by inductive resonance to B_{890}, which seems to be the photosynthetically active pigment system.

Deoxycholate induces changes in the fluorescence pattern of isolated chromatophores, indicative of a disturbance of energy transfer from B_{850} to B_{890}. The detergent, however, does not seem to affect the mutual spatial arrangement of B_{890} and B_{850} (see ref. 2).

In the present paper observations are presented which may permit greater insight into the nature of the bacteriochlorophyll types. Thus, experiments with chromatophores lacking normal carotenoids have revealed that B_{890} and B_{850} are also separable in space. Another consequence of the disorganization of the chromatophoral structure by detergent action, namely, an increased sensitivity of bacteriochlorophyll towards photooxidation, was investigated.

METHODS AND MATERIALS

Chromatium strain D was grown in a malate–thiosulfate medium. For inhibition of carotenoid synthesis, the organisms were cultured and subcultured in the presence of $7 \cdot 10^{-5}$ *M* diphenylamine. This resulted in a markedly retarded growth rate of the organisms, which were blue-green in appearance. Chromatophores from normal red and blue-green cells were prepared as described previously[2].

All preparations used in the experiments were adjusted to an absorbancy of 0.9–1 (1-cm path length) at 800 mμ. Detergent treatment consisted of suspending the chromatophores in a buffered 0.25 *M* KCl medium containing 0.25% deoxycholate. Throughout the experiments 0.05 *M* Tris buffer (pH 8) was used.

Photobleaching was performed by exposing 5-ml samples in test tubes, immersed in a waterbath at 18°, to a 150-W reflector-type incandescent lamp. Light intensity at the level of the tubes was approx. 500 foot candles.

Absorption spectra were recorded with a Beckman DK_2 spectrophotometer. Fluorescence was excited by the 366-mμ mercury line. Emission spectra were obtained by using a Bausch and Lomb grating monochromator and a liquid air-cooled far red-sensitive DuMont 6911 multiplier. The spectra are corrected for grating transmission and photodetector response.

Crystalline horse-heart cytochrome *c* and DPNH were obtained from Sigma Chemical Corporation, and deoxycholate, succinate and diphenylamine from British Drug Houses.

RESULTS AND INTERPRETATIONS

Spectral characteristics of blue-green chromatophores

Although the blue-green chromatophores exhibit the same variability in the relative intensities of the bacteriochlorophyll absorption bands as the normal red ones, their infrared spectra are essentially similar. The shoulder at 890 mμ, however, is usually less pronounced in the former (Fig. 1). Absorption in the 400–550-mμ region demonstrates the absence of normal coloured carotenoids. These findings are in

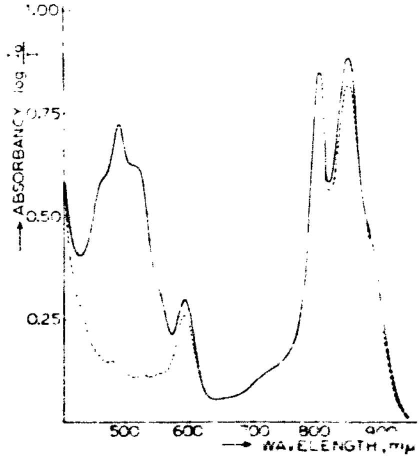

Fig. 1. Absorption spectra of red (solid line) and blue-green (dotted line) chromatophores.

contrast with those of BERGERON AND FULLER[3], who observed a correlation between carotenoid content and the shape of the infrared spectra of *Chromatium*. From this correlation, the authors concluded that in this organism the complex structure of the absorption spectrum is determined by bacteriochlorophyll–carotenoid interactions. A similar suggestion was made by STANIER *et al.*[4] on account of spectral changes observed in a carotenoid-less mutant of *Rhodopseudomonas spheroides*. The absence of any correlation between carotenoid deficiency and the near infrared bacteriochlorophyll absorption spectrum of blue-green *Chromatium* grown in our laboratory, indicates that such interactions are not likely to contribute to the infrared spectrum of this species.

These divergent experimental results are not readily explained. The difference might be due to the use of different strains. In this connection, it should be emphasized that all results presented in this paper apply to *Chromatium* only. Experiments with *Rhodopseudomonas spheroides* revealed that chromatophores isolated from this organism differ markedly from those of *Chromatium* with respect to photobleaching and susceptibility to detergent action, strongly indicating differences in architecture of the respective organelles. It seems reasonable to assume that even within one single species the chromatophoral structure might be dependent on such factors as growth conditions and age of the parent cells. This assumption is supported by electron microscopic observations[5].

Photooxidation of bacteriochlorophyll

Red chromatophores: Upon illumination of a suspension of the particles, a slow bleaching of bacteriochlorophyll occurs. As both light and oxygen are necessary, this bleaching is of a photooxidative nature.

The following course of events is observed. From the start of illumination, there is a steady decrease of the 850-mμ peak, with the ultimate disappearance of B_{850} absorption at this wavelength. During this period no destruction of bacteriochloro-

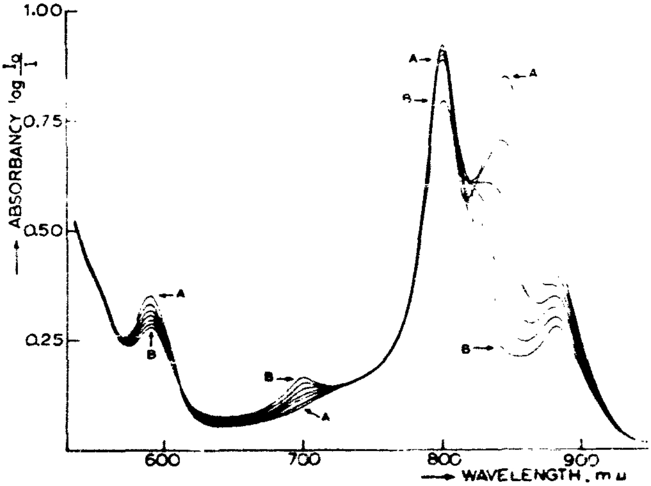

Fig. 2. Photobleaching of red chromatophores in a 0.25% deoxycholate–0.25 *M* KCl medium. The arrows A and B mark some spectral positions before illumination and after an illumination period of 6 h, respectively. The other spectra were measured at hourly intervals.

phyll, responsible for absorption at 800 mμ, takes place. On the contrary, some increase in absorption may be observed. As soon as the B_{850} absorption has disappeared, however, the 800-mμ peak in turn begins to decrease and may vanish upon prolonged illumination. The bacteriochlorophyll type absorbing around 890 mμ, on the other hand, shows a remarkable photostability, as it is not readily bleached at the light intensity used. The shoulder at 890 mμ eventually emerges as a separate peak. This relative photostability is an interesting characteristic of B_{890} in *Chromatium*, which might be due to a special pigment – protein linkage or to an association with some other molecule, *e.g.* cytochrome[6]. These bleaching phenomena in the near infrared are illustrated in Fig. 2. As each of the bacteriochlorophyll-types contributes to the absorption band at 590 mμ (see ref. 1), a gradual decrease of absorption at this wavelength is expected and observed. The increase of absorption around 700 mμ indicates oxidation of pigment to some product absorbing at this wavelength.

Photobleaching is dependent on temperature and light intensity, but not on other external factors, such as pH (from 6 to 9), ionic strength or osmolarity of the medium. Though there is no change in the sequence of bleaching events, the presence of deoxycholate results in an increased rate of photobleaching. Arrows B in Fig. 2 mark the absorption spectrum of red chromatophores after an illumination period of 6 h in the presence of the detergent. To reach the same stage of oxidation in its absence, about 30 h are needed.

Some additonal observations may be mentioned here. During the bleaching of bacteriochlorophyll, at least in the early stages, there is no evidence for carotenoid destruction. The carotenoid spectrum remains intact; no changes in peak height ratios or shifts of the absorption bands could be seen. This observation, too, does not support bacteriochlorophyll – carotenoid interactions.

Further, a rapid quenching of fluorescence precedes bleaching. The fluorescence intensity may drop considerably before measurable bleaching takes place. This phenomenon is also dependent on light and highly sensitive to traces of oxygen.

Though the sequence of bleaching events is always the same, the rate of bleaching

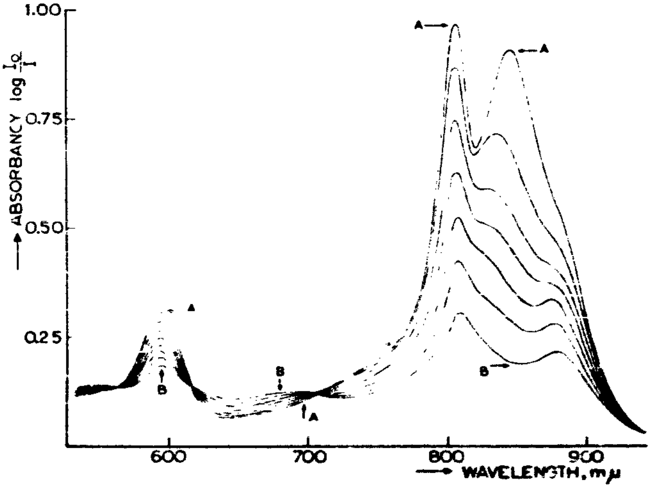

Fig. 3. Photobleaching of blue-green chromatophres in a 0.25% deoxycholate–0.25 *M* KCl medium. For other details, see legend Fig. 2.

may vary from one preparation to another. It was found that bacteriochlorophyll in freshly prepared chromatophores is more sensitive towards photooxidation, than in chromatophores, which have been stored for some days in the refrigerator. This observation is believed to be due to the formation of protective agent(s), such as hydrogen sulfide.

Blue-green chromatophores: The behaviour of the bacteriochlorophyll types in these particles towards photobleaching deviates from that in the red ones in one notable respect. As may be seen from Fig. 3, from the start of illumination there is a decrease of *both* the 800 and 850-mμ peaks. B_{890}, however, has retained its relative photostability. Here also, deoxycholate affects the rate of photobleaching without changing the bleaching pattern.

It cannot be stated that bacteriochlorophyll in the blue-green chromatophores shows an increased photosensitivity compared with that in the red ones in terms of markedly increased rates of photooxidation of the different pigmenttypes, but considerably larger amounts of pigment are photooxidized in a given time.

The absence of increased absorption around 700 mμ indicates that the oxidative process differs from the one occurring in the red chromatophores.

Photobleaching can be observed in intact red and blue-green cells as well. Light-activated pigment destruction, therefore, is not a consequence of some damage to the chromatophoral structure, caused by isolation.

Interpretation of the bleaching phenomena

The following interpretation of the observed bleaching phenomena is suggested. In the chromatophores, bacteriochlorophyll molecules in the excited state may undergo one of various competing processes, three of which are relevant here: (a) their excitation energy may be transferred to other pigment molecules by inductive resonance, (b) they may emit their energy as fluorescence, measurable under suitable conditions, *e.g.* anaerobiosis or in the presence of an antioxidant as described below, (c) their energy may be consumed in a photochemical reaction with molecular oxygen.

Apart from other requirements, the efficiency of energy transfer between molecules by the mechanism of inductive resonance is inversely proportional to the sixth power of their distance[7]. It thus may be imagined that in the normal red chromatophore the average distance between B_{800} and B_{850} is such, that the efficiency of transfer between these types approaches 100%. The spatial positioning of B_{850} and B_{890}, however, would not fully favour this process. Consequently, there is a fair chance for the excited B_{850} molecules either to fluoresce or to react with oxygen, the latter process resulting in an oxidative descruction of the B_{850} molecules. As soon as all, or nearly all, B_{850} molecules have been oxidized and B_{800} can no longer pass on its excitation energy, selfdestruction of these molecules sets in. It is interesting to note that the transfer mechanism, apart from its apparent physiological significance, confers a considerable protection from photooxidation on bacteriochlorophyll in *Chromatium* chromatophores, as the major part of the absorbed energy is transferred to the photochemically stable B_{890} molecules.

The situation in blue-green chromatophores is similar except that here, because of a structural derangement, energy transfer from B_{800} to B_{850} also is not fully effective. Hence B_{800} in these particles is liable to direct photodestruction.

The mechanism of detergent action, resulting in increased rates of photobleaching, is thought to be due to a separation in space of the energetically linked pigment molecules, thus inhibiting the process of energy transfer. As has been shown earlier[2], increasing deoxycholate concentrations result in increasing B_{850} fluorescence, until a constant level is reached. Apparently, the efficiency of energy transfer decreases as the spatial separation of B_{850} and B_{890} proceeds, until distances are too great for the transfer process to take place.

It might be that the detergent acts through some other mechanism, *e.g.* by affecting the mutual orientation of the pigment molecules or their physico-chemical state. The latter possibility might involve a disruption of the pigment–protein linkage or a change in the tertiary structure of the carrier protein. However, several observations, such as the reversibility of the detergent action[2], the unchanged absorption spectra of treated particles and the approximate position of the evoked fluorescence emission bands, render such mechanisms less likely.

Bacteriochlorophyll in the chromatophore can be oxidized chemically in the dark. Controlled oxidation with ferricyanide showed that B_{850} is the most sensitive pigment system, while B_{890} is relatively stable. Here also, the oxidation rate is increased by detergent action. Thus a not unexpected consequence of the structural impairment induced by the detergent seems to be an "exposure" of the pigment molecules, rendering them more readily accessible to oxidants.

Fluorescence emission spectra

Substantiation for the conclusions arrived at in the previous section has been obtained by fluorescence measurements. Disorganization of the chromatophoral structure, caused by detergent or otherwise, might be expected to result in changes of the fluorescence spectra, as has been discussed previously[2].

The emission spectrum of normal intact chromatophores shows a nearly symmetrical band with a maximum around 910 mμ. The assumption that energy transfer

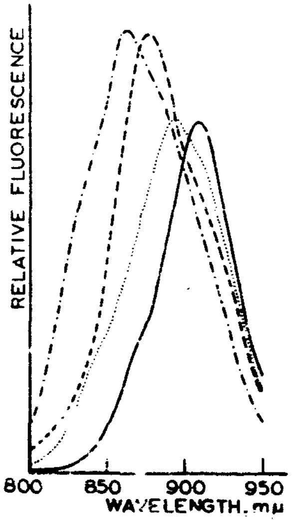

Fig. 4. Fluorescence spectra of chromatophores. ———, red chromatophores; – – – – –, detergent-treated red chromatophores; . . . . ., blue-green chromatophores; –·–·–·–, detergent-treated blue-green chromatophores.

between B_{850} and B_{890} is not fully effective, is confirmed by the finding that a contribution of B_{850} fluorescence consistenly appears as a slight shoulder on the short-wave side of the emission band (Fig. 4). In the presence of deoxycholate, the emission spectrum is considerably changed. The main peak shifts from 910 to 880 mμ. Apparently, under this condition B_{850} fluoresces strongly and its emission band overlaps the reduced B_{890} emission band, now only recognizable as a shoulder. It is interesting to note that the area covered by the emission spectrum of the particles is increased after detergent treatment. This suggests that the lifetime of excited B_{850} molecules is longer than that of excited B_{890} molecules.

In blue-green chromatophores the emission spectrum is clearly asymmetric. This indicates, as might have been expected from the bleaching experiments, that this spectrum is composed of three overlapping bands emitted by each of the pigment types. As transfer between all types is strongly inhibited by deoxycholate, treatment of the blue-green particles with the detergent changes the relative contributions of each type to the emission spectrum. Particularly the relatively high fluorescence intensities observed at wavelengths shorter than 850 mμ are indicative of direct B_{800} fluorescence. These results are summarized schematically in Fig. 5. It has already been mentioned that rapid quenching of fluorescence occurs upon aerobic illumination. As will be seen in the next section, with red particles this quenching is fully prevented by the addition of suitable antioxidants. Since blue-green chromatophores are not so readily protected in this way, the observed fluorescence spectra are slightly distorted by quencing effects, which have been shown to occur during measurements. The results, however, satisfactorily support the suggestions made.

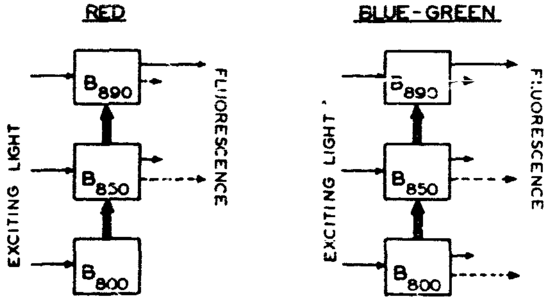

Fig. 5. Energy transfer and fluorescence in red and blue-green chromatophores. Vertical arrows indicate energy transfer between the different bacteriochlorophyll types. Shaded arrows denote interference with transfer by detergent action. Horizontal arrows on the right indicate fluorescence before (full arrows) and after (broken arrows) detergent treatment. The lengths of the arrows are not indicative of actual relative fluorescence intensities, but merely reflect reduction or enhancement of fluorescence of each bacteriochlorophyll type.

Protection against photooxidation and fluorescence quenching

Although all operations involving the isolation of the chromatophores and their subsequent handling were carried out in subdued light, it was desirable to prevent the light-induced effects. This was readily accomplished, both in normal intact and detergent-treated red particles, by the addition of suitable antioxidants, sulfhydryl compounds and ascorbic acid being highly effective (Table I).

When the substance was added to the chromatophore suspension prior to il-

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lumination, neither bleaching nor quenching occurred. Addition of the antioxidant after partial bleaching or quenching has taken place, merely stops the effects produced by light and oxygen. It would appear, therefore, that these effects are irreversible phenomena and that the substances do not protect by reducing oxidized bacteriochlorophyll, but rather by successfully competing with the pigment for oxygen. The antioxidant, then, is oxidized instead of bacteriochlorophyll itself.

The mechanism of sensitized oxidations involving molecular oxygen is not well understood. It is generally assumed, that the excited sensitizer combines with oxygen, probably via the triplet state. All kinds of oxidizable substrates may then react with this "oxy-compound", thereby being oxidized and regenerating the sensitizer in the ground state. In the absence of a suitable substrate, the sensitizer itself might be oxidized.

TABLE I

PREVENTION OF FLUORESCENCE-QUENCHING IN RED CHROMATOPHORES

Additions are given in μmoles (mg for cytochrome *c*) per 5 ml total volume. Fluorescence was measured at wavelengths exceeding 900 mμ. Illumination period, 30 min.

Additions	*Relative fluorescence intensities*
None (dark control)	147
None	26
Cysteine (5)	153
Ascorbic acid (10)	144
Succinate (1.5)	31
DPNH (1.5)	25
Cytochrome *c* (0.5)	29
Cytochrome *c* (0.5) + succinate (1.5)	149
Cytochrome *c* (0.5) + DPNH (1.5)	154

The light-induced effects are also prevented in blue-green chromatophores in this way, but not so readily as in the red ones, even in the presence of excessive amounts of the antioxidants. The reason for this is not clear, but might be connected with the observation that the oxidative destruction of bacteriochlorophyll probably proceeds differently in the two kinds of chromatophores.

Apparently, bacteriochlorophyll in *Chromatium* is effectively protected *in vivo*, if involved in photosensitized oxidations. This statement is illustrated by the photosensitized oxidation of cytochrome *c*. Purple bacteria and their isolated chromatophores are capable of carrying out a light-activated oxidation of exogenous ferrocytochrome *c* by molecular oxygen[8]. It was found that cytochrome *c* was indeed an excellent protective substance, if added together with succinate or DPNH (Table I). Dark reduction of the cytochrome through the chromatophoral succinic dehydrogenase or DPNH–cytochrome *c* reductase and its subsequent sensitized oxidation in the light can be demonstrated by the characteristic absorption changes at 550 mμ.

The physiological significance of such a protection by oxidizable substrates, which may be products or intermediates of normal metabolism, is not evident for obligate anaerobes. The conclusion of DWORKIN[9] that the temporary desensitization of carotenoid-less *Rhodopseudomonas spheroides* acquired upon prior dark aerobic growth is to be ascribed to the synthesis of a photochemical buffer during this period, however, is in accordance with these findings.

DISCUSSION

The described results might bear upon the state of bacteriochlorophyll *in vivo*. The considerable differences in spectral (red shift) and photochemical properties of chlorophyllous pigments, *in vitro* and *in vivo*, indicate some organized state of the pigments in the intact cell. It has been suggested, that the spectral shift might arise from electronic interactions of closely spaced molecules. Shifts of the absorption band of chlorophyllous pigments indeed accompany the transition of monomolecularly dispersed molecules in solution to a variety of condensed states, *e.g.* colloidal suspensions, monolayers, microcrystals, etc.[10,11], the peak position depending on the kind of association and the density of packing of the molecules. As in purple bacteria there is but a single bacteriochlorophyll pigment, KRASNOVSKY *et al.* were led to suggest that different aggregated forms of the pigment are responsible for the complex character of their absorption spectrum[12].

An alternative explanation for the change of energy levels of bacteriochlorophyll *in vivo* is based on the effect of conjugation of the pigment with some chromatophoral macromolecule, probably a protein, as suggested by WASSINK *et al.*[13]. Dyes bound to high-polymer substances differ considerably, both spectrally and photochemically, from the free dye in solution[14]. In addition, a chlorophyll derivative in the bound state has been found to be fluorescent[15], in contrast to the various aggregated forms of the pigment *in vitro* mentioned above. The observations with these model systems of bound dyes and pigment render the suggestion of the occurrence of bacteriochlorophyll–protein complexes rather attractive. Our results show that the three pigment types apparently exist *in situ* as definite entities, separable in space without spectral changes and each one with an intrinsic capacity for fluorescence. The peak position of each type, then, is supposed to be determined by different pigment–protein linkages. Taken together with the individual and different behaviour of each type towards pH (see ref. 16), heat[1] and oxidation, the results incline us to prefer this picture of the state of bacteriochloropyhll *in vivo* to others, based on pigment–pigment interactions exclusively. A contribution of such interactions, however, to the spectral shift of each type is not excluded. The geometrical distribution and arrangement of the three types in two-dimensional lamellae, in such a way that efficient energy transfer may take place cannot readily be visualized. An answer to these problems must await the actual isolation of the complexes, which so far has been unsuccessful with *Chromatium*. The complex responsible for the 890-mμ absorption in chromatophores of *Rhodopseudomonas spheroides* could be sedimented selectively in the ultracentrifuge after detergent treatment[17]. Unfortunately, further purification and characterization of the complex failed, as the material proved to be highly unstable.

The aberrant behaviour of blue-green chromatophores accentuates the significance of the carotenoids. A satisfactory explanation for the universal occurrence of these pigments in photosynthetic structures has not yet been given. Though it has been well established that carotenoids contribute to photosynthesis by transferring their absorbed energy more or less efficiently to chlorophyll[1], it is doubtful whether this is an essential function. The actual participation of carotenoids in photosynthetic processes as electron or oxygen carriers has not been supported by experimental evidence so far.

Still another function, namely, as protective agents against photodestruction and photokilling, was proposed by STANIER *et al.* as a result of their investigations with a blue-green mutant of *Rhodopseudomonas spheroides*[4,18]. The mechanism of this protective action of the carotenoids has not been established, though it does not seem likely that it is effected by actual chemical participation of the pigments, as suggested by these authors. In this context it is tempting to speculate on the possibility that the observed protective action might be related to the capacity of carotenoids to undergo light-induced *cis–trans* isomerization. Recently it has been found that the stereoconfiguration of polyenes might be affected by chlorophyll-absorbed light[19]. Changes in the configuration of chromatophoral carotenoids might result in structural changes of the particle. It is suggested that such light-induced changes are essential for effective energy transfer and for the protection of sensitive sites from damaging photooxidations. When the normal carotenoids are replaced by less conjugated precursors through inhibition of their synthesis by diphenylamine[20], this mechanism would no longer be effective. The changes in the carotenoid spectrum observed upon short-time illumination of purple bacteria[21,22], should then be ascribed to stereoconfigurational changes, rather than to redox reactions. The light-induced isomerization of the carotenoids should be reversible by dark (enzymic) reaction(s), allowing oxygen to enter freely into the chromatophore and thus enabling those cells which are not strict anaerobes to switch over from photometabolism to respiratory metabolism in the dark. This suggestion for the mechanism of the protective action of carotenoids, which might apply to oxygen-evolving photosynthetic structures as well, is highly speculative. It seems justified to state, however, that normal functioning of the chromatophore and its protection against photooxidations are intimately coupled and depend on an intact chromatophoral structure of which the carotenoids are essential constituents. Photodestruction and photokilling, observed in cells lacking normal carotenoids, should then be regarded as secondary manifestations of a deranged structure of the photosynthetic apparatus.

The retarded growth of *Chromatium* in the presence of diphenylamine indicates an impairment of the photosynthetic machinery. The pigments are no longer efficiently linked energetically and consequently there will be a decreased supply of quanta of the B_{890} molecules to be used in photosynthesis. Besides, any change in the organization of specialized organelles might be expected to affect the performance of various functions. This assertion seems to be supported by the finding that the dark enzymic reduction of exogenously added cytochrome *c* by succinate proceeds less readily with blue-green chromatophores.

Furthermore, it should be borne in mind that, apart from the inhibition of carotenoid synthesis, growth in the presence of diphenylamine might have other consequences. In this respect it is significant that diphenylamine-grown *Rhodospirillum rubrum* cells have greatly reduced concentrations of Coenzyme Q, which might be involved in photosynthetic electron transport[23]*.

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[*] During the preparation of this paper, it came to our knowledge that WASSINK AND KRONENBERG[24] obtained results in conformity with the described spectral characteristics of diphenylamine-grown *Chromatium*.

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