Effects of Nitrate, Nitrite and Diphenylamine on the Photosynthetic Apparatus of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*

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We have compared the effect of diphenylamine (DPA) on the pigment composition of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* grown in photoheterotrophic culture with the previously reported effect of nitrate. It was demonstrated that the effect of nitrate is due to the nitrite which is produced during denitrification. Both nitrite and DPA caused a decrease in the synthesis of spheroidene, and the accumulation of more-reduced precursors not normally seen. Nitrate (effectively nitrite) caused a decrease in the amount of bacteriochlorophyll, and the reaction centre from denitrifying cells did not contain the 28 kDal polypeptide (RC-H) subunit. These effects did not occur over a range of DPA additions (4 to 8 µg ml⁻¹) to cells growing in the absence of nitrate. Denitrifying cells also had 40–50% lower activity of δ -aminolaevulinic acid synthase than those grown with or without DPA. Both nitrite and DPA treatments resulted in the loss of the B870 light-harvesting complex because of a failure to synthesize its 12 kDal polypeptide subunit.

INTRODUCTION

The denitrifying phototrophic bacterium *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* can grow in the light under anaerobic conditions or in the dark under aerobic conditions. In the dark it can also generate ATP by nitrate respiration (Satoh, 1981; Kelley *et al.*, 1982; Michalski & Nicholas, 1984). The presence of nitrate in a photosynthetically grown bacterial culture induces the synthesis and expression of the activities of nitrate, nitrite and nitrous oxide reductases, accompanied by a drastic decrease of carotenoid and bacteriochlorophyll (Bchl) contents (Michalski & Nicholas, 1984).

The photosynthetic apparatus of *R. sphaeroides* f. sp. *denitrificans*, which includes the reaction centre (RC) and light-harvesting (LH) complex components (B800-850 and B870), is very similar to that of *R. sphaeroides* wild-type (Glazer, 1983; Michalski & Nicholas, 1984). There is no definitive biochemical evidence about the way in which pigment-protein complexes are organized into photosynthetic units. Spectroscopic measurements indicate the pathway of energy transfer to be B800-850→B870→RC, and suggest a 'lake' arrangement for these complexes; B870 complexes are situated nearest to the RC, with B800-850 antenna complexes situated peripherally (Monger & Parson, 1977).

All three components of the photosynthetic unit contain carotenoids. The carotenoids not only protect the photosynthetic apparatus from photo-oxidative damage (Codgell *et al.*, 1976) but also contribute to the harvesting of light energy between 400 and 550 nm. Biosynthesis of

Abbreviations: Bchl, bacteriochlorophyll; DPA, diphenylamine; LH-complex, light-harvesting complex; LDAO, *N,N*-dimethyldodecylamine-*N*-oxide; LDS, lithium dodecyl sulphate; PMSF, phenylmethylsulphonyl fluoride; RC, photochemical reaction centre; TLCK, *N* α -*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; ALA, δ -aminolaevulinic acid.

carotenoids has been extensively studied in *R. sphaeroides*. The formation of spheroidene, the major carotenoid in *R. sphaeroides* f. sp. *denitrificans* (Michalski & Nicholas, 1984), is believed to involve phytofluene, ζ -carotene and neurosporene as intermediate metabolites (Goodwin, 1980).

Diphenylamine (DPA) has been reported to be a potent inhibitor of carotenoid synthesis in photosynthetic bacteria. The suppression by DPA of normal carotenoid synthesis is accompanied by a massive intracellular accumulation of the more saturated polyenes, which are not detectable in normal cells (Goodwin & Osman, 1952; Goodwin, 1956; Cohen-Bazire & Stanier, 1958). In this paper, using DPA-treated cells as a non-physiological control for comparison with cells grown in the presence of nitrate (effectively nitrite), we show that the two treatments cause similar but not identical changes in the photosynthetic apparatus of *R. sphaeroides* f. sp. *denitrificans*.

METHODS

Organism and growth conditions. *Rhodopseudomonas sphaeroides* f. sp. *denitrificans* strain IL 106 was kindly supplied by Dr T. Satoh (Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo 158, Japan). *R. sphaeroides* NCIB 8523 wild-type (strain 2.4.1) was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, UK. Cells were grown (5%, v/v, inoculum) in liquid culture (Satoh *et al.*, 1976) with modifications as described previously (Kelley *et al.*, 1982). DL-Malate (21.4 mM) and NH_4Cl (12 mM) were included as carbon and nitrogen sources, respectively.

Cells of both strains were grown photoheterotrophically at 30°C under anaerobic conditions in completely filled screw-cap bottles, illuminated with 5000 lx white light (7.35 W m^{-2}). Cell growth was monitored by measuring OD_{660} . Dry weights were determined as described previously (Hillmer & Gest, 1977). When cells were grown in the presence of DPA an appropriate amount of DPA dissolved in ethanol (2.5 mg ml^{-1}) was added to the culture medium immediately before inoculation.

Protein labelling with radioisotopes. Pulse-chase labelling of cell proteins with ^{14}C -labelled amino acids was done as described previously (Michalski & Nicholas, 1984). [$\text{U-}^{14}\text{C}$]Protein hydrolysate ($10 \mu\text{Ci}$; Amersham), specific activity $50 \text{ mCi (mg-atom carbon)}^{-1}$ [$1.85 \text{ GBq (mg-atom carbon)}^{-1}$], was added to anaerobic suspensions of washed cells (20 ml, sparged with argon) grown photosynthetically in the presence or absence of nitrate or DPA. After 5 min incubation (30°C, in the light), 4 ml Casamino acids (casein, acid hydrolysate; 2 mg ml^{-1}) was added to the cell suspensions and incubation continued for a further 5 or 15 min. Then chloramphenicol ($10 \mu\text{g ml}^{-1}$) was added, and the cells were harvested immediately and washed in 20 mM-potassium phosphate buffer, pH 7.4.

Preparation of RC- and LH-complexes. The chromatophore fraction of *R. sphaeroides* f. sp. *denitrificans* was prepared by the method of Barret *et al.* (1978). The RCs were extracted from chromatophore membranes by mild treatment with LDAO at 25°C (pH 7.5), as described by Jolchine & Reiss-Husson (1974). The LH polypeptides were isolated by preparative PAGE in the presence of LDS at 4°C, as described by Broglie *et al.* (1980). Electrophoretically separated LH-complexes were extracted from gel slices by homogenization in 50 mM-Tris/glycine buffer. LH-complex polypeptides were then separated by SDS-PAGE. All operations were done in the dark at 4°C. All buffers contained the protease inhibitors PMSF, TLCK and TPCK (Sigma; all 0.5 mM).

Electrophoresis. Discontinuous electrophoresis was done in the presence of 0.1% (w/v) SDS on slab polyacrylamide gels according to the method of Laemmli & Favre (1973). The ^{14}C -labelled chromatophore fraction was solubilized as described by Chory & Kaplan (1983). The location of electrophoretically separated ^{14}C -labelled proteins was determined by fluorography (Bonner & Laskey, 1974) on Cronex Hi-plus (Du Pont) intensifying screens. Bacterial proteins separated by electrophoresis were calibrated against low molecular weight protein standards (Pharmacia electrophoresis calibration kit). The proteins were stained with Coomassie brilliant blue R250.

Analysis of pigments. Bchl extracted by acetone/methanol (7:2, v/v) from washed cell suspensions was analysed spectrophotometrically ($\epsilon_{772} = 76 \text{ mM}^{-1} \text{ cm}^{-1}$, Cohen-Bazire *et al.*, 1957). Carotenoid pigments were extracted from washed cell suspensions with acetone/methanol (1:1, v/v) (Cogdell *et al.*, 1976). Individual carotenoids were separated by TLC on Eastman Kodak silica gel sheets (containing fluorescein) with the following solvents: benzene/chloroform (1:1, v/v) (spheroidene, spheroidenone, neurosporene), light petroleum (boiling range 40–60°C) (phytofluene R_f 0.12) or benzene/light petroleum (1:1, v/v) (ζ -carotene, phytofluene R_f 0.93). For quantitative analysis of individual carotenoids the following extinction coefficients were used: spheroidene, $\epsilon_{453} = 1.49 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$; spheroidenone, $\epsilon_{483} = 1.29 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$; neurosporene, $\epsilon_{437} = 1.69 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Cogdell *et al.*, 1976); ζ -carotene, $\epsilon_{400} = 1.23 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ($A'_{1\text{cm}}$ 2270 at 400 nm); phytofluene, $\epsilon_{348} = 0.73 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ($A'_{1\text{cm}}$ 1350 at 348 nm) (Davies, 1965).

Spectroscopy. Room temperature absorption spectra of pigment extracts were recorded in a Perkin Elmer Lambda 5 spectrophotometer. Low temperature (77 K) spectra of chromatophore fractions and RCs (in 50%, w/v,

glycerol) were recorded in a Shimadzu multipurpose recording spectrophotometer, model MPS-50L, fitted with low temperature spectroscopy attachments.

Enzyme assays. Nitrate reductase (EC 1.7.99.4) and nitrite reductase (EC 1.7.2.1) activities were assayed spectrophotometrically as described earlier (Michalski & Nicholas, 1984). Nitrite was measured as described by Nicholas & Nason (1954). δ -Aminolaevulinic acid synthase (EC 2.3.1.37) activity in a soluble protein fraction (supernatant 144000 g, 90 min) was assayed according to the method of Yubisui & Yoneyama (1972).

Protein determination. Protein contents of cell fractions were determined by the method of Bradford (1976), with bovine serum albumin as the protein standard.

RESULTS

Effects of DPA and nitrate on growth and pigment synthesis in R. sphaeroides f. sp. denitrificans

The pigment composition of *R. sphaeroides f. sp. denitrificans* is similar to that reported for *R. sphaeroides* when both are grown in the light (Cogdell *et al.*, 1976; Michalski & Nicholas, 1984).

The addition of DPA to photosynthetically grown cultures of *R. sphaeroides f. sp. denitrificans* resulted in a drastic decrease in carotenoid content (Table 1). The specific inhibition of carotenoid synthesis (by 50–60%) occurred over a very narrow range of DPA additions (6–8 $\mu\text{g ml}^{-1}$ culture) but at these concentrations growth and Bchl synthesis continued at the same rate as in the untreated controls. Higher concentrations of DPA ($>10 \mu\text{g ml}^{-1}$) inhibited cell growth in the early stages (results not shown). The extent to which the Bchl:carotenoid ratio can be modified by growth in the presence of DPA is evident from the absorption spectra of pigment extracts obtained from normal and DPA-treated cells (Fig. 1, spectra *a, b, c*). The cells grown photosynthetically in the presence of DPA (8 $\mu\text{g ml}^{-1}$) became pale green, compared with the reddish brown colour of untreated cells.

The addition of nitrate to photosynthetically grown cultures resulted in a decrease (by a half) of both carotenoid and Bchl contents (Table 1; Fig. 1, spectrum *d*). The rate of growth of cultures was not affected by the addition of nitrate.

The suppression of normal carotenoid synthesis in cells grown with DPA or nitrate was accompanied by an accumulation of the more saturated polyenes, which were almost absent from normal cells (Fig. 1). The major component accumulated was ζ -carotene, accompanied by smaller quantities of phytofluene (20–28% and 13% of total, respectively; Fig. 1, spectra *c, d*). Quantitative analyses of the extracted polyenes show that the ζ -carotene and phytofluene contents of cells grown in the presence of DPA or nitrate were about ten times those of normal cells.

A rapid synthesis of coloured carotenoids occurred when cells which had been grown in the presence of DPA were washed and resuspended in fresh DPA-free medium (Fig. 2). After 20

Table 1. *Effects of various amounts of DPA on carotenoid and bacteriochlorophyll a contents of R. sphaeroides f. sp. denitrificans*

DPA added [$\mu\text{g (ml culture)}^{-1}$]	Pigment content* [nmol (mg dry wt) $^{-1}$]		Bchl:carotenoid ratio
	Carotenoid	Bchl	
<i>Cells grown without nitrate</i>			
0	2.49	4.34	1.7
4	2.14	4.22	2.0
6	1.47	4.69	3.2
7	1.26	4.50	3.6
8	0.87	4.65	5.3
10	0.81	4.18	5.2
<i>Cells grown with nitrate (20 mM)</i>			
0	0.79	1.66	2.10

* The values are means of two measurements made in each of two separate experiments, and the values agreed within 10%.

Fig. 1

Fig. 1. Absorption spectra of acetone/methanol extracts prepared from cells grown photoheterotrophically without any additions (*a*), with $4 \mu\text{g DPA ml}^{-1}$ (*b*), with $8 \mu\text{g DPA ml}^{-1}$ (*c*), or with 20 mM-nitrate (*d*). The table below shows the carotenoid composition of extracts [$\text{nmol (mg dry wt)}^{-1}$] corresponding to the absorption spectra (*a-d*). The percentage contribution of individual components to the total carotenoid in each extract is shown in parentheses.

Extract	Spheroidene plus			
	spheroidenone	Neurosporene	ζ -Carotene	Phytofluene
<i>a</i>	2.04 (88)	0.20 (8)	0.04 (2)	0.05 (2)
<i>b</i>	1.61 (78)	0.16 (8)	0.18 (9)	0.10 (5)
<i>c</i>	0.51 (53)	0.07 (7)	0.27 (28)	0.12 (12)
<i>d</i>	0.56 (62)	0.05 (5)	0.18 (20)	0.12 (13)

Fig. 2

Fig. 2. Absorption spectra of the carotenoid extracts of cells grown photoheterotrophically without DPA (*a*), or with $8 \mu\text{g DPA ml}^{-1}$ (*b*). In (*c*) cells were grown photoheterotrophically in the presence of DPA for 12 h, after which they were harvested, washed and transferred into fresh, sterile, DPA-free culture medium for 20 min in the light at 30°C . The table below shows the carotenoid composition (see Fig. 1) of extracts corresponding to absorption spectra (*a-c*). Values in parentheses are as in Fig. 1.

Extract	Spheroidene plus			
	spheroidenone	Neurosporene	ζ -Carotene	Phytofluene
<i>a</i>	2.15 (90)	0.18 (8)	0.03 (1)	0.03 (1)
<i>b</i>	0.58 (60)	0.08 (8)	0.19 (20)	0.11 (12)
<i>c</i>	1.40 (78)	0.14 (8)	0.16 (9)	0.10 (5)

min incubation in the light the cells contained almost normal amounts of the major carotenoid spheroidene (78% of total) and only small quantities of ζ -carotene and phytofluene (9% and 5% of total, respectively).

Fig. 3. Absorption spectra of the carotenoids extracted from cells grown photoheterotrophically without DPA (*a, e*), or with $8 \mu\text{g DPA ml}^{-1}$ (*b, f*). Additionally, cell cultures grown in the presence of DPA were harvested at mid-exponential phase (12 h), washed and then transferred into sterile DPA-free media containing 1 mM-nitrite or 20 mM-nitrate. Growth of these cultures was then continued for 1 or 3 h in the presence of nitrite (*c, d*), or for 3 or 6 h in the presence of nitrate (*g, h*). The table below shows the carotenoid composition (see Fig. 1) of extracts corresponding to absorption spectra (*a*)–(*h*). Values in parentheses are as in Fig. 1.

Extract	Spheroidene plus spheroidenone	Neurosporene	ζ -Carotene	Phytofluene
<i>a, e</i>	2.0 (94)	0.11 (5)	0.02 (1)	0.02 (1)
<i>b, f</i>	0.60 (57)	0.06 (6)	0.24 (24)	0.14 (13)
<i>c</i>	0.65 (61)	0.05 (5)	0.24 (22)	0.12 (12)
<i>d</i>	1.35 (82)	0.10 (6)	0.09 (6)	0.10 (6)
<i>g</i>	1.40 (84)	0.07 (4)	0.12 (7)	0.10 (5)
<i>h</i>	0.70 (64)	0.03 (3)	0.23 (21)	0.13 (12)

Inhibition of carotenoid synthesis by nitrite

Although the cells of *R. sphaeroides f. sp. denitrificans* grown under denitrifying conditions produced a large amount of nitrite (up to 10 mM in approximately 8–10 h after 20 mM-nitrate had been added), they failed to grow photosynthetically when 1 mM-nitrite was added to the culture at early growth stages. The addition of nitrite to photosynthetically grown cultures inhibited carotenogenesis (Fig. 3). The synthesis of spheroidene did not occur when cells which had been grown in the presence of DPA ($8 \mu\text{g ml}^{-1}$) were washed and resuspended in DPA-free medium containing 2 mM-nitrite (Fig. 3*c*). However, an extensive and rapid synthesis of this carotenoid took place after 3 h incubation (Fig. 3*d*), at which time all the nitrite had been removed by nitrite reductase.

Fig. 4. Low temperature (77 K) absorption spectra of RC-complexes (a-c) and LH-complexes (in chromatophores) (d-f). Chromatophores and RC-complexes were isolated from cells grown photoheterotrophically without any additions (a, d), with 8 $\mu\text{g DPA ml}^{-1}$ (b, e), or with 20 mM-nitrate (c, f).

When DPA-treated cells were washed and resuspended in DPA-free medium containing 20 mM-nitrate, synthesis of carotenoids was not affected during 3 h incubation in the light (Fig. 3g). At this time, almost 80% of the original amount of spheroidene was found in the cells. However, after further incubation (4 and 6 h), when the concentration of nitrite produced reached approximately 1 mM, the synthesis of spheroidene again ceased and ζ -carotene accumulated (Fig. 3h, peaks at 425, 400 and 378 nm). These observations confirm that nitrate does not directly inhibit carotenoid synthesis, but are consistent with the view that nitrite, produced by the activity of nitrate reductase, has a specific inhibitory effect.

Spectral and biochemical characterization of pigment-protein complexes

In photosynthetic bacteria, the light-absorbing pigments (Bchl and carotenoid) exist *in vivo* as well-defined RC and light-harvesting antenna protein-pigment complexes (Thornber *et al.*, 1978). *R. sphaeroides* wild-type and *R. sphaeroides* f. sp. *denitrificans* both contain two antenna complexes, one absorbing maximally at 870 nm, termed the B870 complex and the other at 800 and 850 nm, termed B800-850; both of these spectral forms of Bchl can be observed in the 77 K, near-IR spectra of chromatophores (peaks 803, 853 and 885 nm; Fig. 4d). The 885 nm peak (the spectrum of the B870 complex is shifted at low temperature) disappears from spectra of chromatophores of cells grown in the presence of either DPA (Fig. 4e) or nitrate (Fig. 4f). In these chromatophores the absorption peaks at 855 nm, relative to those at 800 nm, are also smaller than in normal cells.

Spectral properties of RCs purified by extraction with LDAO from chromatophores of pigment-depleted cells (grown in the presence of DPA or nitrate) are shown in Fig. 4. There were no changes in the near-IR component of the spectra of RC complexes obtained from either untreated or nitrate- or DPA-treated cells (Fig. 4a, b, c, respectively). However, absorption

Fig. 5. Autoradiograms of electrophoretically separated RC and LH-complex polypeptides after a 'pulse-chase' labelling of the cells with ^{14}C -labelled amino acids for 5 min (lanes 1 and 2) and 15 min (lanes 3–6). Cells grown photoheterotrophically for 20 h in the presence of 20 mM-nitrate (lanes 2 and 4), or 8 $\mu\text{g DPA ml}^{-1}$ (lane 6) were harvested, washed and resuspended in nitrate-free, DPA-free medium. These cells were pulse-labelled as described in Methods and the chromatophores were then prepared. Radioactive proteins (50 μg) were separated by SDS-PAGE and autoradiographed. Lanes 1, 3 and 5 represent the labelling patterns of untreated photoheterotrophically grown cells. All ^{14}C -labelled protein samples were diluted so that they contained approximately 40 000 c.p.m., which corresponds to approximately 40–60 μg protein.

spectra of RC-complexes of cells grown in the presence of nitrate or DPA (8 $\mu\text{g ml}^{-1}$) again indicate that ζ -carotene had accumulated (peak at 400 nm). These results show that both types of carotenoid-depleted cells (i.e. cells grown in the presence of DPA or nitrate) lack the B870 LH-complex. It is, however, noteworthy that the yield of the RC-complexes extracted from the chromatophore membranes of cells grown with nitrate was about 30–40% lower than that of untreated cells.

Biosynthesis of RC- and LH-complex polypeptides in cells grown in the presence of nitrate and DPA

The synthesis of subunits of RC- and LH-complexes was studied by following the incorporation of ^{14}C -labelled amino acids into chromatophore proteins, in 'pulse-chase' labelling experiments (5 min labelling followed by a 5 or 15 min 'chase'). Fig. 5 shows autoradiograms of electrophoretically separated ^{14}C -labelled chromatophore proteins. After a 5 and 15 min 'pulse-chase' labelling, six major polypeptides identified as RC- and LH-complex subunits had been synthesized in cells grown in the light (Fig. 5*a, c, e*). After 15 min 'pulse-chase' labelling of cells grown in the presence of nitrate, subunits of the RC were much less intensively labelled, and no sharp protein bands could be identified in the autoradiogram even after a longer exposure period (Fig. 5*d*). All three subunits of the RC-complex in cells grown in the presence of DPA were labelled, although the intensity was less than that in control cells (Fig. 5*e, f*). The 10 kDal subunit of LH-complexes of cells grown with either nitrate (effectively nitrite) or DPA was less intensively labelled than in control cells. In LH-complexes of the treated cells no 12 kDal polypeptide was present (Fig. 5*d, f*). Since the 12 kDal polypeptide is a subunit only of the B870 LH-complex, these results support the conclusion that nitrite produced during denitrification, as well as DPA, inhibits synthesis and/or assembly of this particular pigment-protein complex.

Fig. 6. Polypeptide composition of RC- and LH-complexes. (a) LDS-PAGE at 4 °C on a 7.5–15% gradient gel of chromatophores (160 µg protein containing 15 µg Bchl) from cells grown without nitrate (lane 1), or with 20 mM-nitrate (lane 2). The LH-complex polypeptides were extracted from lanes 1 and 2 and re-run on LDS-PAGE gels (lanes 3 and 4 respectively). H, M and L are RC subunits of apparent molecular mass 28, 24 and 21 kDal respectively. (b, c) SDS-PAGE of LH-complex polypeptides on a 10–17% gradient gel (30 µg protein in each sample). Lanes 5 and 9, total LH-complex polypeptide extracted from lane 3. Lanes 6 and 7, duplicate samples of the B800-850 complex polypeptides; lane 8, B870 complex; both complexes were prepared by LDS-PAGE according to Broglie *et al.* (1980). Lanes 10, 11 and 12, LH-complex polypeptides separated from chromatophores of cells grown with nitrate, nitrite and DPA respectively.

Polypeptide composition of RC- and LH-complexes in chromatophores of cells grown in the presence of nitrate or diphenylamine

Substitution of LDS for SDS in Laemmli-type electrophoresis enables solubilization and electrophoresis to be done at 4 °C. The separation of polypeptides from the chromatophore membrane preparations (preheated in boiling water for 90 s) is shown in Fig. 6(a). In the presence of LDS, the RC of normal cells (Fig. 6a lane 1), as well as those treated with DPA (not shown), dissociated into three subunits with apparent molecular masses of 28, 24 and 21 kDal (subunits H, M, L, respectively). The RC in chromatophores of denitrifying cells, however, lacked a 28 kDal subunit (Fig. 6a, lane 2).

Although the LH-complex polypeptides were well separated from RC subunits, they could not be fully resolved in this system. Therefore, they were extracted from gel slices, purified on similar LDS-gels (Fig. 6*a*, lanes 3 and 4), then extracted again and characterized by SDS-PAGE on 10–17% (w/v) gradient slab gels. The LH-complex from normal cells (Fig. 6*a*, lane 1) was separated into three polypeptides of apparent molecular masses 12, 10 and 8 kDal (Fig. 6*b*, lane 5), representing subunits of B870 (12 and 8 kDal; Fig. 6*b*, lane 8) and B800-850 (10 and 8 kDal; Fig. 6*b*, lanes 6 and 7). As shown in Fig. 6*c*), complete LH-complexes from chromatophores prepared from either DPA- or nitrate- or nitrite-treated cells lacked the 12 kDal polypeptide (Fig. 6*c*, lanes 10, 11 and 12 respectively). Since this particular polypeptide is a subunit only of the B870 LH-complex, these results support the conclusion that nitrite (produced by denitrification of nitrate or added directly), as well as DPA, inhibits synthesis and/or assembly of this LH-complex (cf. Fig. 5).

DISCUSSION

The structural and biochemical properties of *R. sphaeroides f. sp. denitrificans* IL 106 resemble those of *R. sphaeroides* wild-type (Sato *et al.*, 1976; Michalski & Nicholas, 1984). In both organisms the photosynthetic apparatus contains an RC as well as the two Bchl-protein complexes (B800-850 and B870) of the light-harvesting system. Spheroidene was a major carotenoid component of the photosynthetic membranes. DPA, a potent inhibitor of carotenoid synthesis in many organisms (Goodwin, 1980), inhibited the formation of spheroidene in *R. sphaeroides f. sp. denitrificans* (Table 1). By contrast, DPA at similar concentrations inhibited the growth of *R. sphaeroides* wild-type but without any differential effect on pigmentation (cf. Goodwin, 1956).

As reported by Michalski & Nicholas (1984), the inclusion of nitrate in cultures of *R. sphaeroides f. sp. denitrificans* grown photoheterotrophically resulted in a drastic decrease in both Bchl and carotenoid contents. We now show that the decrease in the formation of carotenoids in these cultures is due to nitrite, a product of nitrate reduction. The synthesis of carotenoids (mainly spheroidene) was restored when DPA-treated cells were washed, transferred to DPA-free medium and then incubated in light in the absence of nitrite (Fig. 3). Since the inhibition of carotenoid synthesis by either DPA or nitrite resulted in an accumulation of ζ -carotene (Figs 1 and 2) it is clear that both compounds inhibit the enzymic desaturation of ζ -carotene to neurosporene, one of the precursors of spheroidene (Goodwin, 1980).

The decrease in carotenoid content of cells grown in the presence of either DPA or nitrate (effectively the reduction product, nitrite) is accompanied by structural changes in the photosynthetic apparatus. Both types of cells lack LH-complex B870 (Figs 4–6) and do not synthesize one of the three subunits (12 kDal polypeptide) needed for this pigment-protein complex (Fig. 6).

The decrease in Bchl and carotenoid contents in cells grown in the presence of nitrate is also accompanied by a limited formation of RC-complexes. These cells contained fewer chromatophores and the number of RC complexes was about 60% of that in untreated cells.

Although the results that we obtained on the effect of DPA and nitrite are consistent with the proposal that the enzymic desaturation of ζ -carotene (possibly a single enzyme reaction) is inhibited, the fact that cells grown in the presence of nitrate have smaller amounts of Bchl, RC-complexes and lack the 28 kDal and 12 kDal polypeptide subunits, whereas DPA-treated cells have normal amounts of Bchl yet also lack the 12 kDal subunit, indicates that this simple explanation may be insufficient. δ -Aminolaevulinic acid (ALA) synthase, which produces 5-aminolaevulinic acid from glycine and succinyl-CoA, is a key enzyme in the synthesis of tetrapyrroles, the reaction common to the biosynthesis of Bchl and haem. Experiments *in vivo*, as well as with purified enzyme preparations, have shown that several factors are involved in photopigment synthesis at this step; light intensity, oxygen pressure, ATP, Mg-protoporphyrine and haem (Jones, 1978). We have measured the ALA synthetase activity in the soluble protein fraction of cells grown under various conditions. Highly active enzyme was found in both normal and DPA-treated cells [4.3 and 4.0 nmol ALA formed min⁻¹ (mg protein)⁻¹, respectively]. However, in denitrifying cells, the enzyme activity was decreased by

approximately a half [$2.4 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. These results, based on several independent experiments, confirm a drastic reduction in soluble cytochrome content of cells grown in the presence of nitrate (W. P. Michalski, D. J. Miller & D. J. D. Nicholas, unpublished results). Thus, we can now offer a plausible explanation for the similar effect of nitrate, nitrite and DPA on carotenogenesis, as well as their differential effects on Bchl formation.

RC- and LH-complexes from chromatophores of denitrifying cells lack the 28 kDal and 12 kDal polypeptide subunits, respectively (Fig. 6). Since the protease inhibitors PMSF, TLCK and TPCK were included in all buffers, the polypeptide compositions of RC- and LH-complexes were not due to proteolytic action, but rather to the inhibition of their synthesis and/or assembly. It is also noteworthy that two smaller subunits (24 and 21 kDal), which remain associated in the RC-complex of denitrifying cells, were photochemically active at room temperature (see spectra in Fig. 4 and cf. Okamura *et al.*, 1974).

In conclusion, it is still not clear, however, whether the carotenoid deficiency is itself responsible for the failure to synthesize the 12 kDal subunit, so that the self-assembly of the light-harvesting components (Sistrøm *et al.*, 1956) is prevented by the absence of one or more essential components. It remains to be established whether nitrite and DPA primarily affect the synthesis of the 12 kDal subunit, in which case a feed back mechanism might come into operation to control extra carotenoid synthesis (Segen & Gibson, 1971) or Bchl synthesis (Lascelles & Hatch, 1969) when this polypeptide is missing. It is unlikely that the 12 kDal subunit is the primary target in view of the known effects of DPA on carotenoid biosynthesis in a variety of photosynthetic and non-photosynthetic organisms (Goodwin, 1980).

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