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ENERGY METABOLISM IN THE CYANOBACTERIUM *PLECTONEMA BORYANUM*

OXIDATIVE PHOSPHORYLATION AND RESPIRATORY PATHWAYS

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The filamentous cyanobacterium *Plectonema boryanum* catalyzes efficient dark oxidative phosphorylation of exogenous ADP during NADPH consumption after a lysozyme treatment of only 30 min and subsequent dilution in hypoosmotic medium. It is shown that the thylakoid membranes and membrane areas bearing the terminal oxidase (presumably the cell membrane with cytochrome $c:O_2$ oxidoreductase) and easily soluble cytoplasmic proteins are involved in KCN-sensitive dark oxidative phosphorylation. The dinitrophenyl ether of 2-iodo-4-nitrothymol, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone and KCN are inhibitors of dark respiratory ATP synthesis. Dependent on the physiological condition, other more or less KCN-insensitive respiratory pathways towards O_2 may be present. A tentative scheme of the respiratory pathways is proposed.

Introduction

Intact cyanobacteria (blue-green algae) are surrounded by a lysozyme-sensitive cell wall and a cell membrane. The relatively simple intracellular thylakoid membranes are either not continuous or only loosely connected with the cell membrane [1,2]. The main energy-generating pathway in cyanobacteria is chloroplast-like photophosphorylation [3,4]. In addition, dark oxidative phosphorylation has been demonstrated in both photoautotrophic and facultative (photo) heterotrophic strains [5–13]. Photophosphorylation has been studied in cell-free preparations of several cyanobacteria and its efficiency (ATP/2e ratio) is not very much impaired in these systems compared to intact cells [14–19]. However, from the few data on dark oxidative phosphorylation in cell-free preparations [6,11,19] it can be calculated that the ATP/2e ratio is rather low [0.1–0.4] in comparison with the estimated values of about 3 in intact cells [8,13].

These differences were further substantiated by comparing the ATP/2e ratios of photophosphorylation and dark oxidative phosphorylation (0.8–1.2 and less than 0.1, respectively) in membrane vesicles of *P. boryanum* [19]. This selective loss of dark phosphorylation efficiency in cell-free preparations may indicate that membranes other than the thylakoid membranes (presumably the cell membranes) being more sensitive to cell fragmentation, participate in dark energy conservation. Peschek and coworkers have recently demonstrated high rates of dark oxidative ATP synthesis

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DNP-INT, dinitrophenylether of 2-iodo-4-nitrothymol; PQ, plastoquinone; Qbc, the redox complex containing plastoquinone, cytochromes *b*-563 and *c*-557 and the Rieske non-haem iron and sulfur protein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

in French-press-isolated cell-free membranes of several cyanobacteria [7].

In the procedure for making cell-free preparations, cell disruption will lead to vesicles consisting of partially separated or associated thylakoid and cell membrane fragments. Osmotically shocked spheroplasts may produce spatially separated thylakoid and cell membrane vesicles [20], whereas repeated French-press treatment, and even more sonication will most likely lead to recomposition of scrambled membrane vesicles. Thus, in order to arrive at conclusions concerning a cooperativity of both membrane types in dark energy conservation, the method of cell-free membrane preparation is of crucial importance. So far, this aspect did not receive much attention in these studies.

In this paper we describe the preparation of osmotically stable but partially permeable cells by a short treatment (30 min) with lysozyme. These cells have retained their structural integrity upon dilution in hypo-osmotic medium, but are apparently rendered permeable to NADPH and ADP and show an appreciable and stable rate of dark oxidative phosphorylation. Our results point to the participation of both thylakoid and cell membranes in this process.

Materials and Methods

Culture. Plectonema boryanum 594 Gomont, according to Rippka et al., properly called LPP 73110 [21], was kindly provided by Dr. E. Padan and was grown in axenic batch culture as described previously [19].

Preparation of spheroplasts and leaky cells. Intact cell filaments were collected by centrifugation of 400 ml of cell culture in the late logarithmic phase of growth at $5000 \times g$ for 1 min and washed twice in buffer A (containing 10 mM Tricine-NaOH, 5 mM sodium phosphate-potassium phosphate, 10 mM $MgCl_2$ and 500 mM mannitol (final pH 7.8) [14]). The cell filaments were resuspended in about 10 ml buffer A (10–20 mg protein per ml corresponding to 0.5–1.0 mg Chl *a* per ml) and 1 $mg \cdot ml^{-1}$ of lysozyme was added. The cells were incubated at 32°C for a standard 90 min or the indicated times (see Results) and stored on ice until use (within 1 h) [19]. Cells treated with lysozyme for only 30 min will be called leaky cells.

Preparation of membrane vesicles. Washed cell filaments in buffer A (see above) were sonicated for 5 times 1 min (1 min intervals) at 4°C with a MSE Soniprep 150, suited with a microtip at an amplitude of 28 μm. Intact cells were centrifuged at $2000 \times g$ for 10 min and the particles were collected by centrifugation of the supernatant for 10 min at $25\,000 \times g$ and finally resuspended in buffer A at a protein concentration of 15 $mg \cdot ml^{-1}$, corresponding to about 0.8 mg Chl *a* per ml^{-1}. The preparation was stored on ice until use (within 1 h).

Preparation of concentrated soluble proteins was done as described in the preceding paper [28].

Dark oxidative ATP synthesis. Spheroplasts or leaky cells were prepared as described above using variable lysozyme treatment times as indicated in the Results section. Samples of 1 ml of the spheroplast solution were diluted in 9 ml of buffer A and centrifuged at $2000 \times g$ for 2 min. The pellet was resuspended in 10 ml buffer B (buffer A minus mannitol [4]) in small Erlenmeyer flasks placed on rotaryshaker in the 'dark' (a Kodak green safe light as used in the photographers dark room, and tested not to activate the cyanobacterial photosystems, was used as working light source). ATP synthesis then started by addition of ADP (1 mM) and NADPH (0.5 mM); inhibitors or concentrated soluble proteins were added as indicated. The experimental solutions remained air-saturated throughout. At 0 and 20 min or at certain times (as indicated in Results), samples of 2 ml were mixed with 2 ml of ice-cold 5 M $HClO_4$. After 10 min these samples were mixed with 2.2 ml of a solution containing 1 M Tris and 5 M KOH and centrifuged for 2 min at $2000 \times g$. Samples of the clear supernatant were kept at 4°C and either directly used for the ATP determination or frozen at −18°C for later determinations [22]. The assays were usually done in triplicate.

Determinations of ATP. ATP was determined via the luciferine/luciferase assay, essentially as described by Webster et al. [23]. We have used a measuring medium containing 100 mM Tris-acetate/3 mM magnesium-acetate/2 mM EDTA (pH 7.75). An internal ATP standard was used to correct for disturbing impurities in each determination. Measurements were made on a luminometer obtained from the Arrhenius Labora-

tory, University of Stockholm.

NADPH oxidation. Spheroplasts or membrane vesicles were diluted in buffer B plus 0.1 mM NADPH (about 0.1 mg Chl *a* per ml). Further additions were as indicated in the Results section. NADPH oxidation was measured with an Aminco DW-2a dual-wavelength spectrophotometer at 340 nm against 400 nm as reference or alternatively through polarographic assay of O_2 uptake [19].

Determination of chlorophyll a and protein. Protein was determined according to Lowry et al. [24]. Chlorophyll *a* was determined according to MacKinney [25], using extensively lysed cells [19].

Chemicals. DBMIB and DNP-INT were generously provided by Dr. A. Trebst. Luciferine, luciferase and lysozyme were obtained from Boehringer. All chemicals used were of analytical grade.

Results

Dark oxidative phosphorylation

In the present work we have studied dark phosphorylation of added ADP by 'spheroplast' preparations of *P. boryanum* in the presence of NADPH as electron donor and O_2 as terminal electron acceptor. A treatment with lysozyme for 30 min and subsequent dilution in hypoosmotic medium gave rise to optimal rates of dark oxidative phosphorylation, linear for at least 1 h (Fig. 1). In a few experiments with these leaky cell preparations we have determined ATP/2e ratios in NADPH respiration from 0.5 up to 2.3. In the absence of NADPH the rate of ATP synthesis decreased to less than 20% of the control (not shown). The rates of NADH oxidation and accompanying ATP synthesis were generally only 40% of the comparable rates with NADPH. As observed by light microscopy, the briefly (30 min) lysozyme-treated cells retain their intact cell shape after dilution in hypoosmotic medium, but apparently, they have become permeable to NADPH and ADP. These preparations will further be indicated as leaky cells. Prolonged treatment with lysozyme (60–90 min) and dilution in hypoosmotic medium resulted in spheroplast lysis and a strong decrease in the rate of dark oxidative phosphorylation. In contrast, however, these preparations are still very active in photophosphorylation as shown before [19]. Interestingly, Fig. 2 shows that the decrease in the dark ATP synthesis after prolonged lysozyme treatment and subsequent spheroplast lysis could be restored by addition of a concentrate of soluble proteins, indicating that

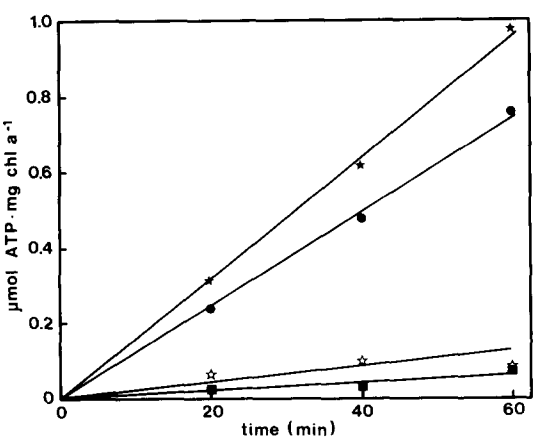

Fig. 1. Dark respiratory ATP synthesis in cells of *P. boryanum* after treatment with lysozyme for different times. The ATP synthesis assay conditions have been described in Materials and Methods. The symbols indicating the lysozyme treatment times were: ■, 0 min; ★, 30 min; ●, 60 min and ☆, 90 min.

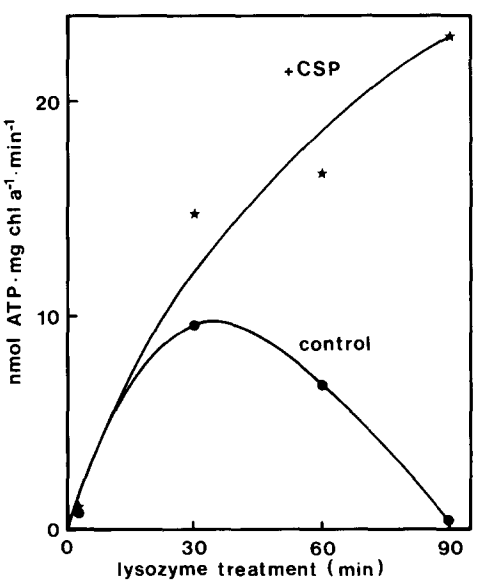

Fig. 2. Effect of concentrated soluble proteins on the rate of dark respiratory ATP synthesis in cells of *P. boryanum* at increasing times of lysozyme treatment. ●, no addition; ★, + 10 $\mu l \cdot ml^{-1}$ concentrated soluble proteins (CSP). Further details in Materials and Methods.

TABLE I

THE EFFECTS OF CONCENTRATED SOLUBLE PROTEINS (CSP) AND KCN ON NADPH OXIDATION IN DIFFERENT MEMBRANE PREPARATIONS

The concentration of concentrated soluble proteins was 10 μg protein per ml and 10 μM of KCN. Two methods of measurements have been used, oxygen electrode measurements (O) and spectrophotometric measurements (S). Further details in Materials and Methods. Single determinations never deviated more than 20% from the mean ($n \geq 3$), deviations are expressed as S.D. values.

Assay condition		Control rate (nmol NADPH/mg Chl *a* per min)	Inhibited rate (nmol NADPH/mg Chl *a* per min)	Inhibition (%)	Method of measurement
preparation	inhibitors added				
Intact cells	KCN	198±17	23±3	88±12	O
Spheroplasts	KCN	113±10	54(22–79) [a]	52(30–80) [a]	S, O
Washed spheroplasts	KCN	64± 6	59±6	8± 6	S, O
Washed spheroplasts	KCN, CSP	70± 6	26(14–30) [a]	63(60–80) [a]	S
Membrane vesicles	KCN	40± 5	8±1	80± 4	S, O
Membrane vesicles	KCN, CSP	43± 5	8±1	83± 4	S

[a] Lowest and highest of five separate measurements.

apparently a soluble protein factor, required for dark energy conservation, but not for photophosphorylation, is released and diluted during lysozyme treatment and spheroplast lysis.

Effects of soluble proteins and KCN on dark NADPH oxidation

Dark respiratory electron transfer in different preparations of *P. boryanum* was tested for its sensitivity to inhibition by KCN (Table I). The preparations probably differ in the degree of scrambling of the thylakoid and cell membranes [28]. O_2 uptake by intact cells was highly sensitive to KCN. NADPH-induced O_2 uptake in spheroplasts was inhibited by KCN at variable degree, washing of these spheroplasts in hypotonic medium resulted in a strong decrease of inhibition. Interestingly, this decrease could largely be overcome by addition of concentrated soluble proteins. However, not quite as expected, concentrated soluble proteins do not stimulate NADPH oxidation very much. Membrane vesicles prepared by sonication presumably consisting of scrambled fragments of both thylakoid and cell membranes were highly sensitive to KCN, regardless the absence or presence of concentrated soluble proteins.

TABLE II

RATES OF ATP SYNTHESIS IN LEAKY CELL PREPARATIONS

Inhibitors were added as indicated. Determinations with one preparation deviated no more than 20% of the corresponding average ($n \geq 3$), deviations are expressed as S.D. values. Further details in Materials and Methods.

Preparation number	Inhibitors added	Control rate (nmol ATP/mg Chl *a* per min)	Inhibited rate (nmol ATP/mg Chl *a* per min)	Inhibition (%)
1	DNP-INT (1 μM) [a]	30± 4	19± 3	35± 4
2	DBMIB (1 μM)	47± 5	9± 2	81± 4
3	DBMIB (10 μM)	203±17	65± 7	68± 6
	DBMIB (10 μM)+KCN (10 μM)	203±17	65± 7	68± 6
	DBMIB (10 μM)+KCN (10 mM)	203±17	49± 5	76± 6
4	KCN (10 μM)	230±27	120±12	48±21
	KCN (10 mM)	230±27	90± 8	61±19

[a] DNP-INT was added prior to NADPH.

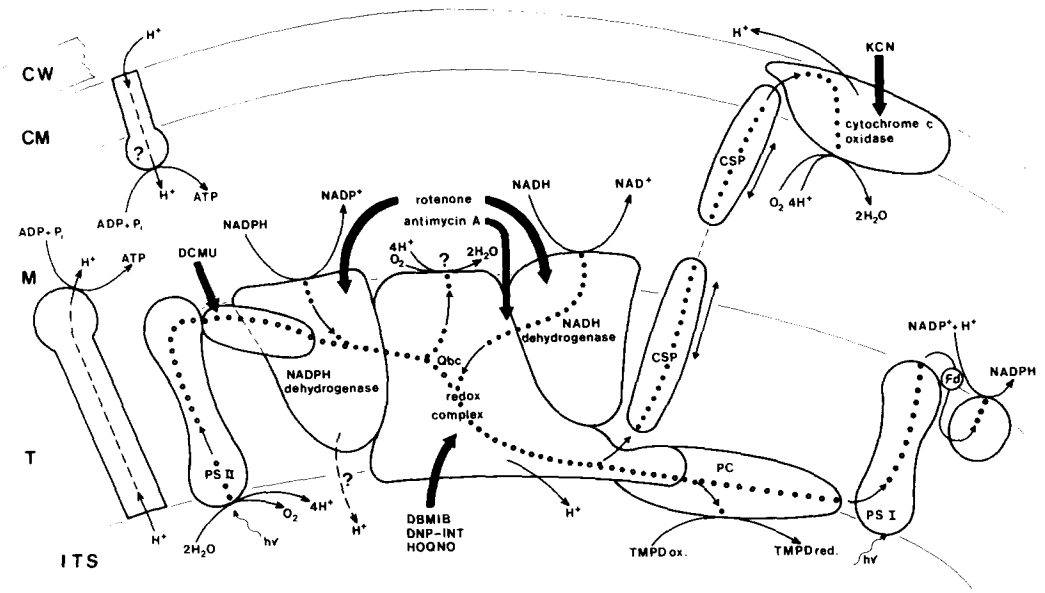

Fig. 3. Scheme of electron transfer pathways in *P. boryanum*. O——O indicates light-mediated, and ●——● dark respiratory electron transfer. CW, cell wall; CM, cell membrane; M, matrix; T, thylakoid membrane; ITS, inner thylakoid space.

Effects of electron transfer inhibitors on dark oxidative phosphorylation

Dark ATP synthesis has also been studied in the presence of electron transfer inhibitors acting at the Qbc redox complex (DNP-INT and DMBIB) and at the terminal oxidase (KCN). These inhibitors alone, or in combination, all lower the rate of dark ATP synthesis (Table II). The control rates usually vary to a large extent between preparations of different cultures.

Discussion

In the present work we have described a method to obtain preparations of *P. boryanum* that actively phosphorylate exogenously-added ADP in the dark during NADPH oxidation. A lysozyme treatment of only 30 min resulted in a preparation that remained osmotically stable but apparently became leaky to added ADP and NADPH, and showed appreciable rates of dark ATP synthesis. The ATP/2e ratois in NADPH respiration of up to 2.3 demonstrate efficiencies that are compatible with the value of 3.0 detected in intact cells of *Aphanocapsa* 6714 by Pelroy and Bassham [8] and the value of 2.8 that was detected recently by Nitschman and Peschek [13] in intact cells of *Anacystis nidulans*.

Oxidative phosphorylation in cell free preparation of cyanobacteria has been studied by Leach and Carr [6]. They observed a phosphorylation associated with NADPH oxidation with an ATP/2e ratio of about 0.4 in sonicated preparations of *Anabaena variabilis*. Peschek [11] reported a slow dark ATP synthesis activity in sonicated preparations of *Anacystis nidulans*. We reported before [19] that treatment of *P. boryanum* with lysozyme for 2 h and subsequent osmotic shock gave membrane vesicles that showed appreciable photophosphorylation and dark NADPH oxidation but only poor oxidative phosphorylation activity. From the latter study it is concluded that neither inhibition of electron transfer nor uncoupling may explain the loss of oxidative phosphorylation activity. In the experiment of Fig. 2 we have shown that the decrease in the rate of dark oxidative ATP synthesis after prolonged treatment with lysozyme (60–90 min) could be reversed by addition of a concentrate of soluble proteins. Moreover, the addition of concentrated soluble proteins also restores the KCN inhibition of NADPH oxidation in washed spheroplasts. From these results it is concluded that a readily soluble protein factor required for dark respiratory ATP synthesis

also restores the sensitivity of respiration to KCN. Although we did not yet further identify this protein, reports on the influence of *c*-type cytochromes in reconstitution of respiration activity of depleted membranes [26] suggests that this protein may be a *c*-type cytochrome. *c*-Type cytochromes have been shown to donate electrons to a cytochrome aa_3-type of terminal oxidase in several cyanobacteria [27].

In the preceding paper [28] on the participation of the photosynthetic electron transfer chain in the dark respiration of NADPH and NADH in cell free preparations of *P. boryanum,* we concluded that O_2 reduction may take place at two different sites, one partly induced in vitro situated near to the NADPH dehydrogenase and another KCN-sensitive one, presumably cytochrome $c : O_2$ oxidoreductase, situated beyond the Qbc redox complex. The conclusions on dark NADPH oxidation pathways are further substantiated in the present work as it was shown in Table II that DNP-INT, DMBIB and KCN also inhibit dark ATP synthesis. The variation in the degree of inhibition (cf. lines 2 and 3) may be explained by the preparation-dependent variation of the use of either of these O_2 reducing pathways.

A tentative scheme of electron transfer pathways is depicted in Fig. 3. This scheme of electron transfer pathways combines the conclusions of the reports that suggest the participation of membrane areas or molecular environments outside of the thylakoid membranes [12,29–31,38] and of those that demonstrate the participation of the thylakoid membranes in dark respiration [31–36]. The polarity of proton gradients is analogous to the scheme of Padan and Schuldiner [15] and corresponds with the proposed cytochrome $c : O_2$ oxidoreductase imposed proton gradient [37] (acidic outside) across the cell membrane in dark respiring spheroplasts [38]. We assume that soluble proteins transfer electrons through the cytoplasm to the cell membrane where they are accepted at or near cytochrome $c : O_2$ oxidoreductase. A similar (cytochrome-*c*-mediated) electron shuttle is known for the outer membrane electron transfer route in mitochondria [39]. The serious problem that the electron-carrying soluble protein has to penetrate through the thylakoid membrane as well as in the cell membrane in order to reach the electron-donating and -accepting sites (cf. Fig. 3) would be solved if the membrane containing cytochrome $c : O_2$ oxidoreductase has the same orientation as or is part of the thylakoids as suggested by Binder [36]. Results in literature [18,38,41] as well as unpublished results from our laboratory justify the localization of cytochrome $c : O_2$ in the cell membrane but do not definitely exclude its presence in the thylakoid membrane. However, further evidence for the scheme of Fig. 3 has been obtained from the determination of a membrane-bound copper containing compound (presumably cytochrome $c : O_2$ oxidoreductase) in the cell membrane fraction of separated thylakoid and cell membranes of *P. boryanum* [40] and from studies on 9-amino-6-chloro-2-methoxy-acridine fluorescence quenching and enhancement in relation to energy transduction in spheroplasts and intact cells of *P. boryanum* (see Ref. 41 and Matthijs, H.C.P., Van Steenbergen, J.M. and Kraayenhof, R., unpublished data). Finally, the observations of Imafuku and Katoh [32] and Webster and Frenkel [42] that respiratory O_2 uptake stops when photoelectron transfer is initiated may be explained by a regulation in the cytochrome *c* and plastocyanin area of the thylakoid membranes. This could result in electron transfer to Photosystem I on the inside of the thylakoid membrane, in the light, or to concentrated soluble proteins on the outside, in the dark (cf. Fig. 3).

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References

1 Golecki, J.R. and Drews, G. (1982) in The Biology of Cyanobacteria (Carr, N.G. and Whitton, B.A., eds.), pp. 125–129, Blackwell Scientific Publications, Oxford
2 Nierzwicki-Bauer, S.A., Balkwill, D.L. and Stevens, S.E., Jr. (1983) J. Cell Biol. 97, 713–722
3 Krogman, D.W. (1977) in Encyclopedia on Photosynthesis I (Trebst, A. and Avron, M., eds.), Springer-Verlag, München, pp. 625–636
4 Stanier, R.Y. (1977) Carlsberg Res. Commun. 42, 77–98
5 Biggins, J. (1969) J. Bacteriol. 99, 570–575
6 Leach, C.K. and Carr, N.G. (1969) Biochem. J. 112, 125–126
7 Peschek, G.A. (1982) Naturwissenschaften 69, 599

8 Pelroy, R.A. and Bassham, J.A. (1973) J. Bacteriol. 115, 937–942
9 Bornefeldt, T. and Simonis, W. (1974) Planta 115, 309–318
10 Ginzberg, D., Padan, E. and Shilo, M. (1976) Biochim. Biophys. Acta 423, 440–449
11 Peschek, G.A. (1980) Arch. Microbiol. 125, 123–131
12 Almon, H. and Böhme, H. (1982) Biochim. Biophys. Acta 679, 279–286
13 Nitschmann, W.H. and Peschek, G.A. (1982) FEBS Lett. 139, 77–80
14 Binder, A., Tel-Or, E. and Avron, M. (1976) Eur. J. Biochem. 67, 187–196
15 Padan, E. and Schuldiner, S. (1978) J. Biol. Chem. 253, 3281–3286
16 Wax, E. and Lockau, W. (1980) Z. Naturforsch. 35c, 98–105
17 Spiller, H. (1980) Plant Physiol. 66, 446–450
18 Ono, T.A. and Murata, N. (1978) Biochim. Biophys. Acta 502, 477–485
19 Matthijs, H.C.P., Scholts, M.J.C. and Schreurs, H. (1981) in Photosynthesis II. Photosynthetic Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 269–278, Balaban International Science Services Philadelphia, PA
20 Lindsey, J.K., Vance, B.D., Keeter, J.S. and Scholes, V.E. (1971) J. Phycol. 7, 65–71
21 Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) J. Gen. Microbiol. 111, 1–61
22 Larsson, C.M. and Olsson, T. (1979) Plant Cell Physiol. 20, 145–155
23 Webster, J.J., Chang, J.C., Manley, E.R., Spirey, H.O. and Leach, F.R. (1980) Anal. Biochem. 106, 7–11
24 Lowry, O.H., Rosebrough, A.L., Farr, R.J. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
25 MacKinney, G. (1941) J. Biol. Chem. 140, 315–322
26 Lockau, W. (1981) Arch. Microbiol. 128, 336–340
27 Kienzl, P.F. and Peschek, G.A. (1982) Plant Physiol. 69, 580–584
28 Matthijs, H.C.P., Ludérus, E.M.E., Löffler, H.J.M., Scholts, M.J.C. and Kraayenhof, R. (1984) Biochim. Biophys. Acta 766, 29–37
29 Scherer, S., Stürzl, E. and Böger, P. (1981) Z. Naturforsch. 36c, 1036–1040
30 Peschek, G.A., Muchl, R., Kienzl, P.F. and Schmetterer, G. (1982) Biochim. Biophys. Acta 679, 35–43
31 Peschek, G.A., Schmetterer, G. and Sleytr, U.B. (1981) FEMS Microbiol. Lett. 11, 121–124
32 Imafuku, H. and Katoh, T. (1976) Plant Cell Physiol. 17, 515–524
33 Hirano, M. Satoh, K. and Katoh, S. (1980) Photosynth. Res. 1, 149–162
34 Eisbrenner, G. and Bothe, H. (1979) Arch. Microbiol. 123, 37–45
35 Peschek, G.A. and Schmetterer, G. (1982) Biochem. Biophys. Res. Commun. 108, 1188–1195
36 Binder, A. (1982) J. Bioenerg. Biomembr. 14, 271–286
37 Krab, K. and Wikström, M. (1979) Biochim. Biophys. Acta 548, 1–15
38 Peschek, G.A. (1983) J. Bacteriol. 153, 539–542
39 Bernardi, P. and Azzone, G.F. (1981) J. Biol. Chem. 256, 7187–7192
40 Matthijs, H.C.P., Van Hoek, A.N., Löffler, H.J.M. and Kraayenhof, R. (1983) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 643–646, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
41 Barsky, E.L., Gusev, M.V., Nikitina, K.A., Samuilov, V.D. (1981) Arch. Microbiol. 129, 105–108
42 Webster, G.C. and Frenkel, A.W. (1952) Plant Physiol. 63–69