JOURNAL OF BACTERIOLOGY, Oct. 1987, p. 4651–4659
0021-9193/87/104651-09$02.00/0

Vol. 169, No. 10

Inhibition of Bacteriochlorophyll Synthesis in *Rhodobacter sphaeroides* subsp. *denitrificans* Grown in Light under Denitrifying Conditions

WOJCIECH P. MICHALSKI AND D. J. D. NICHOLAS*

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, 5064 South Australia, Australia

Received 11 May 1987/Accepted 17 July 1987

The inclusion of nitrate or nitrite in cultures of *Rhodobacter sphaeroides* subsp. *denitrificans* grown heterotrophically in light depressed the formation of bacteriochlorophyll *a*. The pigment biosynthesis was inhibited at the stage of the reduction of chlorophyllide (chlorin) to bacteriochlorophyllide (tetrahydroporphyrin) since 3-hydroxyethylchlorophyllide *a* accumulated in the culture medium. The addition of exogenous 5-aminolevulinic acid to these cultures resulted in a complete restoration of bacteriochlorophyll synthesis accompanied by the accumulation of 3-vinylbacteriopheophorbide. This indicates that under these conditions bacteriochlorophyll was formed via an alternative route, in which the reduction of chlorins to tetrahydroporphyrins precedes modifications of the C-3 side chain. The multiple forms of 5-aminolevulinic acid synthase were purified from cells grown with and without nitrate. Antibodies against these proteins were raised in rabbits and used in enzyme-linked immunosorbent assays for various forms of 5-aminolevulinic acid synthase. In denitrifying cells, the amount and activity of fraction I of the enzyme was reduced by approximately 40 and 30%, respectively. Partly active enzymes from both types of cells were activated by cystine trisulfide.

Tetrapyrrole biosynthesis in photosynthetic bacteria is initiated by the formation of 5-aminolevulinic acid (ALA), the precursor common to the pathways of heme and bacteriochlorophyll synthesis (for a review, see reference 18). *Rhodobacter sphaeroides* synthesizes the iron tetrapyrrole prosthetic groups of the cytochromes involved in both photosynthetic and respiration-dependent electron transport (7, 8), but magnesium tetrapyrroles are produced only for photosynthetic growth. The synthesis of bacteriochlorophyll in this bacterium is regulated by oxygen and by light: either high oxygen tension or high light intensity suppresses pigment synthesis (2, 13). The bacteriochlorophyll biosynthesis pathway is shown in Fig. 1.

Many photosynthetic bacteria, in which bacteriochlorophyll synthesis has been affected (mutants and inhibited cultures), excrete porphyrins and their metal-free derivatives into the medium during growth. A number of these pigments which have been isolated and characterized are probably precursors of bacteriochlorophyll synthesis (18).

In common with other photosynthetic bacteria, *R. sphaeroides* subsp. *denitrificans* can grow in light under anaerobic conditions or in the dark in air. In addition to these two distinct growth regimes, it can also generate ATP by nitrate respiration (19, 24, 32). The presence of nitrate in a photosynthetically grown bacterial culture enhanced the synthesis of nitrate, nitrite, and nitrous oxide reductases (22, 24) but markedly depressed the synthesis of carotenoids, bacteriochlorophyll, and soluble cytochromes (23–26). Denitrifying cells also contain fewer chromatophores, and the number of reaction center complexes is about 60% of that of nondenitrifying cells (26). It has been postulated that these denitrifying cells contain reduced amounts of the cytochrome b-c_1 complexes (23). In this study, we report on a mechanism for the inhibition of bacteriochlorophyll production in *R. sphaeroides* subsp. *denitrificans* grown under denitrifying conditions in light.

* Corresponding author.

MATERIALS AND METHODS

Organism and growth conditions. *R. sphaeroides* subsp. *denitrificans* IL106 was kindly supplied by T. Satoh, Department of Biology, Tokyo Metropolitan University, Tokyo, Japan. It was grown photoheterotrophically in a mineral salt medium as described previously (24).

Analysis of pigments. (i) Extraction and purification. Cells were grown photosynthetically at 30°C with or without 20 mM nitrate. At the end of the log phase of growth (optical density at 660 nm, 2.0 to 2.2), the cells were centrifuged at 20,000 × *g* at 4°C. For preliminary pigment identification, the supernatants of 1-liter cultures were extracted three times with diethyl ether. The ether extracts were washed in water, dried over anhydrous Na_2SO_4, concentrated in a rotary evaporator at 30°C, and then kept under argon at −10°C. For purification of pigments, the supernatants of 5-liter cultures were extracted with ethyl acetate at pH 5.5 as described in reference 30. This procedure resulted also in the extraction of magnesium-free derivatives of the pigments under these mild acidic conditions. The extracts were washed with water in the presence of acetone and NaCl to separate the phases (30). The organic phase evaporated to dryness, and the residue, dissolved in a small volume of 40% (vol/vol) methanol, was loaded onto a polyethylene powder (BDH, Poole, England; chromatography grade [1]) column (2 by 18 cm) equilibrated with 40% (vol/vol) methanol and eluted with 400 ml of methanol gradient (40 to 100% [vol/vol]). The pigments were eluted in sequence at the following concentrations of methanol: 3-hydroxyethylbacteriopheophorbide *a* (Ph-720), 45 to 55%; 3-hydroxyethylpheophorbide *a* (Ph-662), 60 to 70%; 3-vinylbacteriopheophorbide *a* (Ph-730), 80%; divinylpheophorbides and protophorphyrins, 90 to 100%. Aqueous methanol fractions

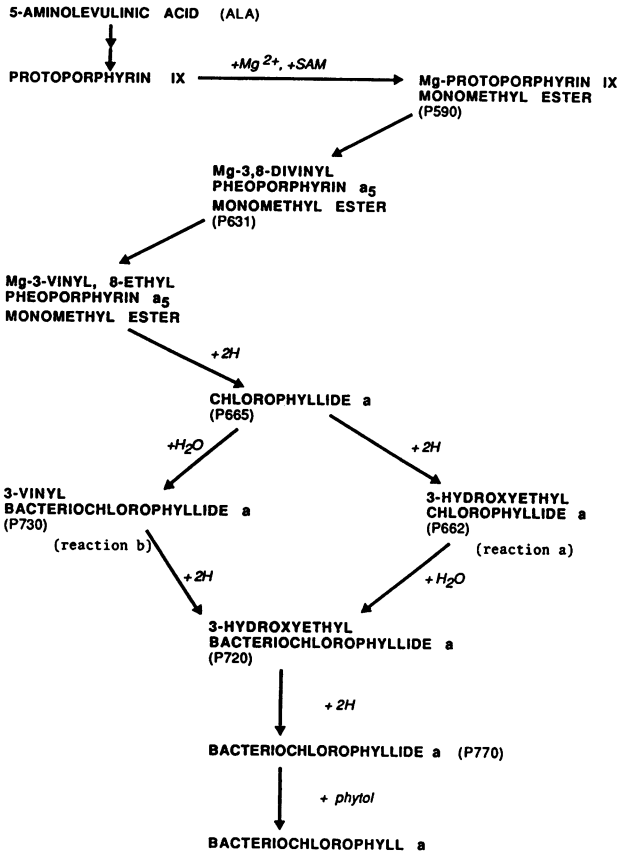

FIG. 1. Bacteriochlorophyll biosynthetic pathway (28, 29). The order of reactions has been inferred from a consideration of the structures of accumulated intermediates. The red-most spectral peaks of the intermediates are indicated in parentheses. SAM, *S*-Adenosylmethionine.

diluted with water to approximately 30% were extracted into diethyl ether, dried over anhydrous Na_2SO_4, and concentrated by evaporation. Individual pigments were then rechromatographed on a small (1 by 10 cm) polyethylene powder column as described above. Aqueous fractions were again extracted with diethyl ether, concentrated, and stored under argon at −10°C. The pigments were also purified by various paper and thin-layer chromatographic techniques described below. Pure compounds, recovered from chromatograms by elution with diethyl ether, were crystallized in a distilled water-diethyl ether system as described in reference 17.

Bacteriochlorophyll *a* was extracted in acetone-methanol (7:2 [vol/vol]) from washed cell suspensions and determined by using an extinction coefficient value of 76 mM cm^{-1} at 772 nm (24). The pigment was also purified from acetone-methanol extracts by powdered-sugar column chromatography. The procedure of Strain and Svec (34) was followed, except that the preparation was scaled down 100-fold.

(ii) Preparation of derivatives. Magnesium-free derivatives of pigments were obtained by shaking the ether extracts with 2 N HCl for 10 min. Acid-treated extracts were then washed with water and dried over anhydrous Na_2SO_4.

Dehydration of hydroxyethyl groups was achieved in vacuum (approximately 100 Pa) at 240°C as described in reference 30. This procedure converted 3-hydroxyethylpheophorbide *a* (Ph-662) into 3-vinylpheophorbide. The visible spectrum of the latter compound in diethyl ether resolved into the following bands, given in nanometers (relative absorption is given in parentheses): 665 (1.00); 607 (0.22); 556 (0.17); 535 (0.27); 503 (0.31); 409 (3.15).

Oxidation of tetrahydroporphyrins to chlorins was achieved in a reaction with 2,3-dichloro-5,6-dicyanobenzoquinone (28). The procedure converted 3-hydroxyethylbacteriopheophorbide *a* (Ph-720) into 3-hydroxyethylpheophorbide *a* (Ph-662), and its spectrum in diethyl ether revealed the following bands, given in nanometers (relative absorption is given in parentheses): 660 (1.0); 600 (0.17); 531 (0.18); 500 (0.19); 405 (2.3). Oxidation of 3-vinylbacteriopheophorbide *a* (Ph-730) resulted in the formation of 3-vinylpheophorbide *a*. Its spectrum in diethylether was resolved into the following bands, given in nanometers (relative absorption given in parentheses): 667 (1.0); 610 (0.20); 534 (0.21); 507 (0.27); 408 (3.42).

Paper and thin-layer chromatography. The ascending chromatographic method on Whatman no. 1 paper (Whatman, Inc., Clifton, N.J.) and with 2,6-lutidine–H_2O (5:3 [vol/vol]) development (5) as well as silica gel thin-layer chromatography (acetone-methanol, 1:1 [vol/vol]) were used to determine the number of unesterified carboxyl groups. Chloroform-kerosene (13:20 [vol/vol]) paper chromatography was used to detect the presence of hydroxyl groups. Samples were esterified by treatment with 5% (wt/vol) H_2SO_4 in methanol at 0°C. Acetylation of hydroxyl groups was accomplished with a mixture of acetic anhydride-pyridine (1:10 [vol/vol]) (3). The pigments were also separated on silica gel thin-layer sheets (Merck & Co., Inc., Rahway, N.J.) developed with benzene-ethyl acetate-ethanol (8:2:2 or 8:2:5 [vol/vol/vol]) as described in reference 33.

Spectroscopy. Visible spectra of pigments in diethyl ether were recorded in a Lambda 5 Perkin-Elmer spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) in which an abscissa calibration was done automatically at the sharp emission line at 656.1 nm emitted by the deuterium lamp. The following extinction coefficients (ε, millimolar per centimeter) for purified pigments in diethyl ether were used: P-590, 18.2; P-662, 51.5; Ph-730, 38.0. Infrared spectra of pigments (KCl disks or film) were recorded in a Perkin-Elmer 983 IR spectrophotometer. Comparative analysis of IR spectra indicated that none of the extracted pigments (except bacteriochlorophyll) was in a phytylated form (19).

Radioactive labeling of pigments with ^{14}C-ALA. In vivo labeling of pigments with 5-amino[4-^{14}C]levulinic acid (^{14}C-ALA) (1.97 GBq/mmol; Amersham Corp., Arlington Heights, Ill.) was achieved as follows. Small cultures (15 ml) grown photoheterotrophically for 24 h without nitrate were used to inoculate (5% [vol/vol]) a series of flasks containing 300 ml of mineral salt medium supplemented with ^{14}C-ALA (30 μCi) and 20 mM nitrate. After 24 h of growth, cells were separated from the culture medium by centrifugation. Radioactively labeled pigments were extracted and purified as described above. These pigments were used in further experiments as radioactive standards. For studies of pigment production by cells grown under various conditions, 15-ml cultures were labeled with ^{14}C-ALA (10 μCi) in the presence of nitrate (10 mM) or nitrite (2 mM). Pigments excreted into the culture medium were then extracted with diethyl ether and separated by thin-layer chromatography as described above. Radioactively labeled compounds were located on chromatograms by autoradiography by using intensifying screens (Cronex Hi-plus, Du Pont Co., Wilmington, Del.). Compounds were extracted from the chromatograms and radioassayed with scintillation fluor (Ready Solv NA; Beck-

man Instruments, Inc., Fullerton, Calif.) in a Packard Instrument Co., Inc. (Rockville, Md.), Tri-Carb liquid scintillation spectrometer (model 460 CD).

The rate of ALA uptake was measured as follows. Cells grown in 8-ml cultures (0.5 mg [dry weight]/ml) for 8 h with or without nitrate were then incubated in light with 2 μCi of ^{14}C-ALA for a further 60 min at 30°C. Samples of cell suspension (100 μl) were taken every 5 min and filtered through Millipore Corp. (Bedford, Mass.) HA filters. Filters were washed with 4 ml of ice-cold 5% (wt/vol) trichloroacetic acid, dried, placed in scintillation vials, and radioassayed as described above. Both types of cell culture (grown with or without nitrate) slowly but steadily accumulated a radioactively labeled precursor at a rate of approximately 0.1 pmol of ^{14}C-ALA per min per mg (dry weight).

Purification of ALA synthase. Cells grown photosynthetically with or without 20 mM nitrate were washed to remove nitrite, suspended in 0.1 M Tris hydrochloride buffer (pH 7.4) containing protease inhibitor-phenylmethylsulfonyl fluoride (0.5 mM), and disrupted by two passages through a French pressure cell at 4°C (25,000 lb/in^2; 0.17 GPa). DNase I and RNase (200 μg each) were added to the homogenate prepared from about 50 g of cells (wet weight), and after 5 min it was centrifuged for 20 min at 20,000 × *g* at 4°C. The activity of ALA synthase (EC 2.3.1.37) present in the high-speed supernatant was stabilized by adding 2 mM 2-mercaptoethanol, and the enzyme was then purified by the method of Inve et al. (16) and Tuboi et al. (37). The supernatant was fractionated with $(NH_4)_2SO_4$ at 4°C, and the protein fraction (containing the enzyme) precipitated at 25 to 40% saturation was centrifuged at 30,000 × *g* for 15 min, dissolved in 25 mM Tris hydrochloride buffer (pH 7.4) containing 2 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride, and dialyzed overnight at 4°C against the same buffer. The dialyzed fraction was concentrated with a YM30 Amicon membrane filter and loaded onto a Sephacryl S200 column (16 by 800 mm) equilibrated with 25 mM Tris hydrochloride buffer containing 100 mM NaCl and 2 mM 2-mercaptoethanol (pH 7.4). Pooled fractions containing the enzyme (molecular mass of 80 to 95 kilodaltons [kDa]) were dialyzed overnight at 4°C against 25 mM Tris hydrochloride buffer (pH 7.4). The dialyzed enzyme fraction was then loaded onto a DEAE-Sephacel (Pharmacia, Uppsala, Sweden) column (3.3 by 30 cm) equilibrated with the same buffer and eluted with 600 ml of a NaCl gradient (0 to 0.2 M) in the buffer, at a flow rate of 40 ml/h. Two fractions (I and II) containing ALA synthase were eluted with 0.04 to 0.045 and 0.06 to 0.07 M NaCl, respectively. They were dialyzed against 25 mM Tris hydrochloride buffer, pH 7.4, and separately concentrated on a small DEAE-Sephacel column (5 by 30 mm) eluted with 0.1 M NaCl in 25 mM Tris hydrochloride buffer (pH 7.4). The enzymes were then finally purified by gel filtration on an S-200 Sephacryl column, as described above, except that 2-mercaptoethanol was omitted from the running buffer.

Enzyme assays. ALA synthase activity was assayed as described by Tuboi et al. (37). Kinetic constants ± standard error were determined using a least-squares fit directly to the original (nonlinear) rate equation as described by Cleland (6). The computer program was run on a Hewlett-Packard 9825A desktop computer (Hewlett-Packard Co., Inc., Palo Alto, Calif.).

Immunological methods. Antibodies against purified fractions I and II of ALA synthase (see Table 2) were raised in rabbits. Samples of electrophoretically homogenous enzymes were adjusted to 400 μg of protein per ml in 0.15 M NaCl, emulsified with 1 ml of complete Freund adjuvant (Sigma Chemical Co., St. Louis, Mo.) and injected subcutaneously into the backs of the animals. Samples containing 380 μg of protein in Freund incomplete adjuvant were administered after 2 weeks intramuscularly into the cervical region. Additional samples of 250 μg of the enzyme fractions in saline were injected after 2 and 4 more weeks. The antibody titer was followed by double immunodiffusion (12) and was appreciable from week 5 onward. Sera prepared before immunization showed no precipitin reaction with purified enzymes. Blood samples were taken during weeks 5, 6, and 7. Immunoglobulin G (IgG) fractions specific for various forms of ALA synthase were purified from antisera as described in reference 12. Specificity of purified IgG fractions was tested by tandem cross-immunoelectrophoresis techniques (21).

ALA synthases were quantified in soluble fractions (S_{240}) by enzyme-linked immunosorbent assay techniques. Indirect noncompetitive enzyme immunoassay procedures with antigens or antibodies immobilized on the solid phase were used (35). The goat anti-rabbit IgG (whole-molecule) peroxidase conjugate (Sigma) was used in all immunoassays.

Synthesis of bis(2-amino, 2-carboxyethyl)trisulfide (cystine trisulfide) from cysteine and sulfur. The reaction conditions of Fletcher and Robson (10) were used, except that the preparation was scaled down threefold. The reaction products were tested by ascending paper chromatography (Whatman no. 1) with butan-1-ol–acetic acid–H_2O (12:30:50 [vol/vol/vol]) as a developing system. The R_f values of the compounds of interest in this system, which reacted with ninhydrin reagent, were: cystine (0.06, blue-grey), bis(2-amino-2-carboxyethyl)trisulfide (0.1, brown), and cysteine (0.34, blue with yellow center).

Electrophoresis. Details of sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis and sample preparations were as previously described (24). Gradient gel electrophoresis was run on Pharmacia PAA 4/30 gels. In each case, when gel electrophoresis was done, the gels were calibrated with low- or high-molecular-weight protein standards (Pharmacia).

Isoelectric points of purified ALA synthases were determined by focusing on polyacrylamide slab gel with Ampholines from pH 3.5 to 10 (LKB, Bromma, Sweden) by the method described by Hallenbeck et al. (11).

Analytical methods. The A_{660} of the cell suspension was measured, and the dry weight was calculated from a calibration graph. In the mid-log phase, the culture density was 0.45 to 0.5 mg (dry weight)/ml (A_{660}, 1.0 to 1.1).

Protein content was determined by the method of Bradford (4), by using bovine serum albumin as a standard. Nitrite was determined as described by Nason and Evans (28). The method of Ellman (9) was used to determine sulfhydryl groups in purified preparations of ALA synthase.

RESULTS

Identification of pigments excreted into the medium in cultures grown under various conditions. (i) Photosynthetic growth. In common with other phototrophic bacteria, *R. sphaeroides* subsp. *denitrificans* grown in light under anaerobic conditions synthesizes both bacteriochlorophyll *a* and carotenoids and derives ATP for growth from photosynthetic electron transport (20, 24, 26). At the late exponential-stationary phase of growth, it excretes into the culture medium a pink-red pigment with an absorption spectrum resolved into three bands: 428, 550, and 590 nm, character-

TABLE 1. Effects of ALA on bacteriochlorophyll content in cells and accumulation of tetrapyrrole pigments in culture media[a]

Growth conditions (anaerobic; with light)	Concn (mM) of added ALA	Pigment content (nmol/mg [dry wt] ± SD; n = 4) in:			
		Cells (Bchl)	Culture medium		
			P-590	P-662	Ph-730
Without nitrate	0	3.83 ± 0.14	0.31 ± 0.07	ND[b]	ND
	0.5	3.91 ± 0.10	0.36 ± 0.07	ND	ND
	1.0	3.89 ± 0.11	0.37 ± 0.06	ND	ND
	3.0	3.81 ± 0.16	0.31 ± 0.03	ND	ND
	5.0	3.79 ± 0.21	0.20 ± 0.03	ND	ND
	10.0	3.60 ± 0.22	tr[c]		
With 20 mM nitrate	0	2.26 ± 0.26	0.29 ± 0.05	0.4 ± 0.09	
	0.5	2.49 ± 0.21	0.21 ± 0.05	0.1 ± 0.03	tr
	1.0	2.70 ± 0.19	tr	tr	0.25 ± 0.09
	3.0	3.06 ± 0.17	ND	tr	0.29 ± 0.05
	5.0	3.87 ± 0.10	ND	ND	0.13 ± 0.03
	10.0	3.78 ± 0.11	ND	ND	tr

[a] ALA was added to the culture medium together with 5% (vol/vol) inoculum. After 20 h of growth in light, cells were separated from the culture medium by centrifugation and pigments were analyzed as described in Materials and Methods. Bchl, bacteriochlorophyll *a*; P-662, 3-hydroxyethylchlorophyllide; P-590, magnesium protoporphyrin IX, 13[3]-monomethyl ester; Ph-730, 3-vinylbacteriopheophorbide *a*.

[b] ND, Not detectable.

[c] Trace, <0.1 nmol/mg (dry weight).

istic of magnesium protoporphyrin or its methyl esters (Table 1; Fig. 2).

The pigment was extracted from the medium, purified and identified by various chemical tests (see Materials and Methods) as magnesium protoporphyrin IX, 13[3]-monomethyl ester (P-590). When phototrophically grown cultures (8 h of growth) were supplemented with ^{14}C-ALA, radioactively labeled pigment was found in the culture medium after a further 12 h of growth (Fig. 3a and b). At the late exponential phase of growth (18 h after addition of the tracer [Fig. 3c]), 54 pmol of radioactive precursor was found to be incorporated per nmol of excreted magnesium protoporphyrin. Under these conditions (18 h), 570 pmol of ^{14}C-ALA was incorporated per nmol of bacteriochlorophyll *a*.

(ii) Photosynthetic growth with nitrate. Cells grown photosynthetically in the presence of nitrate contained reduced

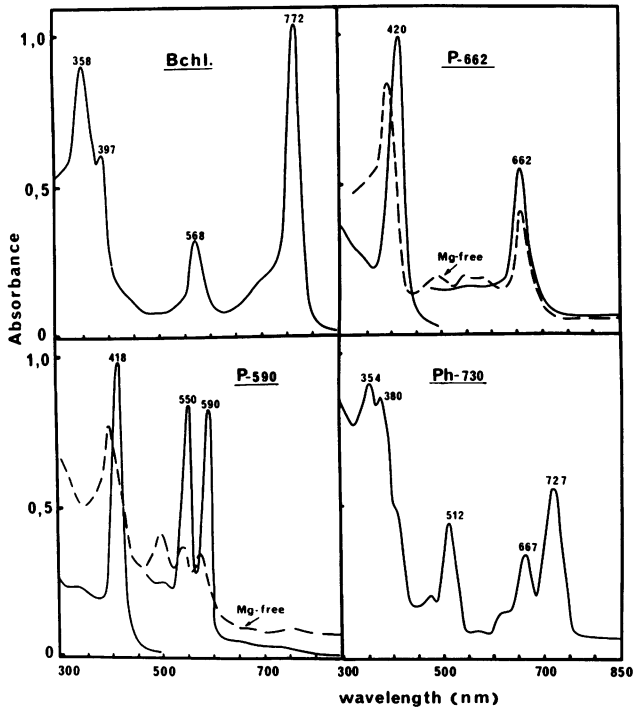

FIG. 2. Absorption spectra in diethyl ester of pigments purified from cells (Bchl, bacteriochlorophyll *a*) and from culture media (see Materials and Methods): P-662, 3-hydroxyethylchlorophyllide *a*; P-590, magnesium protoporphyrin IX, 13[3]-monomethyl ester; Ph-730, 3-vinylbacteriopheophorbide *a*. Extinction coefficients for these pigments are given in Materials and Methods.

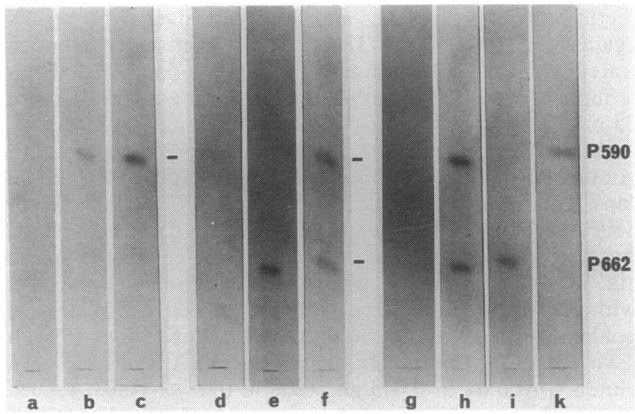

FIG. 3. Separation and identification of ^{14}C-labeled pigments excreted into the culture medium by cells grown under various conditions. Radioactive labeling of cells with ^{14}C-ALA and pigment extraction procedures are described in Materials and Methods. Diethyl ether extracts were spotted onto silica gel sheets and chromatographed with benzene-ethyl acetate-ethanol (8:2:5 [vol/vol/vol]). Radioactive compounds were located by autoradiography and identified by comparing their positions on chromatograms with those of authentic radioactive markers. Lanes: a, b, and c, pigments excreted by nondenitrifying cells labeled for 6, 12, and 18 h, respectively; d, e, and f, pigments excreted by denitrifying cells (grown with 20 mM nitrate) labeled for 4, 10, and 18 h, respectively; g and h, pigments extracted from the medium of nondenitrifying cultures radioactively labeled for 2 and 6 h in the presence of 2 mM nitrite. Cells were grown for 8 h in light before nitrate or nitrite and the radioactive tracer were added; i and k, pure ^{14}C-labeled 3-hydroxyethylchlorophyllide *a* and magnesium protoporphyrin IX, 13[3]-monomethyl ester, respectively, prepared as described in Materials and Methods.

amounts of photopigments (24, 26), but the rate of growth of denitrifying cultures was virtually the same as that of cultures grown in light without nitrate (25). The utilization of nitrate (denitrification) by these cultures was followed by a rapid accumulation of nitrite as well as of red-green pigments (bands of 420, 550, 590, and 660 nm) in the culture medium (Table 1). The pigments extracted from the medium with diethyl ether were identified as magnesium protoporphyrin IX, 13^3 monomethyl ester (P-590) and 3-hydroxyethylchlorophyllide *a*, (P-662) (Fig. 2). In denitrifying cultures grown in the presence of ^{14}C-ALA (added at mid-log phases after 8 h of growth), the accumulation of radioactively labeled chlorophyllide commenced 8 to 10 h after the addition of the tracer and was followed by the accumulation of ^{14}C-labeled magnesium protoporphyrin (Fig. 3e and f). After 18 h, specific incorporation of the radioactive precursor was 210 and 160 pmol of ^{14}C-ALA per nmol of pigment P-590 and P-662, respectively. At that time, the bacteriochlorophyll content in denitrifying cells was depleted by approximately half (24, 26). However, specific labeling of the pigment (490 pmol of ^{14}C-ALA per nmol of bacteriochlorophyll *a* formed) was only 15% lower than that of cells grown without nitrate. These results indicate that the synthesis of ALA was also affected in denitrifying cells, resulting in a smaller endogenous pool of bacteriochlorophyll precursors.

(iii) Photosynthetic growth in the presence of nitrite. Although the cells grown under denitrifying conditions produced a large amount of nitrite (up to 10 mM in approximately 8 to 10 h from 20 mM nitrate added initially), they failed to grow when 3 mM nitrite was added to the culture at the early stages of growth. The addition of nitrite (up to 3 mM) at mid-log phase (8 h growth) did not restrict bacterial growth, and nitrite was slowly removed from the culture by nitrite reductase (25). However, the rate of bacteriochlorophyll synthesis in cells grown under these conditions was reduced by approximately 30%. The labeling of these cells with ^{14}C-ALA (Fig. 3g and h) revealed an accumulation in the culture medium of two radioactive pigments, identified as magnesium protoporphyrin IX, 13^3-monomethyl ester and 3-hydroxyethylchlorophyllide *a*; after 6 h of incubation, the specific incorporation of radioactive precursor was 100 and 127 pmol of ^{14}C-ALA per nmol of pigment, respectively. An extensive synthesis of bacteriochlorophyll took place after a further 3 h of incubation, at which time all the nitrite had been removed by nitrite reductase. After 18 h of growth in the presence of radioactive tracer, specific labeling of bacteriochlorophyll was 605 pmol of ^{14}C-ALA per nmol of pigment formed. However, the pigments which had accumulated in the medium were not taken up by the cells during this period. These observations confirm that nitrate does not directly inhibit bacteriochlorophyll synthesis, but they are consistent with the view that nitrite, produced by the activity of nitrate reductase, has a specific inhibitory effect.

Effects of exogenous ALA on pigment formation. The addition of ALA to cultures grown photosynthetically in the presence of nitrate resulted in a pronounced increase in bacteriochlorophyll content in the cells, as well as a decrease in the amounts of tetrapyrrole pigments excreted into the culture medium (Table 1). Over a range of ALA additions (3 to 10 mM), bacteriochlorophyll content was the same as in cells grown photosynthetically without nitrate. At these concentrations, only very small amounts of 3-hydroxyethylchlorophyllide *a* (P-662) and magnesium protoporphyrin (P-590) pigments were detected in the culture medium. There was, however, another pigment which accumulated in the medium with an optical spectrum characteristic of bacteriochlorophyllides (Table 1). It was extracted from the medium, purified, and identified by various chemical tests (see Materials and Methods) as 3-vinylbacteriopheophorbide *a* (Ph-730; Fig. 2). Growth and bacteriochlorophyll synthesis in nondenitrifying cultures supplemented with ALA continued at the same rate as in the untreated controls, and magnesium protoporphyrin (P-590) accumulated in the culture medium (Table 1). These data indicate that bacteriochlorophyll synthesis in denitrifying cells might be regulated at the level of ALA formation, i.e., by modulation of ALA synthase activity.

To ascertain that pigments excreted under denitrifying conditions (P-662 and Ph-730) did not result from a chemical reaction in the medium, the following experiments were conducted. Cultures (2-liter) were grown photosynthetically without nitrate; at the late exponential phase of growth (18 h), cells were harvested, and the culture medium containing magnesium protoporphyrin (P-590) was transferred into 250-ml glass bottles and made anaerobic with nitrogen gas. The bottles were then supplemented with 20 mM potassium nitrate–10 mM potassium nitrite, potassium nitrate–3 mM ALA, or potassium nitrite–3 mM ALA. They were then illuminated for at least 18 h and tested spectrophotometrically for tetrapyrrole pigments. No pigment was detected other than protoporphyrin (only absorption peaks at approximately 590, 550, and 430 nm were detected). In all bottles, however, this pigment was reduced by approximately 10% (data not shown). These results indicate that pigments extracted from media of cultures grown under various conditions were excreted by bacterial cells and were not the result of chemical reactions in the media.

Activity of ALA synthase in cells grown with or without nitrate: purification of two forms of the enzyme. Highly active ALA synthase was found in cells grown in light without nitrate (0.30 μmol of ALA produced per min per mg of protein). In denitrifying cells, however, the enzyme activity was decreased by about 40% (0.19μmol/min per mg of protein [26]). The addition of 2 mM 2-mercaptoethanol to soluble protein fractions (S_{240}) of both denitrifying and nondenitrifying cells enhanced these activities by 35 and 20%, respectively, and had a stabilizing effect during early stages of enzyme purification (see Materials and Methods for details) (Table 2). The purification of ALA synthase revealed that both types of cells contained two distinct forms of the enzyme, namely fractions I and II, which were separated by a DEAE-Sephacel chromatography (see Materials and Methods). Purified fraction I enzyme had an apparent molecular mass of 78 to 80 kDa as determined by gel filtration and gradient gel electrophoresis techniques. Two protein bands with molecular masses of 42 (a) and 39.5 (b) kDa were detected on SDS gel electrophoresis (Table 3; Fig. 4, lanes 1 and 3). Focusing of fraction I on polyacrylamide gels resulted in the separation of two proteins with pI of 5.55 and 5.45 (Table 3). Similarly, fraction II of the enzyme which had a molecular mass of 85 to 90 kDa, gave two protein bands (a, 46 kDa; b, 42 kDa) on SDS gel electrophoresis (Fig. 4, lanes 2 and 4). On electrofocusing gel, fraction II separated into two proteins with pI values of 5.10 and 5.00 (Table 3). These results indicate that each fraction (I and II) of the enzyme contains two dimeric proteins differing slightly in their isoelectric points and in their molecular masses (Table 3; Fig. 4). These proteins most probably represent interconvertible high-activity (a) and low-activity (b) forms of either fraction of the enzyme, as postulated previously (14, 36; for a review, see reference 18). Both fractions I and II of ALA synthase were purified from cells grown either with or

TABLE 2. Purification of ALA synthases from *R. sphaeroides* subsp. *denitrificans*[a]

Fraction derived from:	Total protein, mg	Total activity, U[b]	Sp act, U/mg of protein	Purification, fold	% Yield
S_{240}	1,005 (987)		0.30 (0.19)		
S_{240} + 2 mM 2-mercaptoethanol	1,005 (987)	352 (286)	0.35 (0.29)	1	100 (100)
$(NH_4)_2SO_4$ fractionation (25–40%)	202 (207)	282 (193)	1.40 (0.93)	4.0 (3.2)	80 (67)
YM30 Amicon membrane concentrate	142 (139)	260 (190)	1.83 (1.37)	5.2 (4.7)	74 (66)
Sephacryl S-200 chromatography	27.7 (24.3)	202 (118)	7.31 (4.84)	20.9 (16.7)	57 (41)
DEAE-Sephacel chromatography					
Fraction I	8.3 (6.9)	114 (54)	13.70 (7.90)	39.1 (27.2)	32 (19)
Fraction II	7.1 (6.5)	70 (56)	9.91 (8.60)	28.3 (29.7)	20 (21)
Sephacryl S-200 chromatography					
Fraction I	4.8 (3.9)	119 (69)	24.93 (17.63)	71.2 (60.8)	34 (24)
Fraction II	3.8 (3.5)	56 (52)	14.70 (15.01)	42.0 (51.8)	16 (18)

[a] The enzymes were purified from cells grown under nondenitrifying and denitrifying (values in parentheses) conditions according to the procedure described in Materials and Methods.
[b] 1 unit = 1 μmol of ALA formed per min.

without nitrate (Table 2), and both contained low- and high-activity forms of the enzyme (Fig. 4, lanes 1 to 4). In denitrifying cells, however, the amount of fraction I was about 40% less than in cells grown without nitrate, and its specific activity was also reduced by a third (Table 2).

Activation of ALA synthase. Although the addition of 2-mercaptoethanol stabilized ALA synthase in crude extracts and in partially purified preparations, it inhibited the purified enzyme (100% inhibition at 1 mM). This indicates a function for cystine residues (di- or trisulfide bridges) in the purified enzyme. It has been shown that the activation of the low-activity form of the enzyme is achieved by the addition of disulfides in the presence of an endogenous activating enzyme (36) or cystine trisulfide (38). Inactivation of the enzyme occurred on incubating with a thiol and a particulate fraction from the cells (36). Similarly, when fractions I and II of the purified enzyme from *R. sphaeroides* subsp. *denitrificans*, grown with or without nitrate, were incubated for 90 min with 50 μM cystine trisulfide their activities were enhanced about fivefold (Table 3). However, after activation, the fraction I enzyme from denitrifying cells had still lower specific activity than that of activated fraction I enzyme from cells grown without nitrate (Tables 2 and 3).

Cystine trisulfide-activated fraction I enzymes, from both nondenitrifying and denitrifying cells, had a molecular mass of 80 kDa as determined by a gradient gel electrophoresis technique (Table 3). The enzyme (fraction I) was composed of two identical subunits since it gave one protein band with a molecular mass of 40 kDa on SDS gels (Fig. 4, lanes 7 and 9). Similarly, fraction II enzymes, from both types of cells, activated with cystine trisulfide had a molecular mass of 85 kDa and were separated into identical subunits of 45 kDa (Table 3; Fig. 4, lanes 8 and 10). Cystine trisulfide-activated fraction I and fraction II enzymes both focused as single protein bands on polyacrylamide gels with pI values of 5.6 and 5.1, respectively.

Thus, it appears that, upon activation, *b*-type subunits of both fraction I and II enzymes were converted into *a*-type subunits, characteristic for a high-activity form of these enzymes (Fig. 4; Table III). Activated fractions I and II also contained fewer sulfhydryl groups than did nonactivated species (Table 3). A similar quaternary structure (dimer with identical subunits) of highly active ALA synthase has been reported by Nandi and Shemin (27).

Immunological determination of ALA synthases. Polyclonal antibodies against purified fractions I and II of ALA synthase (Table 2) were raised in rabbits. IgG fractions were purified from antisera, and their specificity was tested by tandem cross-immunoelectrophoresis techniques (for details, see Materials and Methods). Fractions I and II were immunologically different since anti-fraction I and anti-fraction II immunoglobulins were monospecific, i.e., they reacted only with fraction I and fraction II, respectively. However, each IgG fraction gave positive precipitin reaction

TABLE 3. Molecular properties of ALA synthases purified from *R. sphaeroides* subsp. *denitrificans*[a]

Enzyme	Apparent molecular mass (kDa)			pI[b]	No. of sulfhydryl groups	Activity (U/mg of protein)
	Gel filtration	Gel electrophoresis				
		SDS	Gradient			
Partially active						
Fraction I						
a	80	42	78	5.55	3–4	24.9 (17.6)
b		39		5.45		
Fraction II						
a	90	46	85	5.10	4–6	14.7 (15.0)
b		42		5.00		
Activated (with cystine trisulfide)						
Fraction I a		40[c]	80	5.60	2	109.6 (68.9)
Fraction II a		45[c]	85	5.10	2–3	52.9 (64.5)

[a] All experimental details are given in Materials and Methods. The procedure for enzyme activation with bis(2-amino,2-carboxyethyl)trisulphide is described in the legend to Fig. 4. The unit of enzyme activity is defined as 1 μmol of ALA formed per min. Activity of the enzymes purified from denitrifying cells is given in parentheses. The number of sulfhydryl groups was determined in reaction with 5,5-dithiobis(2-nitrobenzoic acid).
[b] pI values for partially active fractions I and II represent different forms of the enzymes as described in the text.
[c] Samples were not treated with 2-mercaptoethanol before electrophoresis.

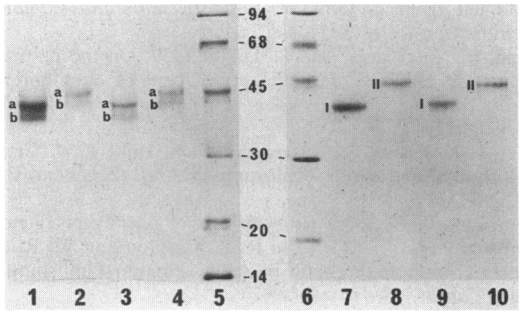

FIG. 4. Subunit composition of purified ALA synthases determined by SDS-polyacrylamide gel electrophoresis (12.5% [wt/wt], lanes 1 to 5; 11% [wt/wt], lanes 6 to 10). The enzyme was purified from cells grown in light, without nitrate: fraction I (30 μg, lane 1), fraction II (20 μg, lane 2), fractions I and II activated with cystine trisulfide (30 and 20 μg, lanes 7 and 8). The enzyme from denitrifying cells: fraction I (20 μg, lane 3), fraction II (20 μg, lane 4), fractions I and II activated with cystine trisulphide (20 μg each, lanes 9 and 10). Lanes 5 and 6: molecular-weight standards (10^3). Activation of the enzyme fractions was achieved by incubation with 50 μM cystine trisulfide for 90 min at 4°C. Note that activated enzymes were separated on an SDS gel in the absence of 2-mercaptoethanol. All samples, however, contained 1% (wt/vol) SDS.

with either nonactivated or activated forms of the respective enzyme fraction (data not shown).

Specific IgG fractions were used in an enzyme-linked immunoassay to quantify various forms of ALA synthase in soluble fractions (S_{240}). Thus, cells grown without nitrate contained 2.15 μg of fraction I and 1.65 μg of fraction II per mg of protein. In cells grown under denitrifying conditions, the amount of fraction I enzyme was reduced by approximately 45%, whereas that of fraction II enzyme was not affected under these conditions.

Kinetic properties of the activated enzyme. ALA synthase requires pyridoxal phosphate as a cofactor for the formation of ALA from glycine and succinyl-coenzyme A. The pH optimum for enzyme activity was 7.5 for fraction I and 7.8 for fraction II, but K_m values for glycine (7.5 ± 0.1 mM) and succinyl-coenzyme A (11.3 ± 0.4 μM) were identical in the two forms. Both fractions I and II of the activated enzyme were inhibited by a 3- and 6-μM magnesium protoporphyrin, IX 13^3-monomethyl ester, but only activity of fraction I enzyme was affected by 3-hydroxyethylchlorophyllide *a* (50% inhibition at 10 μM). The presence of cystine trisulfide (up to 50 μM) did not prevent the inhibition of the enzyme by tetrapyrrole pigments.

DISCUSSION

Because *R. sphaeroides* subsp. *denitrificans* can be grown in the dark anaerobically in the presence of nitrate, it follows that ATP generated during denitrification (respiration to nitrate as a terminal electron acceptor) is sufficient to meet its energy requirements (20, 24, 32). In cells grown photoheterotrophically in the presence of nitrate, however, the energy transformation system appears to be more complex. The inclusion of nitrate or nitrite in cultures grown in light enhanced the synthesis of nitrate, nitrite, and nitrous oxide reductases (24, 25, 28) but markedly depressed the synthesis of carotenoids, bacteriochlorophyll, and soluble cytochromes (23, 24, 26). The decrease in bacteriochlorophyll contents of denitrifying cells was also accompanied by a limited formation of reaction centers and cytochrome b-c_1 complexes, components of the photosynthetic apparatus (23, 26). However, the growth rate of bacteria cultured in the presence of nitrate in light was about three times that of cells grown in the dark. Although a link between photosynthesis and nitrate respiration as energy-transforming systems has been postulated in this bacterium (24, 31, 32), the precise relationship between these systems remains to be established.

Cells of *R. sphaeroides* subsp. *denitrificans*, like other phototrophic bacteria (18), excreted small amounts of magnesium protoporphyrin IX, 13^3-monomethyl ester (P-590) into the culture medium when grown heterotrophically in light (Fig. 3; Table 1). However, in cultures grown photosynthetically in the presence of nitrate or nitrite, bacteriochlorophyll synthesis was partly inhibited, the excretion of protoporphyrin was greater, and this was accompanied by an accumulation of 3-hydroxyethylchlorophyllide *a* (P-662) (Fig. 3; Table 1). This immediate precursor of bacteriochlorophyll *a* and its magnesium-free derivative have been detected in bacteriochlorophyll-less mutants of *R. sphaeroides* and *Rhodobacter palustris* (15, 18, 30) (Fig. 1, reaction a). Two modifications of the tetrapyrrole structure are necessary for the conversion of 3-hydroxyethylchlorophyllide *a* to bacteriochlorophyll *a*. The hydroxyethyl substituent at position 3 must be dehydrogenated to form the acetyl group, and a double bond in ring B of the tetrapyrrole must be reduced to give the bacteriochlorin ring structure (18). Thus, since the reduction of ring B precedes formation of the acetyl group (Fig. 1 [30]), the former reaction appears to be inhibited in denitrifying cells, resulting in an accumulation of 3-hydroxyethylchlorophyllide (P-662) in the culture medium (Fig. 3; Table 1). The addition of ALA to cultures grown under denitrifying conditions resulted in complete restoration of bacteriochlorophyll *a* synthesis accompanied by the accumulation of a new pigment in the medium, identified as 3-vinylbacteriopheophorbide *a* (Ph-730) (Table 1). The magnesium derivative of the bacteriopheophorbide cannot be accommodated in the proposed biosynthetic pathway (18) unless the alternative route is accepted (Fig. 1, reaction b). Such a pathway would be feasible should the necessary side-chain modifications occur, more or less at random, at any of the three states of oxidation of the tetrapyrrole, namely porphyrin, chlorin, and tetrahydroporphyrin (29). Indeed, the formation of 3-vinylbacteriopheophorbide *a* (Ph-730) and possibly its magnesium derivative in denitrifying cells supplied with ALA indicates that under these conditions, reduction from chlorin to the tetrahydroporphyrin stage occurred before hydration of the vinyl substituent to a hydroxyethyl group (Fig. 1, reaction b). Furthermore, it is likely that the enzyme which catalyzes the reduction of a vinyl-substituted chlorin 3-hydroxyethylchlorophyllide *a* (P-665) (29) is different from that reducing 3-hydroxyethylchlorophyllide *a*, since the latter enzyme does not operate under denitrifying conditions.

A requirement for ALA in bacteriochlorophyll synthesis in cells grown with nitrate (Table 1) indicates that formation of the precursor in these cells was rate limiting. Indeed, in denitrifying cells both the amount and activity of ALA synthase (Table 2, fraction I) were lower than those of cells grown without nitrate. Fraction I of the enzyme purified from denitrifying cells and activated by cystine trisulfide was still less active than that of nondenitrifying cells (Table 3). Thus, we can now propose a plausible explanation for the inhibition of bacteriochlorophyll synthesis in *R. sphaeroides* subsp. *denitrificans* grown under denitrifying conditions. Not only is the synthesis of ALA synthase affected by

changes in growth conditions, but also the activity of the enzyme is modulated by the cellular concentration of protoporphyrin (18). The accumulation of the latter compound results from the inhibition of an enzyme responsible for the conversion of chlorophyllide to bacteriochlorophyllide (Fig. 3). It is of interest that the formation of phytol is not a limiting factor for the synthesis of bacteriochlorophyll in denitrifying cells, since the amount of free phytol in these cells was identical to that in cells grown without nitrate (data not shown).

ACKNOWLEDGMENTS

W.P.M. acknowledges with thanks the award of a research associateship by the Australian Research Grants Scheme. The work was supported by a grant to D.J.D.N. by the Australian Research Grants Scheme.

The skillful technical assistance of D. F. Mathew is gratefully acknowledged.

LITERATURE CITED

1. **Anderson, A. F. H., and M. Calvin.** 1962. An improvement for the separation and purification of chlorophyll *a*. Nature (London) **194**:285–286.
2. **Arnheim, K., and J. Oelze.** 1983. Differences in the control of bacteriochlorophyll formation by light and oxygen. Arch. Microbiol. **135**:299–304.
3. **Barret, J.** 1959. Detection of hydroxyl groups in porphyrins and chlorins. Nature (London) **183**:1185–1186.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Chu, T. C., A. A. Green, and E. J.-H. Chu.** 1951. Paper chromatography of methyl esters of porphyrins. J. Biol. Chem. **190**:643–646.
6. **Cleland, W. W.** 1967. The statistical analysis of enzyme kinetic data. Adv. Enzymol. **29**:1–32.
7. **Clément-Metral, J. D., and A. Holmgren.** 1983. Purification and some properties of thioredoxin from a photosynthetic bacterium *Rhodopseudomonas sphaeroides* Y, p. 59–68. *In* P. Gadal (ed.), Thioredoxins, structure and functions. Editions du Centre National de la Recherche Scientifique, Paris.
8. **Connelly, J. L., O. T. G. Jones, V. A. Saunders, and D. W. Yates.** 1973. Kinetic and thermodynamic properties of membrane-bound cytochromes of aerobically and photosynthetically grown *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **292**:644–653.
9. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**:70–77.
10. **Fletcher, J. C., and A. Robson.** 1963. The occurrence of bis-(2-amino-2-carboxyethyl)trisulphide in hydrolysate of wool and other proteins. Biochem. J. **87**:553–559.
11. **Hallenbeck, P. C., C. M. Meyer, and P. M. Vignais.** 1982. Regulation of nitrogenase in the photosynthetic bacterium *Rhodopseudomonas capsulata* as studied by two-dimensional gel electrophoresis. J. Bacteriol. **151**:1612–1616.
12. **Harboe, N., and A. Ingild.** 1973. Immunization, isolation of immunoglobins, estimation of antibody titre, p. 161–164. *In* N. H. Axelsen, J. Krøll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis. Universitetsforlaget, Oslo.
13. **Hauser-Gerspach, I., and J. Oelze.** 1985. Control of the synthesis of bacteriochlorophyll, *c*- and *b*-type cytochromes, and coproporphyrin III in *Rhodopseudomonas sphaeroides* mutant H5. Arch. Microbiol. **185**:151–155.
14. **Hayasaka, S., and S. Tuboi.** 1974. Control of δ-aminolevulinate synthetase activity in *Rhodopseudomonas sphaeroides*. III. Partial purification of the fraction I activating enzyme and the occurrence of two forms of fraction II. J. Biochem. **76**:157–168.
15. **Houghton, J. D., O. T. G. Jones, J. M. E. Quirke, M. Murray, and C. L. Honeybourne.** 1983. 2-(1-Hydroxyethyl)-2-desvinyl chlorophyllide *a*: characterization by nuclear over Hauser enhancement proton magnetic resonance of a novel pigment obtained from mutants of *Rhodopseudomonas sphaeroides*. Tetrahedron Lett. **24**:5703–5706.
16. **Inue, I., M. Oyama, and S. Tuboi.** 1979. On the nature of the activating enzyme of the inactive form of δ-aminolevulinate synthetase in *Rhodopseudomonas sphaeroides*. J. Biochem. **86**:477–482.
17. **Jacobs, E. E., A. E. Vatler, and A. S. Holt.** 1954. Crystalline chlorophyll and bacteriochlorophyll. Arch. Biochem. Biophys. **53**:228–238.
18. **Jones, O. T. G.** 1978. Biosynthesis of porphyrins, hemes and chlorophylls, p. 751–777. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
19. **Katz, J. J., R. C. Dougherty, and L. T. Boucher.** 1966. Infrared and nuclear magnetic resonance spectroscopy of chlorophylls, p. 186–252. *In* L. P. Vernon and G. R. Seely (ed.), Chlorophylls. Academic Press, Inc., New York.
20. **Kelley, B. C., R. H. Dunstan, and D. J. D. Nicholas.** 1982. Respiration-dependent nitrogenase activity in the dark in a denitrifying phototrophic bacterium *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. FEMS Microbiol. Lett. **13**:253–258.
21. **Krøll, J.** 1973. Tandem cross immunoelectrophoresis, p. 57–59. *In* N. H. Axelsen, J. Krøll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis. Universitetforlaget, Oslo.
22. **Michalski, W. P., D. H. Hein, and D. J. D. Nicholas.** 1986. Purification and characterization of nitrous oxide reductase from *Rhodopseudomonas sphaeroides* f.sp. *denitrificans*. Biochem. Biophys. Acta **872**:50–60.
23. **Michalski, W. P., D. J. Miller, and D. J. D. Nicholas.** 1986. Changes in cytochrome composition of *Rhodopseudomonas sphaeroides* f.sp. *denitrificans* grown under denitrifying conditions. Biochim. Biophys. Acta **849**:304–315.
24. **Michalski, W. P., and D. J. D. Nicholas.** 1984. The adaptation of *Rhodopseudomonas sphaeroides* f.sp. *denitrificans* for growth under denitrifying conditions. J. Gen. Microbiol. **130**:155–165.
25. **Michalski, W. P., and D. J. D. Nicholas.** 1985. Changes in guanosine 5′-diphosphate 3′-diphosphate (ppGpp) content of *Rhodopseudomonas sphaeroides* f.sp. *denitrificans* during adaptation for growth under denitrifying conditions. J. Gen. Microbiol. **131**:2199–2207.
26. **Michalski, W. P., D. J. D. Nicholas, and F. R. Whatley.** 1985. Effects of nitrate, nitrite and diphenylamine on the photosynthetic apparatus of *Rhodopseudomonas sphaeroides* f.sp. *denitrificans*. J. Gen. Microbiol. **131**:1951–1961.
27. **Nandi, D. L., and D. Shemin.** 1977. Quaternary structure of δ-aminolevulinate acid synthase from *Rhodopseudomonas sphaeroides*. J. Biol. Chem. **252**:2278–2280.
28. **Nason, A., and H. J. Evans.** 1953. Triphosphopyridine nucleotide-nitrate reductase in *Neurospora*. J. Biol. Chem. **202**:655–673.
29. **Pudek, M. R., and W. R. Richards.** 1975. A possible alternate pathway of bacteriochlorophyll biosynthesis in a mutant of *Rhodopseudomonas sphaeroides*. Biochemistry **14**:3132–3137.
30. **Richards, W. R., and J. Lascelles.** 1969. The biosynthesis of bacteriochlorophyll. The characterization of latter stage intermediates from mutants of *Rhodopseudomonas sphaeroides*. Biochemistry **8**:3473–3482.
31. **Satoh, T.** 1977. Light-activated, -inhibited and -independent denitrification by a denitrifying phototrophic bacterium. Arch. Microbiol. **115**:293–298.
32. **Satoh, T.** 1981. Soluble dissimilatory nitrate reductase containing cytochrome *c* from a denitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*. Plant Cell Physiol. **22**:443–452.
33. **Seliskar, C. J.** 1966. Separation of phytylated and nonphytylated chlorophylls by thin-layer chromatography. Anal. Biochem. **17**:174–177.
34. **Strain, H. H., and W. A. Svec.** 1966. Extraction, separation, estimation, and isolation of the chlorophylls, p. 21–66. *In* L. P. Vernon and G. R. Seely (ed.), Chlorophylls. Academic Press, Inc., New York.
35. **Tijssen, P.** 1985. Quantitative enzyme immunoassay techniques, p. 329–384. *In* R. H. Burdon and P. H. Knippenborg (ed.),

Practice and theory of enzyme immunoassays. Laboratory techniques in biochemistry and molecular biology, vol. 15. Elsevier Biomedical Press, Amsterdam.

36. **Tuboi, S., and S. Hayasaka.** 1972. Control of δ-aminolevulinate synthetase activity in *Rhodopseudomonas sphaeroides*. II. Requirements of a disulfide compound for the conversion of the inactive form of fraction I to the active form. Arch. Biochem. Biophys. **150**:690–697.
37. **Tuboi, S., H. J. Kim, and G. Kikuchi.** 1970. Occurrence and properties of two types of δ-aminolevulinate synthetase in *Rhodopseudomonas sphaeroides*. Arch. Biochem. Biophys. **138**:147–154.
38. **Wider de Xifra, E. A., J. D. Sandy, R. C. Davies, and A. Neuberger.** 1976. Control of δ-aminolevulinate synthetase activity in *Rhodopseudomonas sphaeroides*. Philos. Trans. R. Soc. London B **273**:79–98.