Biochimica et Biophysica Acta, 333 (1974) 279–287

BBA 46698

EPR SIGNALS OF OXIDIZED PLASTOCYANIN IN INTACT ALGAE

J. W. M. VISSER[a], J. AMESZ[a] and B. F. VAN GELDER[b]

[a]*Biophysical Laboratory of the State University, P.O. Box 556, Leiden (The Netherlands) and* [b]*Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, Amsterdam (The Netherlands)*

(Received August 21st, 1973)

SUMMARY

1. An electron paramagnetic resonance (EPR) signal was observed at $g = 2.05$ in the low temperature spectra of intact cells of green, red and blue-green algae and of spinach chloroplasts. The g-value and the shape of the signal were similar to that of purified, soluble plastocyanin.

2. The amount of the copper protein, determined from the EPR signal height, was estimated to be nearly the same in all the studied organisms on the basis of the concentration of chlorophyll. Furthermore, it was found that the amount of the copper protein, determined from the EPR signal height in spinach chloroplasts corresponds with that of plastocyanin as determined chemically by Katoh, S., Suga, I., Shiratori, I. and Takamiya, A. (1961) Arch. Biochem. Biophys. 94, 136–141.

3. Experiments with far-red and red illumination show that the site of the copper protein in vivo is in the electron transport pathway between Photosystems 1 and 2. Plastocyanin is not oxidized by illumination at 77 °K, indicating that no electron transfer occurs between the primary electron donor of Photosystem 1, P700, and plastocyanin at that temperature. Furthermore, the experiments suggest that in the intact cells of the studied algae, plastocyanin is not only reduced by Photosystem 2 but also by cyclic electron transport around Photosystem 1.

INTRODUCTION

In 1960 Katoh [1] reported the discovery of plastocyanin, a copper containing protein, in *Chlorella ellipsoidea*. Subsequently the protein was shown to be present in various higher plants, in green algae, and in the blue-green alga *Anabaena variabilis* [2–4]. However, until recently almost all knowledge about the role of plastocyanin in photosynthesis was based on more or less indirect evidence from experiments with mutants, and with chloroplast fragments from which the protein had been removed,

Abbreviations: P700, chlorophyllous pigment absorbing at 700 nm, primary electron donor of Photosystem 1; P680, chlorophyllous pigment absorbing at 680 nm, presumably the primary electron donor of Photosystem 2; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; tricine, *N*-tris-(hydroxymethyl)methylglycine.

and from experiments with certain inhibitors. Spectrophotometric experiments to directly study oxido-reduction reactions of plastocyanin in vivo have not given clear-cut results [5, 6], because of its broad absorption bands and low extinction coefficients, which made it more difficult to observe than e.g. cytochrome *f*. Studies of the kinetics of known electron transport intermediates in the red alga *Porphyridium aerugineum* gave no evidence for participation of plastocyanin in the electron transport chain (ref. 7 and Amesz, J. and Pulles, M. P. J., unpublished observations). So far, in vitro experiments suggested that plastocyanin is an electron carrier located in the chain between Photosystem 1 and 2, at a site near cytochrome *f*. However, conflicting evidence has been reported about the location relative to cytochrome *f* and P700, a chlorophyllous pigment absorbing at 700 nm and the primary donor of photosystem 1 (e.g. refs. 8–15).

Blumberg and Peisach [16] reported in 1966 the EPR spectrum of soluble plastocyanin, extracted from *Chenopodium album*. Until recently the characteristic EPR bands of the copper protein had not been detected in photosynthetic material, as the concentration of the protein in the organisms is too low to cause detectable electron paramagnetic resonance (EPR) signals at the commonly used measuring temperature of boiling nitrogen, 77 °K. Recently, however, Malkin and Bearden [17] measuring at 25 °K, reported the discovery of the EPR signals of a copper protein in spinach chloroplasts. They identified the measured signals as originating from oxidized plastocyanin on the basis of the parameters of the signals, the amount of the copper protein causing the signals and the sensitivity to illumination of the amplitude of the signals. Their results supported the notion that plastocyanin is located in the electron transport chain between the two photosystems.

In this paper we report measurements at 20 °K of an EPR signal caused by a copper protein and occurring in intact cells of various organisms. It will be concluded that this signal, like the one in chloroplasts, originates from oxidized plastocyanin. The results to be reported indicate that the site of action of plastocyanin in vivo is in the electron transport chain between Photosystems 1 and 2, and that it is also involved in a cyclic electron pathway around Photosystem 1.

MATERIALS AND METHODS

Materials

The algae were grown in liquid culture medium at 25 °C as described elsewhere [18]. *Scenedesmus obliquus* and *Chlorella vulgaris* were grown in the media given by refs 19 and 20 (M.C. medium), respectively; the growth media for *P. aerugineum* and *Anacystis nidulans* are given in ref. 18. All media were modified slightly by omitting $MnCl_2 \cdot 4H_2O$, since otherwise manganese signals were dominating the EPR spectra. No effect of omission of manganese on the growth was observed. Control experiments with *P. aerugineum* grown with and without $MnCl_2$ showed that the light-induced absorbance changes at 552 (cytochrome *f*) and at 703 nm (P700) and the fluorescence change at 683 nm (Q), measured as described in ref. 7 were similar. It should be noted that, since manganese is necessary for photosynthesis [9] presumably some of it was present in the media, originating from the parent cells, which were taken from normal media.

The algae were harvested by centrifugation and stored in concentrated suspen-

sion at 0 °C, generally until about 5 min before use. The absorbances at 680 nm of the suspensions used, corrected for scattering, were 2.2 mm^{-1} (*S. obliquus*), 1.0 mm^{-1} (*P. aerugineum*), 4.2 mm^{-1} (*A. nidulans*), 2.2 mm^{-1} (*C. vulgaris*, Figs 3A, 3B, 3C) and 7.0 mm^{-1} (Figs 3D and 3E). In order to determine these absorbances an aliquot of the suspension was diluted such that accurate measurements could be performed.

Chloroplasts were obtained from market spinach. Leaves (60 g) were washed and ground in a cooled blendor for about 40 s in a solution (80 ml) of pH 7.8 containing 50 mM *N*-tris(hydroxymethyl)methylglycine (tricine), 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$. The homogenate was filtered through four layers of nylon cloth and the filtrate was briefly centrifuged at up to $8000 \times g$. The chloroplast pellet was resuspended in 1 ml of the same buffered solution, and stored at 0 °C in the dark. The concentration of chlorophyll was determined according to Arnon [21]. The stored suspension usually contained about 5 mg chlorophyll per ml. The chloroplasts were resuspended shortly before use in a small volume of the buffered solution to a final concentration of 1.0 mg chlorophyll per ml ($A_{680\,nm} = 3.5\ mm^{-1}$). Usually 0.1 mM methylviologen was added as electron acceptor.

Soluble oxidized plastocyanin, isolated from spinach [22], was a gift of Dr J. S. C. Wessels (Natuurkundig Laboratorium Philips B.V., Eindhoven). The absorbance index, $A_{278/597}$, of the sample was 2.3. The concentration was calculated with $\varepsilon_{597} = 9.8\ mM^{-1} \cdot cm^{-1}$ (ref. 23).

EPR measurements

Electron paramagnetic resonance spectra were recorded using a Varian E-3 EPR spectrometer operating near 9.1 GHz, at 10 mW. The microwave frequency was calibrated by use of a frequency counter (Hewlett–Packard 5246L) with frequency convertor (5255A). The field strength was calibrated using an AEG Magnetfield meter GA 11–22.2. First derivative EPR spectra were obtained by 100 kHz modulation of the magnetic field. Samples in standard quartz tubes (3 mm inner diameter) were cooled to 20 °K by a stream of helium, provided by a liquid transfer system (model LTD-3-100) with automatic temperature controller (OC-20) from Air Product Inc. The temperature was measured with a carbon resistor, previously calibrated against a calibrated germanium resistor. For comparative measurements the same tube was used with different samples in order to avoid effects of different tube sizes and background signals.

Illumination

An Aldis slide projector (500 W) was used for the illumination. The beam passed through a cuvette (5 mm pathway) filled with water, and a filter combination consisting of a Balzers Calflex C heat reflecting filter and additional Schott AL interference or colored glass filters. The following filters and intensities ($mW \cdot cm^{-2}$) were used: AL 705 nm, 25; AL 718 nm, 20; AL 643 nm, 35; AL 617 nm, 50 and RG 630, 300.

Samples were illuminated in EPR tubes. Since rather dense suspensions had to be used, reflectors were placed to illuminate also the rear side of the sample tubes. Chloroplasts were irradiated at room temperature and then frozen in liquid nitrogen in the dark. The freezing procedure took about 20 s. Algae were irradiated before and during the freezing in liquid nitrogen. In some experiments samples were illuminated at 77 °K. The frozen samples were cooled to 20 °K in the cavity of the EPR apparatus.

RESULTS AND DISCUSSION

Occurrence of plastocyanin in algae

The upper spectrum of Fig. 1 shows the larger line ($g_{\perp} = 2.05$) of the EPR spectrum of purified plastocyanin in the oxidized state at 20 °K. At a lower field strength four hyperfine lines of $g_{//}$ are observed (not shown) with similar g-value ($g_{//} = 2.24$) and hyperfine splitting constant ($A_{//} = 190$ Mcycles/s) as reported in the literature [16, 17]. These four lines are too weak to be detected in the intact cells and

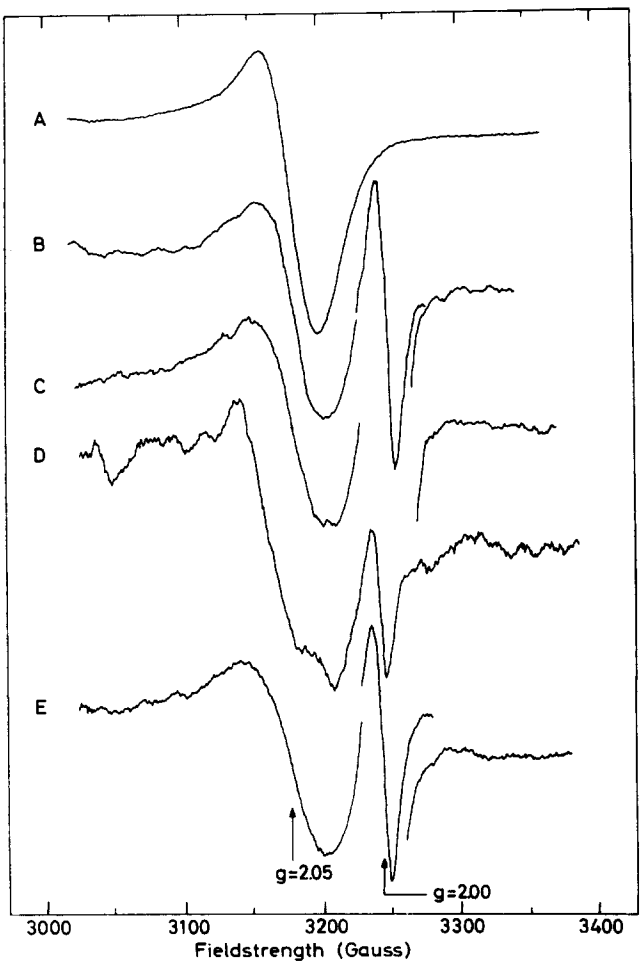

Fig. 1. EPR spectra taken at 20 °K. (A) Oxidized plastocyanin (36 μM) in phosphate Tris buffer, pH 7.8. (B) Spinach chloroplasts, illuminated at room temperature for 10 s with 705 nm light in the presence of 0.1 mM methylviologen, then kept in darkness for 10 s and subsequently frozen. (C) *S. obliquus*, illuminated at room temperature and during the initial freezing for about 30 s with 718 nm light. (D) *P. aerugineum*, illuminated with 705 nm light as for (C). (E) *A. nidulans*, illuminated as for (D). Instrument settings: frequency 9.11 GHz; power 10 mW; modulation amplitude 10 G; time constant 1.0 s; scan rate 1 G/s. Receiver gain settings: (A) $2.0 \cdot 10^4$, (B) $2.0 \cdot 10^5$, (C) $2.5 \cdot 10^5$, (D) $5.0 \cdot 10^5$ and (E) $1.5 \cdot 10^5$. The signal near $g = 2.00$ of (B) and (E) was recorded with modulation amplitude of 5 G.

therefore we will report only about the line at $g = 2.05$. Spectrum B was measured with spinach chloroplasts, illuminated with far-red light at room temperature, then kept in the dark for 10 s and frozen to 20 °K. In this spectrum a signal ($g = 2.05$) can be seen at the same position and similar in shape as in the spectrum of purified plastocyanin. The signal is also observed in illuminated cells of *S. obliquus* (Fig. 1C), *P. aerugineum* (Fig. 1D), *A. nidulans* (Fig. 1E) and *C. vulgaris* (see Fig. 3B). Therefore, it is likely that this signal in spinach chloroplasts and in the organisms tested, originates from plastocyanin. As far as we know, this is the first time that the occurrence of plastocyanin has been observed in a red alga.

In all spectra, except the one of purified plastocyanin, a signal could be seen near $g = 2.00$. This signal is known to be composed of at least two constituents, denoted Signal I and Signal II (ref. 24). Signal I is caused by oxidized P700, the primary electron donor of Photosystem 1 (e.g. ref. 25), whereas Signal II is related to Photosystem 2 (e.g. refs. 26 and 27).

In Fig. 2A the spectrum is given of the signal at $g = 2.00$, measured with the same *S. obliquus* sample as in Fig. 1C, but recorded with half the modulation amplitude. Fig. 2B represents the spectrum of a sample, recorded at a 3.0-fold increased amplification, and which was kept in the dark for one min before and during the freezing. It is evident that the signal height is three times larger in the illuminated than in the dark sample. This difference will be mainly caused by the different redox states of P700 in the two samples; in the illuminated sample P700 is oxidized, whereas in the dark sample P700 is probably reduced. The signal that persists in the latter sample is mainly Signal II [24]. Recent evidence [28] suggests that oxidized P680 (a chlorophyllous pigment absorbing at 680 nm and presumably the primary electron donor of Photosystem 2) also causes an EPR signal near $g = 2.00$. As this signal was only observed in the presence of an oxidant, and no oxidant was added in our experiments, it is unlikely that the difference between the two spectra is caused by P680.

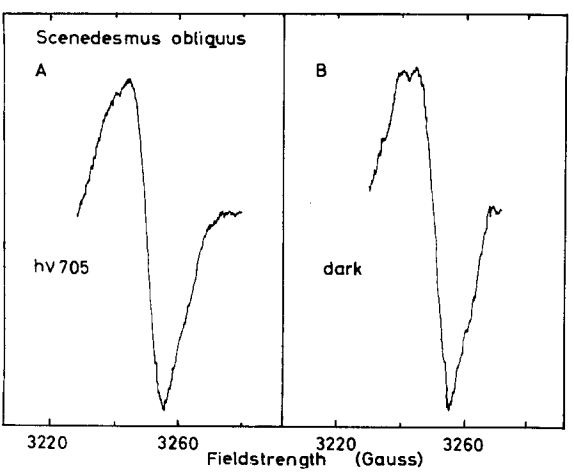

Fig. 2. The EPR signal at $g = 2.00$ of *S. obliquus*. (A) Sample illuminated with 718 nm light for about 30 s before and during the freezing. (B) Dark sample. Conditions and instrument settings as in Fig. 1, except for modulation amplitude, 5 G and receiver gain setting, (A) $5.0 \cdot 10^5$ and (B) $1.5 \cdot 10^6$.

From spectra like those given in Figs 1 and 2 it was possible to correlate the redox states of P700 and of plastocyanin under various conditions in all organisms. The concentration of the copper protein, causing the signal at $g = 2.05$ in chloroplasts was calculated from the height (top—bottom) of the copper signal (see Fig. 1B) and compared with that of a known concentration of purified plastocyanin (Fig. 1A). The amount of plastocyanin was found to be one mole per 400 moles of chlorophyll, in fair agreement with the results of Malkin and Bearden [17] and with the amounts of plastocyanin present in chloroplasts as determined chemically by Katoh and coworkers [2].

The concentration of chlorophyll in the suspensions of the algae was difficult to determine by extraction and therefore the concentration was correlated with that in the chloroplast suspension using the absorbance at 680 nm, corrected for scattering. From this and from the spectra of Figs 1 and 3 the concentration of plastocyanin relative to that of chlorophyll in the algae was calculated (Table I). In the same way spectra of chloroplasts and of some algae were compared with respect to the light-induced signal at $g = 2.00$ of P700 (Table I, right hand column). It is clear from this table that the copper protein causing the signal is present in nearly the same amounts in the algae tested and in spinach chloroplasts. However, since it is not known in what sequence the various electron transport reactions on the oxidizing and reducing side

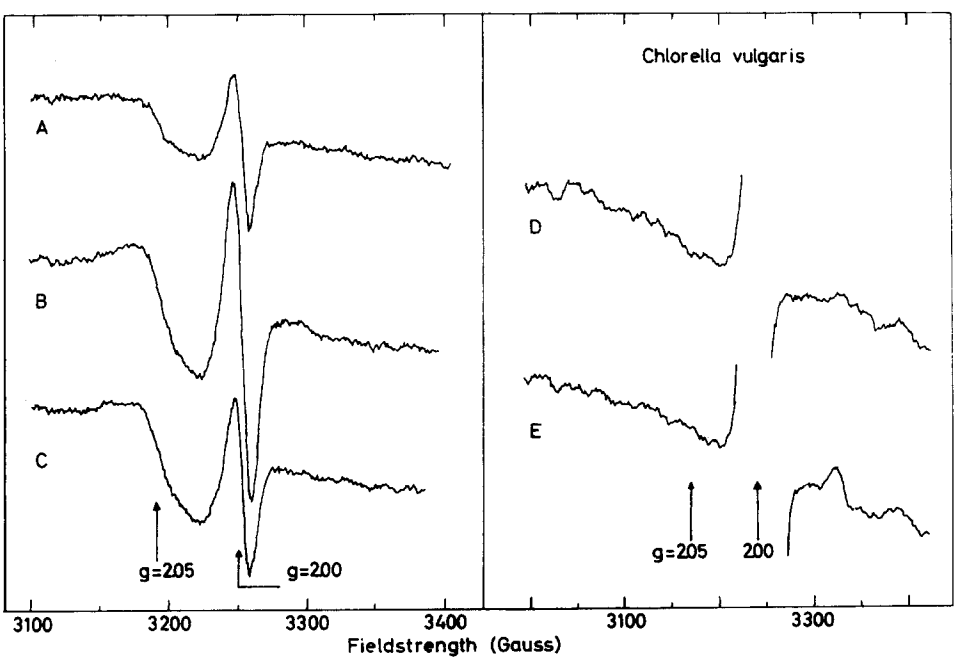

Fig. 3. *Chlorella vulgaris* (A) sample illuminated with 643 nm light, (B) with 705 nm light, (C) with 643 nm light in the presence of 0.1 mM DCMU, (D) sample, kept in the dark for about 1 min before and during the freezing, (E) sample kept in the dark as for (D), and illuminated at 77 °K for about 2 min with white light. Conditions and instrument settings as in Fig. 1, except for (A), (B), (C), receiver gain setting $2.0 \cdot 10^5$, (D) and (E) receiver gain setting $6.2 \cdot 10^5$, temperature 10 °K and frequency 9.13 GHz.

TABLE I

RELATIVE AMOUNTS OF PLASTOCYANIN IN VARIOUS ORGANISMS

Amounts of plastocyanin are calculated relative to the amounts of chlorophyll *a* and P700 for each organism. The values are compared to that of chloroplasts (100 %). The standard errors were estimated from the noise level of the recordings and from the accuracy of the determination of the chlorophyll absorbancy.

Organism	Plastocyanin (% per chlorophyll a)	Plastocyanin (% per P700)
Spinach chloroplasts	100	100
C. vulgaris	100 ± 7	90 ± 8
S. obliquus	100 ± 7	80 ± 8
P. aerugineum	120 ± 12	120 ± 20
A. nidulans	90 ± 7	70 ± 7

of Photosystem 1 are inhibited during the cooling process, we cannot be sure that plastocyanin and P700 were completely oxidized.

Site of action of plastocyanin in vivo

In Figs 1 and 3B it is shown that plastocyanin is in the oxidized state, when spinach chloroplasts and cells of *S. obliquus*, *P. aerugineum*, *A. nidulans* and *C. vulgaris* were illuminated with Photosystem 1 light at room temperature. In *P. aerugineum* plastocyanin was oxidized after far-red illumination as well as after a dark period of 15 min. In contrast to this in the other algae plastocyanin was reduced after some seconds of darkness, as is shown in Fig. 3D for *C. vulgaris*. Therefore, these algae must be illuminated during the freezing in order to observe the signals of oxidized P700 and plastocyanin. This is not the case with spinach chloroplasts, since in a sample, which was illuminated for 10 s with far-red light and then after a few min frozen in the dark, P700 and plastocyanin were in the oxidized state. However, after a dark period of 30 min, plastocyanin and P700 were fully reduced. Unlike in the experiments of Malkin and Bearden [17] addition of a reductant was not needed to convert plastocyanin to the reduced state in the dark. This difference between the rates of dark reduction of plastocyanin in the dark after far-red illumination, can be explained by a cyclic electron flow around Photosystem 1, which normally occurs in algae [29, 30], but not in chloroplasts unless a redox mediator is added [31, 32]. This suggests that plastocyanin is reduced in intact cells via cyclic electron transport by a reductant formed by Photosystem 1.

Plastocyanin is also reduced by Photosystem 2. This reaction is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) as can be seen in Fig. 3 for *C. vulgaris*. It is clear that plastocyanin is oxidized after far-red illumination (Spectrum B), but that it is partly in the reduced state in the absence of DCMU after 643 nm illumination (Spectrum A). In the presence of DCMU, however, plastocyanin is found to be oxidized after red illumination (Spectrum C). Similar results were obtained with spinach chloroplasts (see also ref. 17) and with *P. aerugineum*. These results indicate that plastocyanin is a component of the electron transport chain between Photosystems 1 and 2 in vivo, and that it is located at the Photosystem 1 side of the DCMU block. In order to obtain more definite proof about this site of action of plastocyanin

it will be necessary to measure rates of reduction and oxidation, as has been done for other components by spectrophotometric methods [33].

The high efficiency of cytochrome *f* photooxidation and reduction in *P. aerugineum* [33] has been taken as evidence that the electron transport chain does not contain other components of about the same redox potential as cytochrome *f* [7]. Therefore the present results may indicate that, although in vitro the redox potentials of cytochrome *f* and plastocyanin are almost equal, they are different when bound to the photosynthetic membrane (cf. ref. 34).

As is shown in Fig. 3E plastocyanin is not oxidized upon illumination at 77 °K in *C. vulgaris*, but at these conditions the oxidation of P700 is not inhibited, as indicated by the increase of the signal at $g = 2.00$ (not shown), and by the occurrence of the line at $g = 1.94$, which is caused by reduced ferredoxin (cf. ref. 35). This shows that P700 does not oxidize plastocyanin at 77 °K. The same results, but not shown, were obtained with spinach chloroplasts and with *A. nidulans*. Since cytochrome *f* is also not oxidized at 77 °K (ref. 36), no information concerning the sequence of cytochrome *f* and plastocyanin in the electron transport chain can be obtained from these experiments.

ACKNOWLEDGEMENTS

We wish to thank Mr B. van Swol and Miss W. van der Plas for technical assistance. The plastocyanin was a kind gift of Dr J. S. C. Wessels.

The investigation was supported in part by the Netherlands Foundation for Chemical Research (S.O.N.), financed by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

REFERENCES

1 Katoh, S. (1960) Nature 186, 533–534
2 Katoh, S., Suga, I., Shiratori, I. and Takamiya, A. (1961) Arch. Biochem. Biophys. 94, 136–141
3 Gorman, D. S. and Levine, R. P. (1966) Plant Physiol. 41, 1637–1642
4 Lightbody, J. J. and Krogman, D. W. (1967) Biochim. Biophys. Acta 131, 508–515
5 De Kouchkovsky, Y. and Fork, D. C. (1964) Proc. Natl. Acad. Sci. U.S. 54, 232–239
6 Fork, D. C. and Urbach, W. (1965) Proc. Natl. Acad. Sci. U.S. 53, 1307–1315
7 Amesz, J., Visser, J. W. M., van den Engh, G. J. and Pulles, M. P. J. (1972) Physiol. Vég. 10, 319–328
8 Boardman, N. K. (1968) Adv. Enzymol. 30, 1–79
9 Bishop, N. I. (1971) Ann. Rev. Biochem. 40, 197–226
10 Avron, M. and Shneyour, A. (1971) Biochim. Biophys. Acta 226, 498–500
11 Knaff, D. B. and Arnon, D. I. (1970) Biochim. Biophys. Acta 223, 201–204
12 Fork, D. C. (1972) Biophys. J. 12, 909–921
13 Knaff, D. B. (1973) Biochim. Biophys. Acta 292, 186–192
14 Plesničar, M. and Bendall, D. S. (1973) Eur. J. Biochem. 34, 483–488
15 Kimimura, M. and Katoh, S. (1972) Biochim. Biophys. Acta 283, 279–292
16 Blumberg, W. E. and Peisach, J. (1966) Biochim. Biophys. Acta 126, 269–273
17 Malkin, R. and Bearden, A. J. (1973) Biochim. Biophys. Acta 292, 169–185
18 Hoogenhout, H. and Amesz, J. (1965) Arch. Mikrobiol. 50, 10–24
19 Kessler, E., Arthur, W. and Bruggen, J. E. (1957) Arch. Biochem. Biophys. 71, 326–335
20 Watanabe, A. (1960) J. Gen. Appl. Microbiol. 6, 283–292
21 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
22 Borchert, M. T. and Wessels, J. S. C. (1970) Biochim. Biophys. Acta 197, 78–83

23 Katoh, S., Shiratori, I. and Takamiya, A. (1962) J. Biochem. 51, 32–40
24 Weaver, E. C. (1968) Ann. Rev. Plant Physiol. 19, 283–294
25 Warden, J. T. and Bolton, J. R. (1972) J. Am. Chem. Soc. 12, 4351–4353
26 Lozier, R. H. and Butler, W. L. (1973) Photochem. Photobiol. 17, 133–137
27 Kohl, D. H., Wright, J. R. and Weissman, M. (1969) Biochim. Biophys. Acta 180, 536–544
28 Malkin, R. and Bearden, A. J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 294–297
29 Urbach, W. and Simonis, W. (1964) Biochem. Biophys. Res. Commun. 17, 39–45.
30 Amesz, J., Pulles, M. P. J., Visser, J. W. M. and Sibbing, F. A. (1972) Biochim. Biophys. Acta 275, 442–452
31 Arnon, D. I., Whatley, F. R. and Allen, M. B. (1954) J. Am. Chem. Soc. 76, 6324–6339
32 Forti, G. and Jagendorf, A. T. (1961) Biochim. Biophys. Acta 54, 322–330
33 Amesz, J., Visser, J. W. M., van den Engh, G. J. and Dirks, M. P. (1972) Biochim. Biophys. Acta 256, 370–380
34 Malkin, R., Knaff, D. B. and Bearden, A. J. (1973) Biochim. Biophys. Acta 305, 675–678
35 Evans, M. C. W., Reeves, S. G. and Telfer, A. (1973) Biochem. Biophys. Res. Comm. 51, 593–596
36 Boardman, N. K., Anderson, J. M. and Hiller, R. G. (1971) Biochim. Biophys. Acta 234, 126–136