The Effect of Antibodies to Neoxanthin on Electron Transport on the Oxygen Evolving Side of Photosystem II and the Reactions of this Antiserum with Various Chloroplast Preparations

Alfons Radunz and Georg H. Schmid

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut), Abteilung Menke, Köln-Vogelsang

(Z. Naturforsch. **30 c**, 622–627 [1975]; received June 6/ July 12, 1975)

Photosystem II, Neoxanthin, Antibodies, Chloroplasts

Antibodies to neoxanthin agglutinate *stroma-free swellable chloroplasts* from tobacco (*Nicotiana tabacum* var. John William's Broadleaf) and *Antirrhinum* (*Antirrhinum majus*) whereas *stroma-freed chloroplasts*, which have lost the swellability are not agglutinated despite the fact that antibodies to neoxanthin are specifically adsorbed. In this latter case the agglutination is hindered for sterical reasons. From this it is concluded that neoxanthin is located in the outer surface of the thylakoid membrane. The antiserum to neoxanthin inhibits the ferricyanide photoreduction in chloroplasts when water is the electron donor by 15%. With diphenylcarbazide in tris-treated chloroplasts no inhibition is observed. Hence, just as in the case of the antiserum to lutein the site of inhibition is on the donor side of potosystem II namely between water and the site of electron donation of diphenylcarbazide. Benzidine/ascorbate is another artificial electron donor system of photosystem II reported in the literature. The photoreduction of anthraquinone-2-sulphonate with this donor system is inhibited. In contrast to the antiserum to lutein the antiserum to neoxanthin inhibits DCMU-sensitive photophosphorylation reactions in the system $H_2O \rightarrow$ ferricyanide and benzidine/ascorbate $\rightarrow$ anthraquinone-2-sulphonate. Therefore, the electron transport coupled to photophosphorylation is inhibited by the antiserum.

Neoxanthin is one of the main carotenoids in chloroplast of higher plants and algae [1]. In comparison to lutein, violaxanthin and neoxanthin other carotenoids are present in only small amounts [1]. The function which is mainly attributed to carotenoids is threefold: a. Photoprotection against photosensitized oxidations [2], b. transfer of absorbed light energy to chlorophyll and c. that of intermediates in the generation of oxygen [3].

As reviewed by Khan and Kasha [4] and by Foote [5] the photoprotective mechanism of carotenoids includes interference of carotenoids with singlet oxygen which is a frequent intermediate in photosensitized oxidations and other reactions thereby protecting chlorophyll from photooxidation. Foote has shown that singlet oxygen is quenched by carotenoids [6, 7]. Interaction of singlet oxygen with certain carotenoids is thought to lead amongst other effects to oxidation products and formation of allenic carotenoids such as the widely distributed neoxanthin [8].

The ability of carotenoids to transfer light energy to chlorophyll is well established and reviewed for example by Krinsky [9]. On the other hand, the role of carotenoids or certain epoxides as intermediates in photosynthetic oxygen evolution has been ruled out by the work of Shneour and Calvin [10].

The present paper reports on serological reactions of an antiserum to neoxanthin with various chloroplast preparations and on the effect of antibodies to neoxanthin on photosynthetic electron transport in *stroma-free swellable chloroplasts* from wild type tobacco. The antiserum inhibits electron transport on the donor side of photosystem II as well as DCMU-sensitive photophosphorylation.

Materials and Methods

Chloroplast preparations: Stroma-freed chloroplasts from *Antirrhinum* and wild type tobacco *Nic. tabacum* var. John William's Broadleaf were prepared according to Kreutz and Menke [11]. Stroma-free swellable chloroplasts from wild type tobacco were prepared according to Homann and Schmid [12].

The antiserum agglutinates *stroma-free swellable* publication [13].

Requests for reprints should be sent to Priv.-Doz. Dr. Georg H. Schmid, Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut), *D-5000 Köln 30.*

Abbreviations: DPIP, 2,6-dichlorphenol-indophenol; PMS, phenazine methosulphate; DCMU, dichlorophenyldimethylurea.

Light reactions were carried out as described earlier [12, 14, 15]. Photophosphorylation reactions were carried out according to the Avron procedure using [^{32}P]phosphate [16]. The photooxidation of diphenylcarbazide was carried out according to an earlier publication [17] using either ferricyanide or DPIP as the acceptor. When using ferricyanide the reaction was run at a pH lower than 7.0 in order to reduce the chemical reaction of the donor with the acceptor [18]. In order to destroy the oxygen evolving capacity the chloroplasts were washed with 0.8 M Tris pH 8 according to Yamashita and Butler [19]. Gramicidin was purchased from Serva/Heidelberg and benzidine from Sigma/St. Louis, Mo.

Pigment analyses [20] and light conditions are as described earlier [15].

Isolation of neoxanthin: Ether soluble lipids from *Urtica dioica* were saponified at room temperature with 5 per cent sodium ethylate. The carotenoids were extracted with a mixture of diethyl ether/petrol ether 1 v : 1 v. The carotenes and subsequently lutein and violaxanthin were removed by chromatography on cellulose (MN 2100 ff Macherey, Nagel & Co., Germany) using petrolether (hp 40 – 60 °C)/aceton (100 v/10 v) and (100 v/20 v) for elution. The main portion of the neoxanthin remained on the column and was eluted with methanol. The obtained neoxanthin fraction was purified by thin layer chromatography (aluminium oxide type E. Merck) and recrystallized 3 times from benzene/methanol.

The spectrum of neoxanthin and the derivative spectrum were taken with a Cary Model 118 Spectrophotometer. The spectrum of the crystallized neoxanthin dissolved in ethanol had maxima at 466, 437.5 and 413.5 nm as described in the literature [21, 22].

Immunization of rabbits with neoxanthin was carried out as described for the antiserum to lutein [17]. Two rabbits were treated in a two days interval with an injection mixture containing 2 mg neoxanthin and 1 mg methylated bovine serum albumin in a volume of 2 ml physiological saline. After the 12th injection the treated animal reacted with the formation of antibodies. Agglutination reactions or the Coombs test were described in earlier publications [23, 24].

Results

Characterization of the neoxanthin used for injection into rabbits

Neoxanthin which was recrystallized several times from benzene/methanol yielded the spectrum and the derivative spectrum shown in Fig. 1. The spectrum exhibits all the characteristics of neoxanthin described in the literature [22].

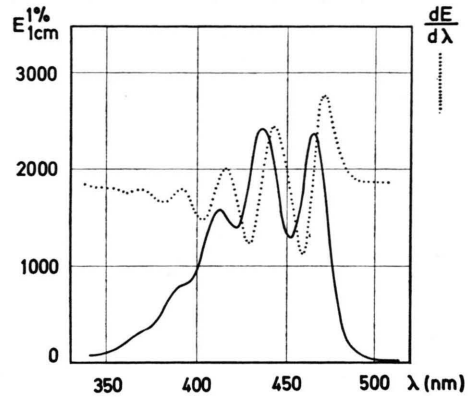

Fig. 1. Solid line: spectrum of the neoxanthin preparation used for injection into the rabbis. Dashed line: derivative spectrum of neoxanthin.

Serological reactions of the antiserum to neoxanthin with different chloroplast preparations

The antiserum agglutinates *stroma-free swellable chloroplasts* [12] from tobacco and Antirrhinum whereas *stroma-freed chloroplasts* [11] specifically adsorb the antibodies (Table I). Thus, just as described earlier wether agglutination occurs or not depends on the state of the molecular structure of the thylakoid membrane [13, 25]. The passive heme-agglutination test does not work for reasons already described for the antiserum to lutein [17].

Table I. Agglutination reactions of the antiserum to neoxanthin with chloroplasts from *Antirrhinum majus* and *N. tabacum.*

Chloroplast type	*Antirrhinum majus*	Wild type tobacco
Strome-free swellable chloroplast [12]	agglutination	agglutination
Stroma-freed chloroplasts [11]	specific adsorption (no agglutination)	specific adsorption (no agglutination)

Chloroplasts were prepared according to reference 11 and 12.

Effects of the antiserum to neoxanthin on photosynthetic electron transport and on photophosphorylation

Just as the earlier described antiserum to lutein the antiserum to neoxanthin inhibits the ferricyanide- and DPIP-Hill-reaction (Table II). When diphenylcarbazide is the electron donor no inhibi-

Table II. Inuence of the antiserum to neoxanthin of the ferricyanide Hill reaction or the DPIP Hill reaction in tobacco chloroplasts, as well as the photoreduction of DPIP when diphenylcarbazide is the electron donor.

Reaction	μmol Ferricyanide or $DPIPH_2$/mg Chl·h	Inhibition [%]
$H_2O \rightarrow K_3Fe(CN)_6$		
0.2 ml antiserum	415	12
0.2 ml control serum	472	
$H_2O \rightarrow$ DPIP		
a. without methylamine		
0.2 ml antiserum	99	14
0.2 ml control serum	115	
b. with methylamine		
0.2 ml antiserum	256	2.5
0.2 ml control serum	263	
Diphenylcarbazide → DPIP *		
0.2 ml antiserum	38.5	4.5
0.2 ml control serum	40.4	
without serum	37	
without serum and without diphenylcarbazide	6.2	

* Chloroplast were tris-washed. All values represent an average of at least 10 measurements.

tion is observed. Again, as reported earlier for the antiserum to lutein or an antiserum obtained to a photosystem-II activity exhibiting chlorophyll protein fraction prepared by Koenig *et al.* [15] the average degree of inhibition is ≈ 15 per cent. However, in contrast to the antiserum to lutein the antiserum to neoxanthin inhibits photophosphorylation in the system $H_2O \rightarrow$ ferricyanide to a comparable degree whereas photosystem I-dependent PMS-mediated photophosphorylation is unaffected (Table III). In order to further characterize the inhibition site by antibodies to neoxanthin the artificial donor couple benzidine/ascorbate was used [26].

Table III. Effect of the antiserum to neoxanthin on cyclic and noncyclic photophosphorylation in chloroplasts from wild type tobacco.

Additions	μmol $[^{32}P]$ATP formed/mg Chl·h	Inhibition [%]
PMS	644	
PMS + antiserum to neoxanthin	632	0
PMS + control serum	630	
$K_3Fe(CN)_6$	61	
$K_3Fe(CN)_6$ + antiserum to neoxanthin	51	17
$K_3Fe(CN)_6$ + control serum	62	
$K_3Fe(CN)_6 + 10^{-5}$ M DCMU	0	

The reaction was carried out in a thermostated plexiglass container at 14–15 °C in open test tubes illuminated for 4 min with 120 000 lx white light through 10 cm of water.

Harth, Reimer, and Trebst have observed that the water splitting reaction at high outside pH is inhibited by uncouplers. An artificial donor system to photosystem II, benzidine/ascorbate, reverses this inhibition. Removal of the uncoupler makes the water splitting reaction operative again [26]. Application of this electron donor system to our type of chloroplast preparation leads to the results shown in Figs 2 and 3. Under these conditions the reaction with *stroma-free swellable* chloroplasts is quantitatively different from that of Harth *et al.*'s [26] broken chloroplast preparation but the result may be interpreted

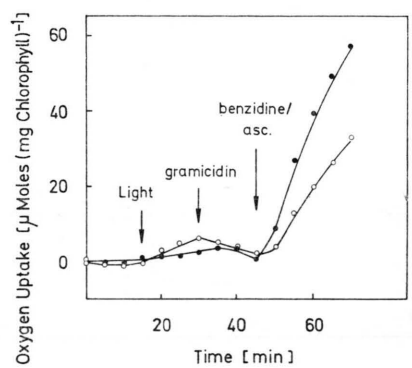

Fig. 2. Dependence of photosystem II activity in tobacco chloroplasts on the pH of the reaction mixture. The reaction was measured as oxygen uptake in an anthraquinone-2-sulphonate dependent Mehler reaction with water as the electron donor or with benzidine/ascorbate as indicated. (○), Time course of the reaction at pH 7.6±0.1. (●), Time course at pH 8.75±0.15. Illumination with 24 000 ergs·sec^{-1}·cm^{-2} of red light 575 nm$<\lambda<$700 nm at 20 °C in a Warburg apparatus.

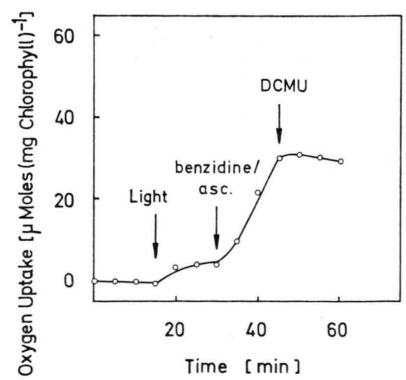

Fig. 3. Control reaction to Fig. 2 at pH 7.6 to show DCMU-sensitivity.

in the same way. At the high pH of the suspension medium electron transport from water to anthraquinone-2-suphonate is lower than the rate at the lower pH. The uncoupler gramicidin inhibits both reactions but the artificial donor couple benzidine/ascorbate restores the rates of anthraquinone-2-sulphonate photoreduction much over the original level. Clearly, at the higher pH where the water splitting reaction was lower, this time the rate of anthraquinone-2-sulphonate reduction is enhanced. This demonstrates that it is not the capacity of the photosystem II reaction itself which is affected but the water splitting reaction which does not work at an unfavourable pH of the suspension medium. The pH dependence of the photoreaction $H_2O \rightarrow$ anthraquinone-2-sulphonate with *stroma-free swellable chloroplasts* from tobacco is shown in Fig. 4 and

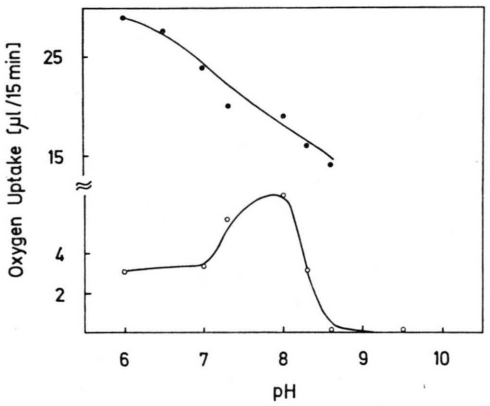

Fig. 4. Dependence of the water splitting reaction in tobacco chloroplasts on the pH of the reaction mixture (○) with anthraquinone-2-sulphonate als electron acceptor. (●), pH-dependence when benzidine/ascorbate is the electron donor instead of water. All other conditions as in Fig. 2.

shows an optimum at pH of about 8 – 8.5 of the suspension medium which agrees with the findings of Bamberger, Rottenberg and Avron [27]. This together with the results of Figs 2 and 3 could now indeed mean that the water splitting reaction occurs inside the thylakoid membrane [26]. Fig. 4 also shows that the pH dependence of the benzidine/ascorbate mediated photoreduction of anthraquinone-2-sulphonate is different from the reaction when H_2O is the electron donor. The antibody to neoxanthin inhibits at a pH 7.6 the photosystem II mediated photoreduction of anthraquinone-2-sulphonate with benzidine/ascorbate (Fig. 5). However, at pH 8.9 a stimulation of the benzidine/ascorbate mediated photo-

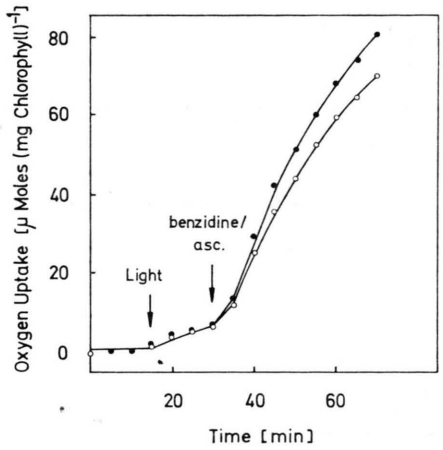

Fig. 5. Effect of the antiserum to neoxanthin on the benzidine/ascorbate mediated anthraquinone-2-sulphonate Mehler reaction at pH 7.6 ± 0.1. (●), Reaction with 0.2 ml control serum. (○), Reaction with 0.2 ml antiserum to neoxanthin. Other conditions as in Fig. 2.

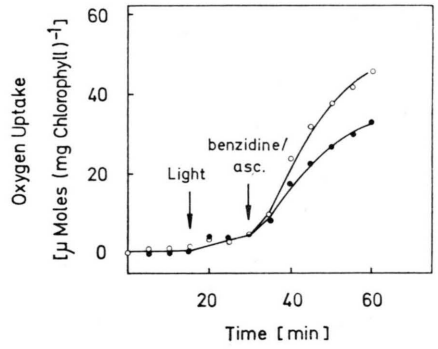

Fig. 6. Effect of the antiserum to neoxanthin on the benzidine/ascorbate mediated anthraquinone-2-sulphonate Mehler reaction at pH 8.75 ± 0.15. All other conditions as in Fig. 2. (●), Reaction with 0.2 ml control serum .(○), Reaction with 0.2 ml antiserum to neoxanthin.

reduction of anthraquinone-2-suphonate is observed (Fig. 6). The stimulation is 40%. If this result is not due to an inhibition by the control serum at this unfavourable pH it is obvious that binding of the antibody at this pH leads to a state of the thylakoid membrane where the photosystem II-reaction is enhanced. By agglutination reaction it was shown that the antibodies to neoxanthin do bind to the lamellar system at this pH. The result is that the site of action of antibodies to neoxanthin differs from the site of action of antibodies to lutein.

The system benzidine/ascorbate→ anthraquinone-2-sulphonate supports DCMU-sensitive photophosphorylation which is inhibited by the antiserum to neoxanthin (Table IV).

Table IV. Effect of the antiserum to neoxanthin on the photosystem II-mediated photophosphorylation reaction in the system benzidine/ascorbate → anthraquinone-2-sulphonate in wild type tobacco chloroplasts.

Additions	μmol [^{32}P] ATP formed/mg Chl·h
–	45
Antiserum to neoxanthin	40
Control serum	45
Antiserum to lutein	34
Control serum	32
0.1 μg Gramicidin	23.6
10^{-5} M DCMU	0
H_2O → anthraquinone-2-sulphonate +0.1 μg gramicidin	21 *

Assay conditions as in Table III. Chloroplasts were tris-washed with the exception of *.

Discussion

The present paper shows that neoxanthin is located in the outer surface of the thylakoid membrane and that antibodies to neoxanthin inhibit photosynthetic electron transport on the oxygen evolving side of photosystem II between the site of electron donation of benzidine/ascorbate and diphenylcarbazide. As a difference to the earlier reported antiserum to lutein [17] the antiserum to neoxanthin inhibits also DCMU-sensitive noncyclic photophosphorylation reactions. The observed inhibition of electron transport by antibodies to neoxanthin can have several reasons:

1. Neoxanthin is electron donor to photosystem II which is thinkable since the compound is autoxydizable but which is not too probable for reasons discussed below.
2. Binding of antibodies to neoxanthin leads to an isomerization of the carotenoid and then to a conformational change of a protein attached to neoxanthin. This would require the proof that neoxanthin is bound to a protein.
3. Neoxanthin is located between protein in the surface of the thylakoid membrane. Binding of antibodies to neoxanthin changes the position of neoxanthin in relation to protein thus resulting in an alteration of the molecular structure of the thylakoid membrane.

The result of Figs 5 and 6 shows that antibodies to neoxanthin inhibit electron transport at pH 7.6 but stimulate at pH 9. Binding of antibodies at the high pH leads apparently to a condition of the thylakoid membrane in which electron transport is enhanced. From this it would appear that point 2 or 3 applies. This does not mean, however, that the effect of the antiserum to neoxanthin is unspecific because differentiated effects are observed with the antisera to neoxanthin and lutein (Tables III and IV). Even though both antisera inhibit on the oxygen evolving side of photosystem II the mode of inhibition is clearly different. Both specific antisera have in common that the average degree of inhibition is rather low which obviously can mean that there is only a limited accessibility of the two carotenoids in the lamellar system.

Investigations by Braun and Govindjee [28, 29] with antisera not defined with respect to the chemical nature of the injected antigen, have also shown inhibition of the electron transport on the oxygen evolving side of photosystem II with a similar low degree of inhibition as reported by us here in this paper and earlier [14, 15, 17]. Moreover, labelling of the lamellar system with [^{35}S] *p*-diazoniumbenzenesulfonic acid (DABS) in the light has led to an inhibition of electron transport on the oxygen evolving side of photosystem II. Incorporation of DABS in the dark preferentially labelled photosystem I. The reaction is thought to affect only surface exposed proteins [30]. All these observations also show a surface exposed site in the thylakoid membrane by which the donor side of photosystem II can be affected. In comparison to these reports our antisera are specific and defined with respect to the chemical nature of the antigen used for injection into rabbits, a fact which permits a subtile differentiation between the mode of inhibition of antibodies to neoxanthin and the inhibition site of antibodies to lutein.

The authors would like to thank Professor W. Menke for stimulating discussions and for taking the neoxanthin spectra. The authors also thank Mrs. M. Russo and Mrs. M. Emons for skillful technical assistance.

[1] B. C. L. Weedon, Carotenoids (O. Isler, ed.), Birkhäuser Verlag 1971.
[2] H. Gaffron, Proc. 5th International Congr. Biochem. 1961, Vol. 6, p. 233, Pergamon Press, New York 1963.
[3] P. Karrer, Fortschr. Chem. Org. Naturst. **5**, 1 [1948].
[4] A. U. Khan and M. Kasha, J. Amer. Chem. Soc. **92**, 3293 [1970].
[5] C. S. Foote, Accounts Chem. Res. **1**, 104 [1968].

[6] C. S. Foote, Y. C. Chang, and R. W. Denny, J. Amer. Chem. Soc. **92**, 5216 [1970].
[7] C. S. Foote and R. W. Denny, J. Amer. Chem. Soc. **90**, 623 [1968].
[8] M. Mousseron-Canet, J.-P. Dalle, and J.-C. Mani, Photochem. Photobiol. **9**, 91 [1969].
[9] N. J. Krinsky, Carotenoids (O. Isler, ed.), Birkhäuser Verlag 1971.
[10] E. A. Shneour and M. Calvin, Nature **196**, 439 [1962].
[11] W. Kreutz and W. Menke, Z. Naturforsch. **15 b**, 402 [1960].
[12] P. H. Homann and G. H. Schmid, Plant Physiol. **42**, 1619 [1967].
[13] G. H. Schmid, A. Radunz, and W. Menke, Z. Naturforsch. **30 c**, 201 [1975].
[14] A. Radunz, G. H. Schmid, and W. Menke, Z. Naturforsch. **26 b**, 435 [1971].
[15] F. Koenig, W. Menke, H. Craubner, G. H. Schmid, and A. Radunz, Z. Naturforsch. **27 b**, 1225 [1972].
[16] M. Avron, Biochim. Biophys. Acta **40**, 257 [1960].
[17] A. Radunz and G. H. Schmid, Z. Naturforsch. **28 c**, 36 [1972].
[18] G. Harnischfeger, Z. Naturforsch. **29 c**, 705 [1974].
[19] T. Yamashita and W. Butler, Plant Physiol. **44**, 435 [1969].
[20] G. H. Schmid, Methods in Enzymology (A. San Pietro, ed.), **Vol. 23**, p. 171, Acad. Press, New York 1971.
[21] N. J. Krinsky and T. H. Goldsmith, Arch. Biochem. Biophys. **91**, 271 [1960].
[22] B. H. Davies, Chemistry and Biochemistry of Plant Pigments (T. W. Goodwin, ed.), p. 489, Acad. Press, New York 1965.
[23] A. Radunz, Z. Naturforsch. **27 b**, 822 [1972].
[24] A. Radunz, Z. Naturforsch. **26 b**, 916 [1961].
[25] G. H. Schmid and A. Radunz, Z. Naturforsch. **29 c**, 384 [1974].
[26] E. Harth, S. Reimer, and A. Trebst, FEBS Letters **42**, 165 [1974].
[27] Elchanan, S. Bamberger, H. Rottenburg, and M. Avron, Eur. J. Biochem. **34**, 557 [1973].
[28] B. Z. Braun and Govindjee, FEBS Letters **25**, 143 [1972].
[29] B. Z. Braun and Govindjee, Plant Science Letters **3**, 219 [1974].
[30] R. T. Giaquinta, R. A. Dilley, and B. J. Anderson, Biochem. Biophys. Res. Commun. **52**, 1410 [1973].