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Cyclic Photophosphorylation in the Mykotrophic Orchid *Neottia nidus-avis*

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ABSTRACT

The mykotrophic orchid *Neottia nidus-avis* (L.) Rich. is not able to evolve oxygen in the light. Plastid preparations from the lip (labellum) of the orchid perform a photosystem I-dependent photoreduction of methylviologen with the artificial electron donor couple 2,6-dichlorophenol indophenol ascorbate. Photosystem II reactions such as the ferricyanide Hill reaction or the photoreduction of 2,6-dichlorophenol indophenol with diphenylcarbazide as the electron donor are not functioning. The plastids exhibit phenazine methosulfate-mediated cyclic photophosphorylation. After infiltration with ^{32}P-labeled phosphate the labellum forms ^{32}P-ATP in the light. This rate of ATP formation is enhanced by additional infiltration of phenazine methosulfate prior to illumination. The brown color of the plant is caused by an absorption shift of carotenoids to longer wavelength. By comparison of absorption spectra with the fluorescence excitation spectra of plastid preparations and of the extracted pigments we show that no appreciable energy transfer from carotenoids to chlorophyll occurs.

The mykotrophic orchid *Neottia nidus-avis* has been reported unable to catalyze photosynthesis despite the fact that the plant contains Chl *a* and xanthophyll (15, 17, 18). The plant appears yellowish brown because part of the carotenoid absorption is shifted into the green spectral region (4, 14). Just as in the case of brown algae (Pheophyceae, Bacillariophyceae), this absorption shift to longer wavelengths is probably due to the binding of carotenoids to proteins (12). However, an absorption shift to longer wavelengths is also shown by colloidal solutions of carotenoids. Plastids of *Neottia* are spindle shaped and contain rolled up thylakoids which are not in contact with each other (11, 13, 14). The present paper shows that *Neottia*, although unable to evolve O_2 in the light, uses light energy for the formation of ATP. Furthermore, it is shown that light energy absorbed by carotenoids in the green spectral region is not transferred to Chl as in the case of brown algae (3, 6, 8).

MATERIALS AND METHODS

Plant Material. The investigations were carried out with wild-grown specimens of the orchid *Neottia nidus-avis* (L.). Rich. For the experiments we used the lips from the flowers in various stages. Adult plants do not have any leaves that could be used. *Antirrhinum majus* (L.) strain 50 was grown in the greenhouse. *Laminaria hyperborea* (Gunn.) Fosl. grew near Helgoland in a depth of approximately 2 m.

Plastid Preparations. Plastid preparations from flower lips of 200 to 500 mg fresh weight were prepared as described earlier for tobacco chloroplasts (9). The lips were placed in a test tube and gently mashed with a glass rod in the presence of a small volume of 0.4 M sucrose, containing 50 mM tris-HCl (pH 7.5), 10 mM NaCl, 0.2% serum-albumin, and 0.2% pectinase (Serva, Heidelberg). The photochemical activities were tested within 1 hr after homogenization of the tissue. Plastid preparations for the spectroscopic measurements were obtained by fractionating centrifugation of aqueous extracts (10).

Light Reactions and Photophosphorylation. Photosystem I activity of the plastids was measured as methyl viologen reduction with the artificial electron donor couple DCPIP[1]/ascorbate (16). Methyl viologen reduction was measured manometrically as O_2 uptake in a Mehler type reaction. The reaction was carried out in a thermostated Warburg apparatus at 30 C illuminated from below with 24,000 ergs·sec^{-1}·cm^{-2} of red light transmitted through a red Plexiglas filter between 580 nm and 750 nm. Photophosphorylation reactions were measured as [^{32}P]ATP formation according to Avron (1). The reaction was carried out in open test tubes which were kept in a water bath at 15 C and illuminated from the side with 20,000 ft-c white light. Other light reactions such as the ferricyanide or DCPIP Hill reaction were studied as described earlier (9).

***In Vivo* Photophosphorylation.** Several flower lips from *Neottia* were floated on 65 mM K phosphate buffer, pH 7.8, supplied with [^{32}P]phosphate. In order to bring the phosphate in contact with the cells, the air was removed from the intercellular spaces by brief evacuation. The tissue pieces were washed with distilled H_2O, floated on 1 ml of 0.014 M Tricine, pH 7.8, and illuminated for 2 min with a projector lamp providing 20,000 ft-c. white light. The reaction was stopped by adding 0.1 ml of 20% trichloroacetic acid and by quickly mashing the tissue with a glass rod that was flattened at the end. The brei was processed for esterified Pi according to the Avron procedure (1). Nonilluminated samples processed in the same way served as controls.

Spectroscopy. For the spectra shown in Figure 4a the chloroplast concentration of 0.164 mg dry weight/ml was used. The absorption spectra of the pigments in solution were taken from the acetone extract of a suspension with the same chloroplast concentration which makes these absorption spectra directly comparable. The extract for the fluorescence excitation spectrum was diluted to $^1/_{10}$ with acetone.

For the extraction of fresh *Neottia* lips, equivalent to 0.217 mg dry weight, 1 ml of acetone was used. For the excitation spectrum this solution was diluted with acetone to $^1/_{25}$. The plastid preparation contains beside Chl at least one more component which fluoresces in the visible region. The thallus piece of *Laminaria* used for spectroscopy came from a young, approximately 7 cm long phylloid. The absorption spectra of the thallus and the acetone extract are quantitatively comparable. For the measurement of the fluorescence excitation spectrum the extracts were diluted. Fluorescence intensities were measured in relative units. For the purpose of comparison the fluorescence curves are shifted towards the base line.

[1] Abbreviations: DCPIP: 2,6-dichlorophenolindophenol; PMS: phenazine methosulfate.

The absorption spectra were recorded with a Cary Model 118 spectrophotometer equipped with a Model 1862050 scattered transmission accessory. For the fluorescence spectra a Perkin Elmer Model MPF-3 fluorescence spectrophotometer equipped with a Hitachi corrected spectra accessory was used. In suspensions and extracts fluorescence was measured in a 90° angle to the exciting beam. With the *Laminaria* thallus the measurements were carried out at 45°.

RESULTS

***In Vitro* Capacity of Plastids from *Neottia* for Photosystem I Reactions.** Plastid preparations from the labellum of the orchid perform a DCPIP/ascorbate-mediated methyl viologen reduction (Fig. 1) whereas typical photosystem II reactions such as the ferricyanide Hill reaction or the photoreduction of DCPIP with diphenylcarbazide as the electron donor are not observed. In line with this observation plastid preparations from flower lips (the labellum) or from the other sepals and petals exhibit only the photosystem I-dependent PMS-mediated phtophosphorylation, whereas the noncyclic type of photophosphorylation with ferricyanide or methylviologen as the electron acceptors is inoperative (Table I). The comparison between the electron transport rate (Fig. 1) and the rate of ATP synthesis (Table I) shows

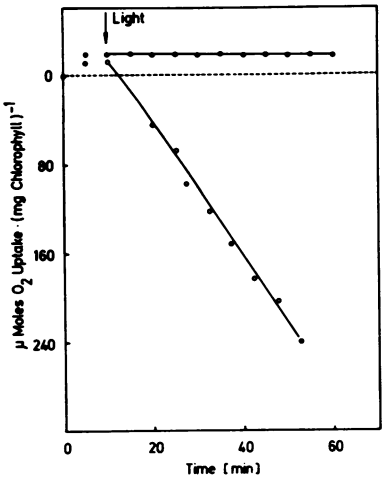

FIG. 1. Photosystem I-mediated photoreduction of methyl viologen with the electron donor couple DCPIP/ascorbate by plastids isolated from the lips of the orchid (○). The same reaction but with plastids that have been heated for 5 min at 90 C (●).

that our plastid preparation has a photosystem I which is mainly uncoupled.

***In Vivo* Capability of *Neottia* Flowers to Form [^{32}P]ATP in the Light.** The capacity of isolated plastids to perform only cyclic photophosphorylation, does not imply that cyclic photophosphorylation would also occur under *in vivo* conditions. The following experiment gives evidence that in the live plant a photophosphorylation reaction takes place (Table II). Lips from *Neottia* were floated on a [^{32}P]labeled phosphate solution. After infiltration of the phosphate, esterified phosphate is isolated from the illuminated tissue. This esterification of inorganic phosphate is insensitive to DCMU.

Energy Transfer from Carotenoids to Chlorophyll. The question whether light energy absorbed by carotenoids in the blue and green spectral region is transferred to Chl is answered by comparison of the fluorescence excitation spectra with the absorption spectra (5, 7). In the following we compare the absorption and fluorescence excitation spectra of *Neottia* plastids with those of *Antirrhinum* chloroplasts and of the thallus from the brown algae *Laminaria hyperborea*. The absorption spectrum of a *Neottia* plastid preparation (Fig. 2a) shows an absorption band at approximately 520 mm, which is missing in the absorption spectrum of the acetone extract (Fig. 2b). The absorption spectrum of a *Laminaria* thallus shows, like *Neottia*, a high absorption in the green and blue-green region (Fig. 3a), which is not seen with *Antirrhinum* chloroplasts (Fig. 4a). In comparison to this, the absorption spectra of the corresponding acetone extracts do not differ appreciably in this spectral region (Fig. 2b, 3b, 4b). Consequently, the brown color of *Neottia* and *Laminaria* is not caused by a green light-absorbing pigment but by a shift of absorption bands. With *Neottia*, the fluorescence yield is relatively low in the wavelength region from 450 to 550 nm (Fig. 2a), whereas the *Laminaria* thallus shows a fluorescence peak in that region (Fig. 3a). The fluorescence excitation spectrum of *Laminaria* exhibits a pronounced maximum at 525 nm (Fig. 3a), which corresponds to the shoulder at approximately 520 nm in the absorption maximum of *Neottia* (Fig. 2a). The excitation spectrum of *Neottia* shows an emission minimum at this point (Fig. 2a). But also between 450 and 500 nm, where the excitation spectra of *Laminaria* and *Antirrhinum* show two overlapping bands, the excitation spectrum of *Neottia* has a minimum despite a relatively high absorption by the plastids. The excitation spectra of the acetone extracts (Figs. 2b, 3b, and 4b) do not differ significantly despite the different absorption spectra. They correspond reasonably well to the absorption spectra of the Chl. Therefore, energy transfer does not occur. By comparison of the excitation spectra of the preparations and the thallus (Figs. 2a,

Table I. *Photophosphorylation Reactions by Plastids from Neottia nidus-avis*
The data represent 10 determinations on 3 plastid preparations.

Photophosphorylation Reaction	Specific Activity: μ moles [^{32}P]-ATP formed / g Fresh Wt $x^{-1}h^{-1}$ [1]	Specific Activity: μ moles [^{32}P]-ATP formed / mg Chlorophyll $x^{-1}h^{-1}$
PMS (cyclic)	5.4 - 10.7	16 - 32
$H_2O \rightarrow K_3Fe(CN)_6$ (non-cyclic)	0	0
Ascorbate → Methylviologen (non-cyclic)	0	0

[1] Refers to the fresh weight of the original plant material used for the plastid preparation.

Table II. *Uptake of [^{32}P]phosphate into the Labellum of the Orchid Neottia nidus-avis*

The flower lips contained 0.33 mg of Chl *a*/g fresh weight.

Reaction	P_i esterified	
	dpm per mg Chl per hr	
Infiltration of $[^{32}P]$-P_i	light	127 700
	dark	12 960
Infiltration of $[^{32}P]$-P_i + 10^{-4} M PMS	light	164 800
	dark	13 200

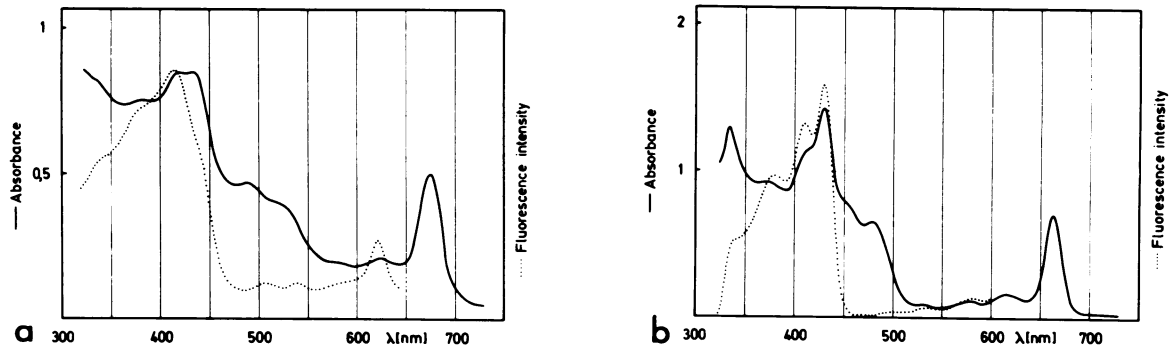

FIG. 2. *Neottia nidus-avis*. a: Plastids: absorption spectrum (zero suppression 0.1) (——); corrected fluorescence excitation spectrum (emission wavelength 688 nm; excitation and emission slit 10 nm) (- - -). b: Acetone extract: absorption spectrum (——); corrected fluorescence excitation spectrum (emission wavelength 670 nm; excitation slit 4 nm; emission slit 10 nm) (- - -).

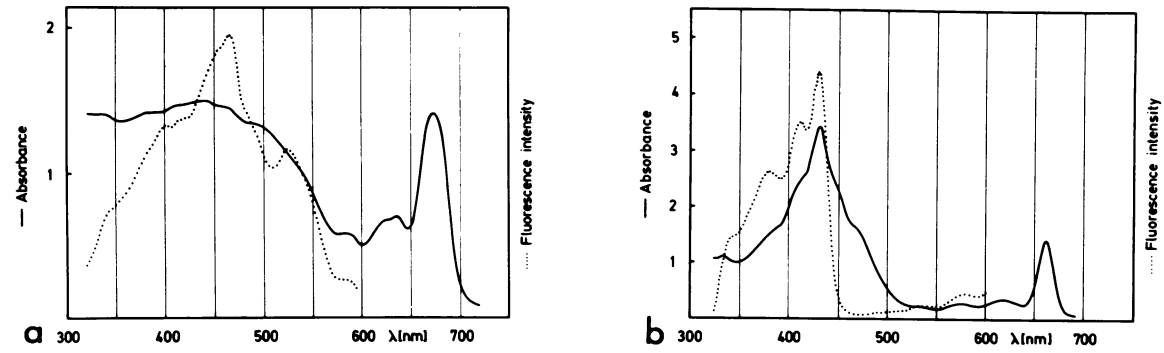

FIG. 3. *Laminaria hyperborea*. a: Thallus: absorption spectrum (zero suppression 0.1) (——); corrected fluorescence excitation spectrum (excitation wavelength 686 nm; excitation slit 10 nm; emission slit 38 nm) (- - -). b: Acetone extract: absorption spectrum (——); corrected fluorescence excitation spectrum (emission wavelength 668 nm; excitation and emission slit 5 nm) (- - -).

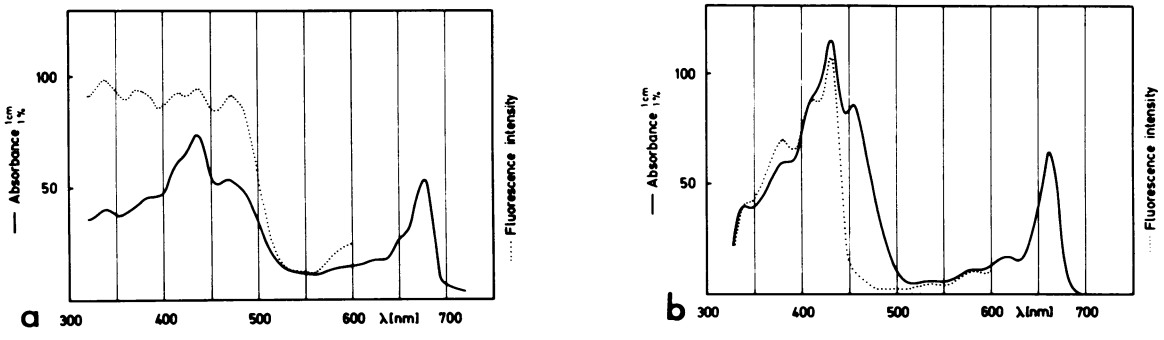

FIG. 4. *Antirrhinum majus*. a: Stroma-freed chloroplasts: absorption spectrum (——). Concentration 0.164 mg dry wt/ml; no zero suppression; corrected fluorescence excitation spectrum (emission wavelength 686 nm; emission and excitation slit 8 nm) (- - -). b: Acetone extract: absorption spectrum (——). The acetone extract contained the same amount of Chl as the chloroplast suspension; corrected fluorescence excitation spectrum (emission wavelength 676 nm; emission and excitation slit 5 nm) (- - -).

3a, and 4a) with those of the corresponding acetone extracts (Figs. 2b, 3b, and 4b) the extent of energy transfer of light absorbed by carotenoids to Chl is seen. This comparison shows that in *Neottia* at best only a small portion of the carotenoid molecules transfers its excitation energy to Chl *a*.

DISCUSSION

It must be assumed that *Neottia* has evolved from an autotrophic ancestor by defect mutations. According to the literature, the plant contains Chl *a* and xanthophyll but lacks β-carotene and Chl *b* (17). In contrast to brown algae, the shift of the carotenoid absorption (probably that of lutein) to longer wavelengths appears to be of no advantage to the plant, because in *Neottia* no energy transfer from carotenoids to Chl was observed.

As already reported in the literature, the orchid is unable to catalyze photosynthesis (17). The functional defect must be localized on the photosystem II side, since isolated plastids do not have the ability to evolve O_2 nor to reduce DCPIP with diphenylcarbazide as the electron donor. On the other hand electron transport reactions involving photosystem I are functioning (Fig. 1) and the plastids perform PMS-mediated cyclic photophosphorylation.

The capacity of isolated plastids to perform cyclic photophosphorylation does not prove, strictly speaking, that cyclic photophosphorylation is also operative under *in vivo* conditions. The data shown in Table II, presents the evidence that a photophosphorylation reaction also occurs *in vivo*. Unlike the *in vitro* experiment, this reaction takes place without the addition of an external mediator. The nature of the native mediator remains unknown. The question whether cyclic photophosphorylation is functioning under natural conditions in higher plants has not been fully resolved (19). The experiments described above are interpreted to mean that in *Neottia* a photosystem I-mediated cyclic photophosphorylation is operative.

No relationship is evident between the described spectroscopic properties of the *Neottia* plastids and the absence of photosystem II activity. The reaction center complex of photosystem II was suggested to contain β-carotene (2) and Chl *b* is thought to play a role as one of the light harvesting pigments in photosystem II. Plastids of an earlier described system II-defective tobacco mutant were characterized by single isolated thylakoids which did not have any grana or touching areas (9). The same is true for *Neottia* plastids which also contain only single thylakoid which do not touch each other.

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LITERATURE CITED

1. Avron, M. 1960. Photophosphorylation by swiss-chard chloroplasts. Biochim. Biophys. Acta 40: 257-276.
2. Butler, W. L. 1973. Primary photochemistry of photosystem II of photosynthesis. Accounts Chem. Res. 6: 177-184.
3. Dutton, H. J., W. M. Manning, and B. M. Duggar. 1943. Chlorophyll fluorescence and energy transfer in the diatom *Nitzschia closterium*. J. Physic. Chem. 47: 308-313.
4. French, C. S. 1960. The chlorophylls *in vivo* and *in vitro*. *In:* Encyclopedia of Plant Physiology, Vol. 1. Springer, Berlin. pp. 252-297.
5. Fork, D. C. and J. Amesz. 1969. Action spectra and energy transfer in photosynthesis. Annu. Rev. Plant Physiol. 20: 305-328.
6. Goedheer, J. C. 1970. On the pigment system of brown algae. Photosynthetica 4: 97-106.
7. Goedheer, J. C. 1972. Fluorescence in relation to photosynthesis. 23: Annu. Rev. Plant Physiol. 23: 87-112.
8. Haxo, F. and L. R. Blinks. 1950. Photosynthetic action spectra of marine algae. J. Gen. Physiol. 33: 389-421.
9. Homann, P. H. and G. H. Schmid. 1967. Photosynthetic reactions of chloroplasts with unusual structures. Plant Physiol. 42: 1619-1632.
10. Kreutz, W. and W. Menke. 1960. Strukturuntersuchungen an Plastiden. Z. Naturforsch. 15b: 402-410.
11. Mangenot, G. and S. Mangenot. 1966. Les corps pigmentés des chloroplasts de *Neottia nidus-avis*. C. R. Acad. Sci. Paris 262: 2456-2457.
12. Menke, W. 1940. Über den Zustand der Carotenoide in den Plastiden. Naturwissenschaften 28: 31.
13. Menke, W. 1965. Feinbau und Entwicklung der Plastiden. Ber. Dtsch. Bot. Ges. 77: 340-354.
14. Menke, W., and B. Wolfersdorf. 1968. Über die Plastiden von *Neottia nidus-avis*. Planta 78: 134-143.
15. Montfort, C. and G. Küsters. 1940. Saprophytismus und Photosynthese I. Bot. Arch. 40: 571-633.
16. Radunz, A., G. H. Schmid, and W. Menke. 1971. Antibodies to chlorophyll and their reactions with chloroplast preparations. Z. Naturforsch. 26b: 435-446.
17. Reznik, H. 1958. Vergleich einer weissen Mutante von *Neottia nidus-avis* (L.) L. C. Rich. mit der braunen Normalform. Planta 51: 694-704.
18. Willstätter, R. and A. Stoll. 1918. Untersuchungen über die Assimilation der Kohlensäure. Springer, Berlin.
19. Zelitch, I. 1971. Photosynthesis, Photorespiration and Plant Productivity. Academic Press, New York. pp. 77-78.