The Effect of an Antiserum to Plastocyanin on Various Chloroplast Preparations

Georg H. Schmid, Alfons Radunz, and Wilhelm Menke

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

(Z. Naturforsch. **30 c**, 201–212 [1975]; received December 9, 1974)

Photosynthesis, Plastocyanin, Antibodies, Chlorophyll-deficient Tobacco Mutants

A monospecific antiserum to tobacco plastocyanin agglutinates *stroma-free swellable chloroplasts* from wild type tobacco, *(Nicotiana tabacum* var. John William's Broadleaf) from the tobacco aurea mutant Su/su^2, *(Nicotiana tabacum* var. Su/su^2) from *Antirrhinum majus* and spinach *(Spinacia oleracea)*. In this condition the antiserum inhibits linear photosynthetic electron flow in tobacco and spinach chloroplasts. This inhibition of electron transport as well as the agglutination are not observed if the chloroplasts have been sonicated prior to antiserum addition. This is due to the fact that plastocyanin is removed by ultrasonication. The antiserum stimulates a number of photophosphorylation reactions in tobacco chloroplasts. This stimulation is always larger in the aurea mutant chloroplasts and in chloroplasts from yellow leaf patches of a variegated tobacco mutant *(N. tabacum*, var. NC 95) than in the green type chloroplasts. The stimulation appears to be a consequence of the inhibition of linear electron transport. The antiserum does not affect PMS-mediated cyclic photophosphorylation in tobacco chloroplasts from the wild type whereas the reaction appears stimulated in the tobacco mutant chloroplasts. However, menadione-mediated cyclic photophosphorylation is inhibited upon addition of the antiserum. The same is true for noncyclic photophosphorylation coupled to electron transport in the aerobic system diaminodurene/ascorbate → methylviologen in the presence of N-tetraphenyl-*p*-phenylenediamine in spinach chloroplasts.

If the lamellar system of *Antirrhinum* and spinach has lost its swellability neither agglutination nor inhibition of electron transport is observed. However, also in this state antibodies to plastocyanin are specifically adsorbed onto the surface of the thylakoid membrane. This state which is characterized by a morphologically well preserved lamellar system is realized in chloroplast preparations from *Antirrhinum* and spinach and is termed *stroma-freed chloroplasts*.

In both states of the molecular structure of the thylakoid membrane, plastocyanin is located in the outer surface of the thylakoid. However, it cannot be excluded that functioning plastocyanin is also located in the interior of the thylakoid membrane.

Katoh has discovered and characterized plastocyanin [1, 2]. It is a blue copper protein which occurs in chloroplasts of higher plants [3] and algae [4, 5]. The protein has electron carrier properties and is readily released from the lamellar system of chloroplasts by ultrasonication and detergent treatment. These treatments cause inhibition of light-dependent $NADP^+$-reduction. In addition, Katoh and San Pietro were able to demonstrate stimulation of light-dependent $NADP^+$-reduction upon addition of plastocyanin in chloroplasts which had been exposed to sonic treatment [6] and the addition of plastocyanin also stimulated $NADP^+$-reduction in detergent treated chloroplasts [7–10]. Uncertainty exists in the literature about the exact site of action of plastocyanin in photosynthetic electron transport. Especially, whether plastocyanin reduces or is reduced by cytochrome f or whether these two components do act in parallel.

Hauska, McCarty, Berzborn und Racker [11] have reported on the effect of a monospecific antiserum to plastocyanin on electron transport and photophosphorylation. As the antiserum did not agglutinate their chloroplast preparations they concluded that plastocyanin *in situ* is not accessible to antibodies. Moreover, the authors were able to make a general distinction between plastocyanin *in situ* and plastocyanin added to a chloroplast preparation. The latter type of plastocyanin induced an artifical electron flow through photosystem I which was not coupled to photophosphorylation and which in turn was sensitive to external antibodies. The authors concluded from their studies that natural plastocyanin *in situ* was functioning in noncyclic electron

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-dichlorophenol-indophenol; PMS, phenazine methosulphate; DAB, 3,3′-diaminobenzidine; DAD, diaminodurene; TMPD, N-tetra-methyl-*p*-phenylenediamine; DCMU, dichlorophenyldimethylurea.

flow between the two photosystems as well as in cyclic photophosphorylation.

As we had observed earlier that the effect of specific antibodies depended on the state in which the lamellar system was in, we wondered how antibodies to plastocyanin would behave in this respect. We therefore, investigated chloroplast preparations which had been obtained by different preparation procedures as well chloroplasts from various chlorophyll-deficient tobacco mutants [12].

Mainly two chloroplast isolation procedures were used which yielded chloroplasts in two states differing with respect to their molecular structure of the thylakoid membrane. The one type was prepared according to Homann and Schmid [12]. The preparation is characterized by being composed originally of stroma-containing chloroplasts which loose the stroma during the assay. The resulting lamellar system of this preparation is reversibly swellable and exhibits high rates of any type of electron transport and photophosphorylation reactions [12]. This chloroplast preparation is designated in the following as *stroma-free swellable chloroplasts*. The second type was prepared according to Kreutz and Menke [13]. These chloroplasts were already freed from the stroma during the isolation procedure and the resulting lamellar system has lost most of its swellability. In the case of *Antirrhinum majus* the lamellar system is morphologically very well preserved but photosynthetic electron transport and rates of photophosphorylation reactions are low. As already described, this type of chloroplasts is designated in the following as *stroma-freed chloroplasts*.

Materials and Methods

Chloroplast preparations: Stroma-freed chloroplasts from the tobacco mutant Su/su^2 and the green control *Nic. tabacum* var. John William's Broadleaf as well as from *Antirrhinum majus* strain 50 were prepared according to Kreutz and Menke [13]. A second type of preparation was prepared according to Homann and Schmid [12] in a buffer containing 0.4 M sucrose, 0.05 M tris, pH 7.8, 0.01 M NaCl, 0.02 M ascorbate, 0.2% serum albumin and 0.2% pectinase (Serva, Heidelberg). The chloroplasts were washed twice and suspended in the same buffer but without ascorbate. As a difference to the *stroma-freed chloroplasts* prepared according to Kreutz and Menke [13] in which the stroma has been removed from the chloroplast suspension, the latter type contains originally stroma-containing chloroplasts. The enveloping membrane usually pops during the assay. The result is a suspension of stroma-free chloroplasts in the assay buffer containing released stroma. Thus, these chloroplasts are in fact *swellable stroma-free* chloroplasts. The variegated tobacco mutant NC 95 has been described earlier [14]. The dominant aurea mutant Su/su^2 originated from a seed population of the earlier described Su/su [15, 16]. The mutant is a more pronounced type of regular Su/su. Its properties will be described elsewhere.

Light reactions

a. *$NADP^+$-reduction* was carried out as described before [12] or with the more sensitive Ellmann procedure [17] where $NADP^+$-reduction in the light is coupled to the reduction of glutathion in the presence of glutathion-reductase (both reagents were purchased from Boehringer Mannheim, W. Germany). The reduced glutathion was brought to reactions with 5-5′-dithiobis-(-2-nitrobenzoic acid) and the concentration of the yellow reaction product was measured at 412 nm in a digital Zeiss Spectralphotometer type PM2D. The assay conditions were exactly as described earlier [18]. When using the donor couple DPIP/ascorbate inhibition was dependent on the concentration. For Figs 7 – 9 the DPIP concentration was at 0.14 mM and ascorbate at 3.3 mM. If methylviologen was used as the terminal acceptor instead of $NADP^+$ concentrations between 3×10^{-5} M and 1.5×10^{-4} M were used. In this case the reaction contained 3 μmol NaCN/3 ml *i. e.* 10^{-3} M NaCN.

b. *Photophosphorylation reactions:* In order to be sure of our observations we used three different methods for the determination of ATP: Most frequently the Avron procedure was used [19]. For direct comparison purpose the identical assay mixture as described by Avron was used. In PMS-mediated photophosphorylation the assay mixture contained in 3 ml, 44 μmol tris pH 7.8; 60 μmol NaCl; 12 μmol $MgCl_2$; 30.5 μmol ascorbate, 12 μmol K_2HPO_4 containing 2 μCi P^{32}; 12 μmol ADP, 0.1 μmol PMS and chloroplasts from green tobacco equivalent to 30 – 40 μg of chlorophyll, from Su/su^2 equivalent to 10 μg of chlorophyll and chloroplasts from the yellow leaf patches of variegated NC 95 equivalent 50 μg of chlorophyll. All assays were routinely carried out in the presence of 10^{-5} M DCMU. Illumination was done for 4 min in a thermostated plexiglas container at 15 °C with 200 000 lx of white light with a Leitz Prado Univer-

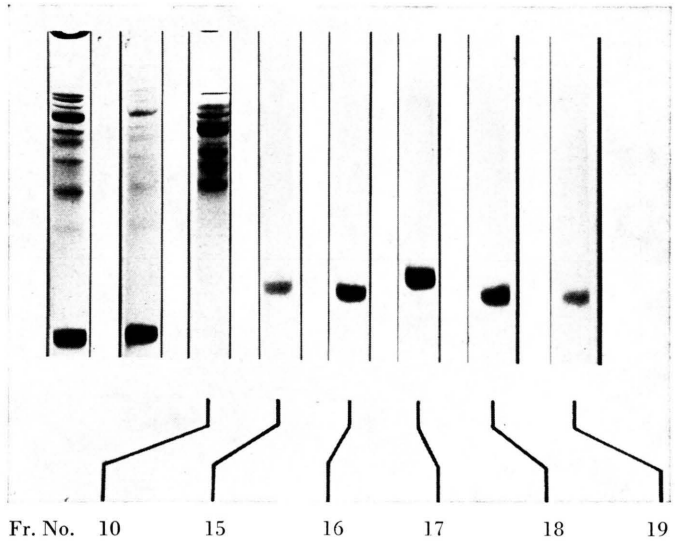

Fig. 1. Polyacrylamide gel electrophoresis of plastocyanin fractions obtained by separation on Sephadex G-75. Fraction 16 was used for injection. The two not numbered gels contained the starting material for the final step of purification.

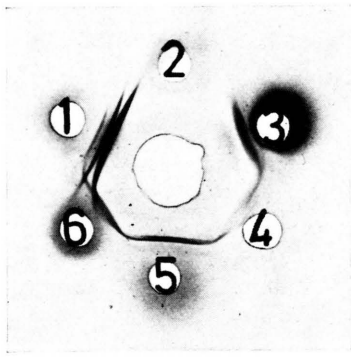

Fig. 2 a

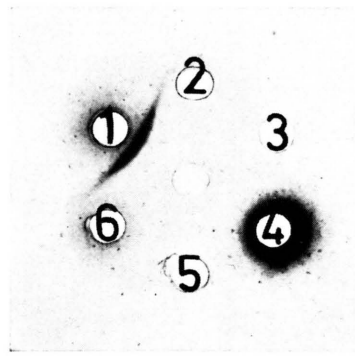

Fig. 2 b

Fig. 2. Serological test for purity of the plastocyanin preparation from tobacco with different complex antisera. a. The center well contained an impure preparation (composition shown in the first gel of Fig. 1) of plastocyanin in order to test the capacity of the complex antisera used. (1) Complex antiserum which contains antibodies to plastocyanin; (2) Monospecific antiserum to tobacco reductase; (3) Antiserum to broken chloroplasts from spinach; (4) Antiserum to broken chloroplasts from *Antirrhinum*; (5) Early antiserum to the lamellar system of *Antirrhinum*; (6) Late antiserum to the lamellar system of *Antirrhinum*. b. The center well contained a pure plastocyanin fraction. (1) Complex antiserum containing antibodies to plastocyanin; (2) Null serum to (1); (3) Monospecific antiserum to tobacco reductase; (4) Complex antiserum to broken chloroplasts from spinach; (5) Complex antiserum to broken chloroplasts from *Antirrhinum*; (6) Antiserum to the lamellar system of *Antirrhinum*.

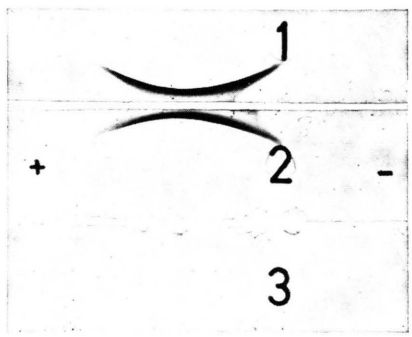

Fig. 3. Test for purity of the plastocyanin preparation from tobacco by means of immunoelectrophoresis. The well between (1) and (2) contained a complex antiserum containing antibodies to plastocyanin; the well between (2) and (3) contained an antiserum to broken chloroplasts from *Antirrhinum*. Holes (1), (2) and (3) contained the pure plastocyanin preparation.

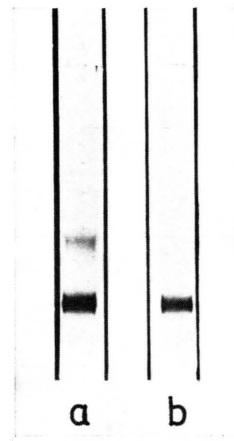

Fig. 4. Sodium dodecylsulphate gel electrophoresis of the pure plastocyanin preparation.

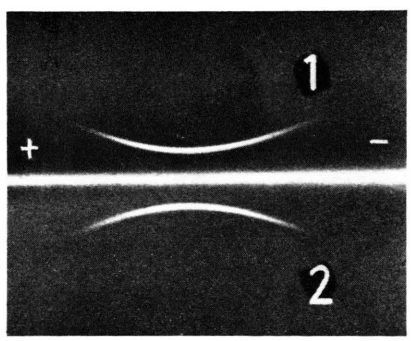

Fig. 6 a

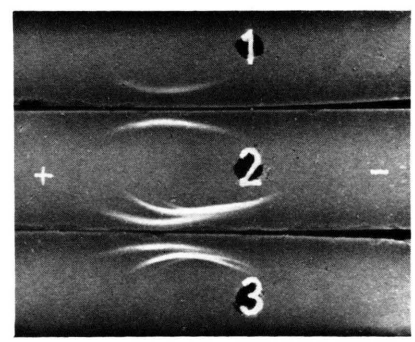

Fig. 6 b

Fig. 6. Test for monospecificity of the antiserum to tobacco plastocyanin by means of immune electrophoresis. a. Well between (1) and (2) contains the monospecific antiserum, holes (1) and (2) the pure antigen. b. The well between (1) and (2) contains the monospecific antiserum; the well between (2) and (3) contains a complex antiserum which amongst others contains antibodies to plastocyanin. Holes (1), (2) and (3) contain a crude plastocyanin containing chloroplast extract.

sal projector. Phosphorylation in the system DPIP/ascorbate → methylviologen or diaminobenzidine → methylviologen was done in the same assay as above without PMS but in the presence of 2.6 μmol DPIP, 61 μmol ascorbate, 3 μmol NaCN and 0.1 μmol methylviologen. In the case of 3-3′-diaminobenzidine (Schuchardt, München), 5 μmol/3 ml assay were used. Our assay in the system DAB → methylviologen gave the same phosphorylation rates as described by Goffer and Neumann [20], however, our ATP/2e ratios were consistently lower. The processing of the illuminated samples was done exactly as described by Avron [19]. The counting was done in a Packard model 3375 Tricarb Liquid Scintillation Spectrometer.

For comparison purpose also the test in which photophosphorylation is followed by measuring the inorganic phosphate content as described by Jagendorff and Avron [21] was used. If all the necessary controls were included in one test which are the assays minus ADP plus PMS or mediator, the reference minus ADP minus P_i and the complete assay minus mediator practically the same values as with the ^{32}P-method were obtained, even though, the absolute 660 nm reading may be greatly displaced by reducing agents such as ascorbate. Again, an enhancement by the antiserum to plastocyanin was demonstrated.

In the case of PMS-mediated cyclic photophosphorylation also the Boehringer/Mannheim "Test-combination for the enzymic determination of blood ATP" was used. The method gave principally the same results as the Avron procedure [19]. Cyanide interfers with the assay, so that pseudocyclic photophosphorylation could not be tested by this method. *Pigment analyses,* light conditions are described earlier [22]. *Protein determinations* were carried out according to the Lowry procedure [23]. In one case a protein determination of pure plastocyanin was made by determination of the dry weight (Fig. 5 b). In aliquots from the same preparation Lowry determinations were made, thus calibrating the Lowry determination against pure plastocyanin instead of calibrating against the usual bovine serum albumin.

Copper determinations were carried out with sodiumdithiocarbamate practically as reversal of the determination method for dithiocarbamate residues on tobacco, all as described earlier [24, 25]. The extinction of the yellow, filtered toluene solution was measured at 425 nm.

Electrophoresis: The polyacrylamide gel electrophoresis was carried out as described previously [18]. Sodium dodecylsulphate gel electrophoresis was run as described and referred to in an earlier paper [26].

Preparation of plastocyanin from green N. tabacum var. John William's Broadleaf

Chloroplasts from 9 kg of green *N. tabacum* var. John William's Broadleaf were processed as described by Vambutas and Racker [27] and Anderson and McCarty [28]. At the point where the Anderson and McCarty procedure finishes, our preparation from tobacco was not pure as shown by polyacrylamide gel electrophoresis (Fig. 1 * the first 2 gels) nor in the Ouchterlony immunodiffusion test with complex antisera (Fig. 2 a). We, therefore, concentrated these fractions again on a DEAE-cellulose column (DE 23 Whatman, ⌀ 3 cm, 5 cm high). The column was equilibrated as before with 0.03 M phosphate buffer pH 7. Elution was obtained by using 1 M phosphate pH 8.

The peak fractions containing a total of 30 mg protein were dialysed for 5 h against 0.03 M phosphate. Addition of a few drops of $K_3[Fe(CN)_6]$ solution developed the blue color. The solution was then applied onto a Sephadex G-75-column (⌀ 2.6 cm, 70 cm high). The column was equilibrated with 0.02 M tricine pH 8 and eluted with the same buffer and 6 ml fractions were collected. The numbered fractions 10 – 19 in Fig. 1 * show the obtained separation. The immunodiffusion test of fraction 16, the fraction which was finally used for immunization gave with a complex antiserum which contained amongst others antibodies to plastocyanin only one single band. No bands are seen with the antisera to the lamellar system of *Antirrhinum* or to broken chloroplasts from spinach [29]. The same was valid for the immunoelectrophoresis (Fig. 3). The fraction had an extinction ratio $E\frac{597}{278}$ of 1.1 and a molar extinction coefficient for the 597 nm peak of $4.5 \cdot 10^3$/M/cm if a molecular weight of 9800 was assumed. Extinction values were measured upon preparation in the Zeiss digital spectrophotometer PM2D.

Spectra were taken with a Cary Model 14R Spectrophotometer. The protein had been treated in the following way: For the visible region the sample had been dialysed for 3 days against three changes of 5 l 0.02 M tricine pH 8 containing 32 μg $K_3[Fe(CN)_6]$/ml. The buffer of the last dialysis was used as the reference. 5 ml of this solution were dialysed for 3 days against running destilled water, then lyophylized and the dry weight determined. Another aliquot was used for a Lowry protein determination and all other protein determinations made by the Lowry procedure [13], which then were referred to this dry weight determination. For the near UV

* Figs 1 and 2 see Table on page 202 a.

region the protein was dissolved in 0.02 M tricine pH 8 with 0.02 M tricine for reference.

Immunization of rabbits with plastocyanin: 0.4 mg of plastocyanin dissolved in 1 ml 0.06 M Sørensen phosphate buffer pH 7.8 were emulgated with 1 ml of Freund's adjuvant (Complete, Difco Laboratories, Detroit, USA) and injected subcutaneously into the rabbit. After 4 weeks 0.4 mg plastocyanin in 2 ml phosphate buffer were injected intravenously into the same rabbit followed by the same treatment after 5 days. 10 days after the last injection blood was withdrawn in intervalls of 7 days.

Three animals have been treated in the above described way. Agglutination tests, antiglobulin tests as well as antibody adsorption tests are described earlier [30].

Results

Characterization of the injected plastocyanin and of the antiserum to plastocyanin

Plastocyanin was prepared from chloroplasts of normal green tobacco, according to a modified procedure of Anderson and McCarty, as described in methods [28]. The preparation was purified until one single band was seen in the polyacrylamide gel electrophoresis (Fig. 1). The same preparation showed one single immunoprecipitation band when tested in the Ouchterlony diffusion test (Fig. 2 b) or in the immunoelectrophoresis against a complex antiserum which contained antibodies to plastocyanin (Fig. 3).

In the sodium dodecylsulphate polyacrylamide gel electrophoresis also only one single band was seen (Fig. 4 **). The apparent molecular weight of this component was determined by comparison with test proteins [26] and found to be 9800. If the preparation was not incubated long enough with the mercaptoethanol and sodium dodecylsulphate containing phosphate buffer a second band was observed. The apparent molecular weight of this larger component was 19000 (Fig. 4). Presumably, this corresponds to the apparent molecular weight of the protein in chloroplasts [2]. The absorption spectrum of the compound used for injection into the rabbit is shown in Fig. 5. It exhibits all the characteristics including those in the near UV described for spinach plastocyanin by Katoh [2]. The molar extinction coefficient for the 597 nm band of our best preparation

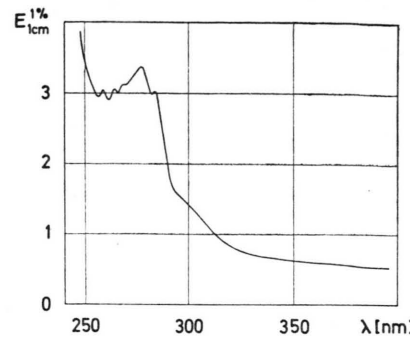

Fig. 5 a

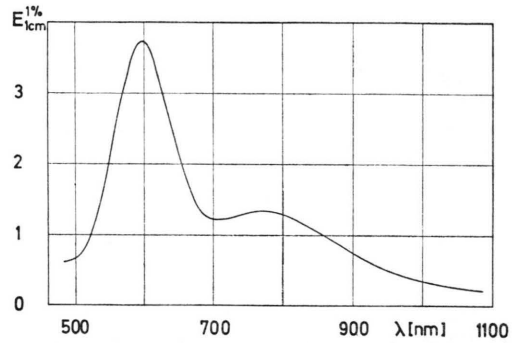

Fig. 5 b

Fig. 5. Spectrum of the pure plastocyanin preparation a. UV-region; b. visible region.

with an assumed molecular weight of 9800 is calculated to be 4.5/mM/cm and the extinction ratio E^{597}_{297} of our preparation was 1.1. The high extinction coefficient value was obtained when the 597 nm extinction was measured immediately after preparation. The value deduced from the spectrum is 4/mM/cm which might be explained by the necessary prolonged dialysis prior to the recording of the spectrum. The extinction ratio E^{597}_{297} is in both independent measurements the same. The copper content as determined with sodium diethyldithiocarbamate was 1 g atom copper per 10500 g protein, corresponding to a copper content of the protein of 0.6 per cent. Consequently, also the tobacco protein contains two copper atoms in the molecule, that is one copper atom per polypeptide chain. This preparation was injected into a rabbit and the withdrawn antiserum showed one single immunoprecipitation band with pure plastocyanin and significantly with a crude plastocyanin preparation (Fig. 6). From all purity tests we conclude that the used antiserum is monospecific.

** Figs 3, 4, and 6 see Table on page 202 b.

Table I. Agglutination reactions of the antiserum to tobacco plastocyanin with chloroplasts from two types of *N. tabacum* and with chloroplasts from *Antirrhinum majus* and *spinach*.

Chloroplast type	*N. tabacum* Wild type	*N. tabacum* Aurea mutant Su/su^2	*Antirrhinum majus* Strain 50	*Spinacia oleracea*
Stroma-freed chloroplasts [13] +antiserum to plastocyanin	agglutination	agglutination	specific adsorption (no agglutination)	specific adsorption (no agglutination
Amount of *stroma-freed chloroplasts* in mg dry weight by which 1 ml of antiserum to plastocyanin appears exhausted	18	19	36	15
Stroma-free swellable chloroplasts [12] +antiserum to plastocyanin	agglutination	agglutination	agglutination	agglutination
Amount of *stroma-free swellable* chloroplasts by which 1 ml of antiserum appears exhausted	20	4	36	7

Chloroplasts were prepared according to references 12 and 13.

Serological reactions of the antiserum to plastocyanin with different chloroplasts preparations

As can be seen from Table I various chloroplast preparations differ with respect to their reaction with the antiserum to plastocyanin. All preparations specifically adsorb antibodies but antibodies are adsorbed to different degrees. Moreover, the adsorption leads to agglutination in some cases whereas in others it does not. The fact that despite the not observed agglutination binding of antibodies had occured was demonstrated by a positive Coombs' test [31] and a positive "mixed antigen agglutination" with plastocyanin according to Uhlenbruck [32]. The specificity of the antibody binding is seen by comparison with control serum. By ultrasonication [33] the agglutinability is abolished. Occurence or nonoccurence of agglutination depends as reported earlier on the state in which the thylakoid membrane is in. This is caused by different preparation procedures and depends as shown here also on the species. For a quantitative evaluation of the exhaustion tests it should be noted that in comparison to agglutination, theoretically twice the amount of antibodies is to be bound for a specific adsorption, a condition which is apparently realized with spinach chloroplasts (Table I). Therefore, in the case of *Antirrhinum* swelling of the lamellar system has led to a loss of plastocyanin even though the exhaustion values are the same. This loss does not seem to occur with tobacco. As a difference to all this an increase in accessible plastocyanin is observed upon swelling in the aurea mutant. This has possibly several reasons: During swelling amongst other effects partition regions of the lamellar system become accessible to antibodies to different extents. On the other hand, due to the different preparation procedures, *stroma-free swellable chloroplasts* [12] contain in the suspension amongst stroma proteins also released plastocyanin whereas stroma-freed chloroplasts got their stroma and the released plastocyanin removed prior to the test. For these and other reasons we do not wish to give a quantitative interpretation at this point.

Finally, we should like to note that complex antisera to the lamellar system and to broken chloroplasts from either *Antirrhinum* and spinach do not contain antibodies to plastocyanin (Fig. 2 b).

Effect of the antiserum to plastocyanin on photosynthetic electron transport and on photophosphorylation

Normal green tobacco chloroplasts prepared according to Homann and Schmid [12] are inhibited by the antiserum with respect to the $NADP^+$-Hill reaction and their DPIP/ascorbate dependent $NADP^+$-reduction (Fig. 7). The maximal inhibition observed was between 40 to 50 per cent. We note that this chloroplast type is also directly agglutinated by the antiserum (Table I). It appears that this inhibition is only observed if the lamellar system is in a swollen condition. If methylamine is added to the chloroplast suspension prior to antiserum incubation neither agglutination nor inhibition of the

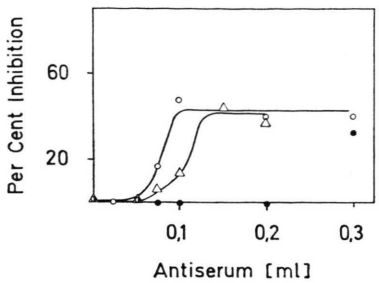

Fig. 7. △—△, Inhibition of photosystem-I mediated photoreduction of $NADP^+$ with the electron donor couple DPIP/ascorbate by the antiserum in tobacco chloroplasts from the green wild type. Chloroplasts corresponding to 52.6 μg total chlorophyll per assay were used. ○—○, Inhibition of the $NADP^+$ Hill reaction with chloroplasts corresponding to 155 μg total chlorophyll per assay. (●), Inhibition of the $NADP^+$ Hill reaction with chloroplasts corresponding to 45 μg total chlorophyll per assay. Note the large lag period.

$NADP^+$-reduction occurs. According to the literature [34] addition of methylamine causes shrinkage of the lamellar system. Hence, just as described earlier [18] direct agglutination and the degree of inhibition of photosynthetic electron transport depends on the condition or state in which the thylakoid membrane is in. Chloroplasts from the tobacco aurea mutant Su/su^2 contain a simplified lamellar system with only small grana and extended intergrana regions (lamellar system similar to Fig. 1 b in ref. 35). The activity of their photosystem-I-mediated methylviologen reduction is also impaired by the antiserum (Fig. 8). Comparison of the degree of inhibition with the amount of chloroplasts which is needed to exhaust 1 ml of antiserum, leads to a possible conclusion that this mutant contains tighter bound plastocyanin in comparison to the wild type.

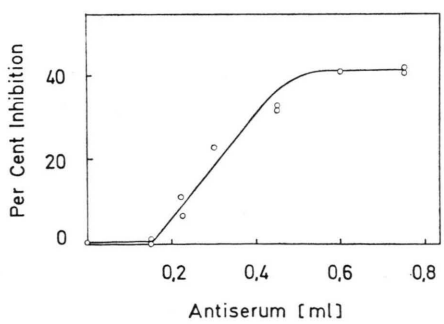

Fig. 8. Inhibition of the methylviologen Mehler reaction in the presence of DCMU with the DPIP/ascorbate donor couple in chloroplasts from the tobacco aurea mutant Su/su^2 corresponding to 10 μg total chlorophyll per assay.

In spinach chloroplasts the photoreduction of $NADP^+$ was also inhibited by the antiserum. The same was true for the DPIP/ascorbate mediated photoreduction of methylviologen (Fig. 9 a). As is already the case for tobacco chloroplasts a lag period is observed, that is, a certain amount of anti-

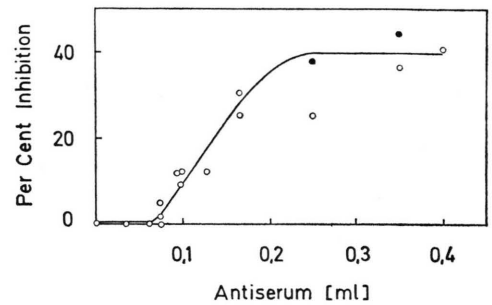

Fig. 9 a

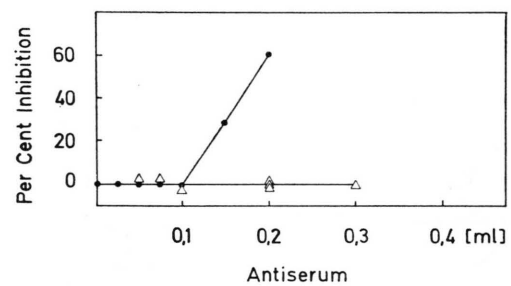

Fig. 9 b

Fig. 9. a. ○—○, Inhibition of the methylviologen Mehler reaction in spinach chloroplasts with the DPIP/ascorbate donor couple. The points are derived from 7 independent curves normalized to the amount of chloroplasts corresponding to 7 μg total chlorophyll. All points are corrected for the effect of the control serum. The points ● are those not corrected for the effect of the control serum. b. ●—●, Inhibition of the DPIP/ascorbate mediated $NADP^+$ reduction by the antiserum with spinach chloroplasts corresponding to 17.8 μg total chlorophyll. △—△, Same test with the same preparation 2 hours later. The control rate was unchanged, 63 μmol $NADP^+$ reduced · (mg chlorophyll)$^{-1}$ h^{-1}.

serum must be added before the onset of inhibition is observed. The resulting sigmoid shaped curves hint at some co-operative effect. In spinach chloroplasts and less frequently with tobacco chloroplasts yet uncontrollable conditions seem to exist in which the antiserum gives no inhibition although the reference rates of the photoreactions of the chloroplasts appear normal. In Fig. 9 b a chloroplast preparation from spinach is inhibited in the DPIP/ascorbate mediated $NADP^+$ reduction in the presence of antiserum by 60 per cent. Two hours

later the same preparation is not inhibited anymore even though the control rate had not changed at all (Fig. 9 b). By this time the chloroplasts had also lost their agglutinability.

Moreover, the antiserum to plastocyanin affects several types of photophosphorylation reactions in tobacco chloroplasts. The main observation is a stimulation which on a per cent basis appears highest in chloroplasts from yellow leaf patches of the variegated tobacco mutant NC95. The mutant contains as described earlier only single isolated thylakoids (Fig. 1 c in ref. 35) and exhibits only photosystem-I reactions [12]. Table II shows the effect of the antiserum on PMS-mediated cyclic and on pseudocyclic photophosphorylation in tobacco chloroplasts.

Table II. Effect of the antiserum to plastocyanin on PMS-mediated cyclic and on pseudocyclic photophosphorylation in tobacco chloroplasts.

Additions	*N. tabacum* Wild type	Aurea mutant Su/su²	Variegated NC 95 Yellow leaf patches
	[μmol ATP formed · (mg chlorophyll)$^{-1}$ · h^{-1}]		
PMS	178 ± 4	604	1075
PMS, 0.2 ml antiserum	172	920	2010
PMS, 0.2 ml null serum	178	708	1485
Methylviologen	21.5 ± 5	152 ± 34	100
Methylviologen, 0.2 ml antiserum	69	356	630
Methylviologen, 0.2 ml null serum	49	149	215
Methylviologen, 10^{-5} M DCMU	0 ± 4.6	0	100

fect of the antiserum on PMS-mediated cyclic photophosphorylation in three types of tobacco chloroplasts. In the same table the photophosphorylation reaction in the presence of antiserum to plastocyanin in the system $H_2O \rightarrow$ methylviologen is seen. Clearly, there is an enhancement of ATP formation in the presence of antiserum. Noteworthy is the fact that methylviologen mediated phosphorylation is DCMU-insensitive in chloroplasts from yellow leaf patches of the variegated tobacco mutant NC95. Obviously, in this case an endogenous donor feeds in electrons behind the inhibition site of DCMU. This is in agreement with what has been said before, namely that this mutant has lost the capacity for water splitting [12].

As plastocyanin is supposed to be located in the interior of the thylakoid membrane [11] we ultrasonicated green tobacco chloroplasts and assayed then for their phosphorylating activity in the system $H_2O \rightarrow$ methylviologen and ascorbate $\rightarrow$ methylviologen. The per cent stimulation by the antiserum was indeed increased although it was also observed that the reference rate decreases (Table III). Comparing this data with the result in Fig. 7 the stimulation might be considered a consequence of the inhibition of linear electron transport. In addition, we observed that the DPIP/ascorbate mediated methylviologen Mehler reaction in the presence of ADP, P_i and $MgCl_2$ was inhibited to a larger extent by the antiserum than under non-phosphorylating conditions (Table IV). This inhibition was completely absent if the chloroplasts were ultrasonicated prior to serum incubation (Table V).

Table III. Influence of ultrasonication on the stimulation of pseudocyclic photophosphorylation in wild type tobacco chloroplasts.

Additions	Ascorbate→Methylviologen		H_2O→Methylviologen	
	no sonication	45 sec sonication	no sonication	45 sec sonication
	[μmol ATP formed · (mg chlorophyll)$^{-1}$ · h^{-1}]			
Control	26.5	15	21.5	1.7
0.2 ml Antiserum to plastocyanin	30.6	42.5	69	22
0.2 ml Null serum	26	15	49	5

Table IV. Effect of the antiserum to plastocyanin on oxygen uptake in the DPIP/ascorbate-driven methylviologen Mehler reaction in tobacco chloroplasts.

Additions	*N. tabacum* Wild type	Aurea mutant Su/su²
	[μmol O_2 Uptake · (mg chlorophyll)$^{-1}$ · h^{-1}]	
–	616	1265
0.2 ml Antiserum to plastocyanin	704	1430
0.2 ml Null serum	752	1350
0.2 ml Antiserum to plastocyanin, 0.13 M CH_3NH_2	1006	1620
ADP, P_i, Mg^{2+}, 0.2 ml antiserum to plastocyanin	649	1330
ADP, P_i, Mg^{2+}, 0.2 ml null serum	1035	1770
ADP, P_i, Mg^{2+}, 0.2 ml antiserum to plastocyanin, 0.13 M CH_3NH_2	960	1620

The reaction was carried out in thermostated Warburg vessels at 25 °C, illuminated from below with 24000 ergs; $sec^{-1}\,cm^{-2}$ of red light 580 nm $< \lambda <$ 700 nm at 25 °C. The pH was 7.2.

Table V. Effect of the antiserum to plastocyanin on oxygen uptake in the DPIP/ascorbate-driven methylviologen Mehler reaction in sonicated chloroplasts from the tobacco mutant Su/su^2.

Additions	*N. tabacum* Aurea mutant Su/su^2 [μmol O_2 Uptake · (mg chlorophyll)$^{-1} \cdot h^{-1}$]
—	1235
0.2 ml Antiserum to plastocyanin	1220
0.2 ml Null serum	1255
0.2 ml Antiserum to plastocyanin, 0.13 M CH_3NH_2	1130
ADP, P_i, Mg^{2+}, 0.2 ml antiserum to plastocyanin	1430
ADP, P_i, Mg^{2+}, 0.2 ml null serum	1255
ADP, P_i, Mg^{2+}, 0.2 ml antiserum to plastocyanin, 0.13 M CH_3NH_2	1185

Conditions as in Table IV. Chloroplasts have been sonicated for 1 min prior to serum addition.

Disregarding the question whether photophosphorylation in the system DPIP/ascorbate → methylviologen is of the pseudo-cyclic type or rather of the cyclic type [36] we observed that under conditions described in methods in the presence of DCMU the phosphorylation reaction is also enhanced by the antiserum (Table VI). The phosphorylation reaction in the system diaminobenzidine → methylviologen (in the presence of DCMU) is according to Goffer and Neumann [20] of the noncyclic type and is also enhanced (Table VI).

This stimulation by the antiserum appears specific and is definitely existent because the stimulation is observed not only with different ATP-measuring assays (see methods) but also because conditions can be chosen in which only in the presence of the antiserum phosphorylation is observed (Table VI). On the other hand the Avron-procedure of ATP-measurement appears straight-forward and sensitive, making an experimental error improbable. If the stimulation effect was an artifact it must be caused by a not understood interference of this antiserum with the donor systems used. At this point we should comment on the unspecific stimulation of the control serum which is sometimes observed. The stimulation is unspecific because the ATP/2e ratio

Table VII. Effect of the antiserum to plastocyanin on the stoichiometry of ATP-formation to electron transport in the system 3,3′-diaminobenzidine (DAB) → methylviologen in normal green tobacco chloroplasts.

Additions	pH	Electron transport *	ATP **	ATP/2e
none (basal rate)	**7.2**	**115**	**0**	—
ADP, P_i, Mg^{2+}	7.2	205	100	0.49
ADP, P_i, Mg^{2+} antiserum to plastocyanin	7.2	222	187	0.84
ADP, P_i, Mg^{2+} normal rabbit serum	7.2	308	154	0.5

* μmol O_2 uptake · (mg chlorophyll)$^{-1} \cdot h^{-1}$; ** μmol ATP formed · (mg chlorophyll)$^{-1} \cdot h^{-1}$.

Table VI. Effect of the antiserum to plastocyanin on photosystem-I-mediated photophosphorylation reactions in tobacco chloroplasts.

Reactions	Wild type	*N. tabacum* Aurea mutant Su/su^2	Variegated NC 95 (yellow leaf patches)
	[μmol ATP formed · (mg chlorophyll)$^{-1} \cdot h^{-1}$]		
Diaminobenzidine (DAB) → methylviologen (MV)	1.3	9.4	0.5
DAB minus MV	0	0	0
DAB/MV + 0.2 ml antiserum, 2nd blood withdrawal	13.3	147	19
DAB/MV + 0.2 ml null serum	4.3	14.55	5.5
DAB/MV + 0.2 ml antiserum, 7th blood withdrawal	11.4	18.9	—
Dichlorophenol indophenol (DPIP)/ascorbate → methylviologen (MV)	0	33.6	68
DPIP/ascorbate minus MV	0	19.0	49
DPIP/ascorbate/MV + 0.2 ml antiserum, 2nd blood withdrawal	20.4	—	—
DPIP/ascorbate/MV + 0.2 ml null serum	7	103	47
DPIP/ascorbate/MV + 0.2 ml antiserum, 7th blood withdrawal	**8.9**	122	87
DPIP/ascorbate/MV + 0.2 ml antiserum from 2nd animal	—	85	—
DPIP/ascorbate/MV + 0.2 ml null serum	—	68	—

Assay as in methods but under light limiting conditions.

in the presence and absence of the control serum remains the same whereas under the same conditions the ATP/2e ratio is increased in the presence of antiserum (Table VII), which means that the electron transport rate and ATP formation are enhanced by the control serum. The control rates of photophosphorylation of our tobacco chloroplasts as shown in Table VII, compare to those reported by Goffer and Neumann [20], however, our ATP/2e-ratios in the controls without and with control serum were usually 0.5.

The control serum belonging to the antiserum to plastocyanin affects the linear electron transport reactions of chloroplasts in different ways depending on the amount of control serum present. Small amounts of null serum stimulate electron transport reactions whereas high amounts are inhibitory (Fig. 10). This appears to be also a property of purchased rabbit anti γ-globulin.

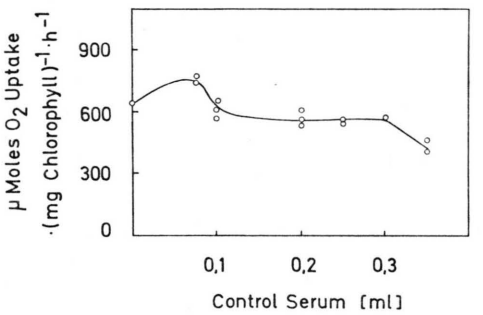

Fig. 10. Effect of the rabbit control serum on the DPIP/ascorbate mediated methylviologen Mehler reaction with spinach chloroplasts.

Concerning the stimulation of photophosphorylation reactions by the antiserum we used for better comparison with work of others in the following experiments * spinach chloroplasts and mediators like menadione or diaminodurene (DAD) under the conditions described by Hauska, Reimer, and Trebst [37]. In Table VIII it is seen that menadione-mediated cyclic photophosphorylation in which the electron flow cycle is closed up with the donor site of photosystem I with plastoquinone [37] is inhibited by 50 per cent. The poising of the system was done with N-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate as described by Hauska *et al.* [37]. Consequently, plastocyanin involved in this reaction is equally accessible to antibodies. Photophosphorylation which

* Experiments suggested by Prof. Trebst.

Table VIII. Effect of the antiserum to tobacco plastocyanin on photophosphorylation reactions in spinach chloroplasts.

Additions	[μmol ATP · (mg chlorophyll)$^{-1}$·h^{-1}]	Inhibition [%]
Menadione, antiserum to plastocyanin	12.2	54
Menadione, normal rabbit serum	26.7	—
DAD/methylviologen	234	—
DAD antiserum to plastocyanin	374	0
DAD normal rabbit serum	363	—
DAD/TMPD/methylviologen	283	—
DAD/TMPD antiserum to plastocyanin	310	13
DAD/TMPD normal rabbit serum	357	—

The values are averages of 5 individual determinations. Light intensity 200 000 lx white light. The assay for menadione mediated photophosphorylation was illuminated for 7 min at 15 °C under anaerobic conditions. The system was poised with TMPD as described [37]. The chloroplasts were preilluminated in buffer in the presence of menadione and ascorbate prior to completion of the assay mixture. Photophosphorylations with DAD, DAD/TMPD were run at room temperature under aerobic conditions and illuminated for 2 min. Methylviologen concentration 10^{-5} M, ascorbate 5 mM, DAD 10^{-4} M, DAD/TMPD $5 \cdot 10^{-5}$ M each.

accompanies electron transport between DAD and methylviologen was not effected at the high light intensity used in the experiments of Table VIII. In the presence of TMPD this photophosphorylation reactions was inhibited by a low 13 per cent. But at lower light intensities (26 000 lx white light) ATP-formation and electron transport in the system DAD/TMPD → methylviologen were clearly inhibited by the antiserum (Table IX). The inhibited electron flow is coupled to photophosphorylation as evidenced by the unchanged ATP/2e ratios. These ratios agree

Table IX. Effect of the antiserum to plastocyanin on the stoichiometry of ATP-formation to electron transport in the system diaminodurene/N-tetramethyl-*p*-phenylenediamine → methylviologen in spinach chloroplasts.

Additions	Electron transport *	ATP **	ATP/2e
ADP, P_i, Mg^{2+}	554	148.5	0.27
ADP, P_i, Mg^{2+}, antiserum to plastocyanin	437	99.5	0.24
ADP, P_i, Mg^{2+}, normal rabbit serum	546	126.5	0.23

Light limiting conditions; 26 000 lx white light for 2 min, assay at room temperature. * μmol O_2 uptake · (mg chlorophyll)$^{-1}$·h^{-1}; ** μmol ATP formed · (mg chlorophyll)$^{-1}$·h^{-1}. The reaction was run under aerobic conditions as described by Hauska *et al.* [37]. The assay contained: DAD 5×10^{-5} M, TMPD 5×10^{-5} M, methylviologen 10^{-5} M, 5 mM ascorbate and 2×10^{-5} M DCMU.

well with those observed by Hauska *et al.* [37]. Under the same conditions photophosphorylation in the system DAD → methylviologen is inhibited by only 10 per cent (experiments not shown).

The initial rates of linear electron transport in the system DAD/TMPD → methylviologen (Table X) are inhibited to nearly the same maximal extent as are those in the linear electron transport reaction in the system DPIP/ascorbate → methylviologen shown in Fig. 9 a.

Table X. Effect of the antiserum to tobacco plastocyanin on noncyclic electron transport between diaminodurene and methylviologen in the presence of N-tetramethyl-*p*-phenylenediamine in spinach chloroplasts.

Additions	[μmol O_2 uptake (mg chlorophyll)$^{-1} \cdot h^{-1}$]	Inhibition [%]
ADP, P_i, Mg^{2+}	1780	—
ADP, P_i, Mg^{2+}, antiserum to plastocyanin	1310	30
ADP, P_i, Mg^{2+}, normal rabbit serum	1880	—

Illuminated at room temperature with 26 000 lx white light for 0.2 min. Conditions as in Table IX.

Discussion

From our experiments it appears that the lamellar system of tobacco, *Antirrhinum* and spinach chloroplasts contains plastocyanin which is accessible to antibodies. In one state of the molecular structure of the lamellar system agglutination and inhibition of linear photosynthetic electron flow is observed upon addition of the antiserum to plastocyanin. In another condition in which the lamellar system has lost its swellability or when it is under the effect of methylamine neither agglutination nor inhibition of linear electron transport (Table IV) is observed. Nevertheless, also in this state plastocyanin is accessible to antibodies as demonstrated by a positive Coombs' test. Our observation that plastocyanin is located in the outer surface of the thylakoid membrane is in agreement with observations by several authors. Selman, Johnson, Dilley, and Voegeli observe that ^{35}S-labelled *p*-diazoniumbenzene sulfonic acid reacts with amino acids of plastocyanin. Due to the molecular size and the properties of this reagent it is supposed to react only with compounds of the thylakoid membrane, accessible from the outside [38]. The same conclusion was reached by use of other chemical protein modifiers [38]. Polysine inhibits photosynthetic electron flow which is according to the interpretation of Brand, San Pietro and Mayne due to an inhibition of plastocyanin [39]. Compared to these reactants an antiserum has the advantage of specificity. Our results, however, do not agree with those of Hauska *et al.* who concluded from studies with an antiserum to plastocyanin that plastocyanin should be located in an inaccessible location inside the thylakoid membrane [11]. The different findings of ours and those of Hauska *et al.* might be due to the shape of the inhibition curve seen in Figs 7 – 9. Most inhibition curves obtained by antisera have hyperbolic shapes whereas in the case of antibodies to plastocyanin sigmoid shapes are observed. Hauska *et al.* might not have used sufficient concentrations of antibodies to overcome the lag. In addition, we observed that the chloroplasts eventually loose the ability to agglutination and inhibition of electron transport by the antiserum (Fig. 9 b). It should be emphasized again at this point that this state is not necessarily characterized by a loss of the plastocyanin since specific adsorption *i. e.* a positive Coombs' test is observed. Finally, there remains the possibility that for unknown reasons the antibody by Hauska *et al.* is directed towards different antigenic determinants of the plastocyanin molecule than our antibody. In this case the positive observation would be decisive.

In contrast to our earlier observation with ferredoxin-$NADP^+$ reductase, inhibition of electron transport by antibodies to plastocyanin is only observed in the swollen chloroplast condition. In this condition partition regions are partially opened up [18]. From this we conclude that the plastocyanin involved in linear electron transport might well be located in the partition regions of the lamellar system, but, nevertheless, on the outer surface of the thylakoid membrane. The observed maximal inhibition of electron transport by 40 to 60 per cent would mean that only 40 to 60 per cent of the plastocyanin present participates in electron transport or that 40 to 60 per cent of the total amount of plastocyanin is accessible. Since not all partition regions are opened up the first assumption is absurd. The view of Hauska *et al.* that externally added plastocyanin is readsorbed unspecifically onto the thylakoid membrane would infer that our plastocyanin has leaked out prior to its readsorp-

tion. This view is difficult to dispute but would in turn infer that a chloroplast condition without agglutination by the antiserum would be rather difficult to obtain. We find agglutination (Table I). Hauska *et al.* do not [11].

We observed that antibodies to plastocyanin inhibit linear photosynthetic electron transport, but we observed no effect of the antiserum on PMS-mediated cyclic photophosphorylation in wild type tobacco chloroplasts (Table II). The latter observation fits the view of Witt, Rumberg, and Junge [40], Trebst [41] and Hauska, Reimer, and Trebst [37] namely that PMS-mediated photophosphorylation can proceed without plastocyanin, closing the electron flow cycle directly with P_{700}. In the mutant chloroplasts a slight stimulation of PMS-mediated photophosphorylation is observed (Table II). In contrast to the PMS-mediated cyclic photophosphorylation we observe an effect of our antiserum on various other photophosphorylation reactions in undisrupted chloroplasts, the general effect being a stimulation which is especially high in the mutant chloroplasts (Tables II, III, VI, and VII). This stimulation is a consequence of the inhibition of linear electron flow. In Table VII it is seen that the stoichiometry of ATP-formation is changed mainly because there is inhibition of electron flow. Consequently, when linear electron flow is inhibited by antibodies to plastocyanin certain photophosphorylation reactions are accelerated, may be because a linear or basal electron flow competes for a common intermediate or acceptor (in the assay) with an electron flow coupled to photophosphorylation [42]. In fact, some of these stimulated phosphorylation reactions might, like the PMS-mediated type, be reactions in which no plastocyanin is involved [43]. In Table VI the stimulation was observed in a system with high DPIP concentrations [44] and DAB at high concentrations might also feed in the electrons behind the plastocyanin site. Since ATP forming rates of these systems are not high one could visualize the stimulation as being caused by a competitive reaction mentioned above in which the functionability of plastocyanin would exert a kind of regulatory influence on these reactions. In contrast, in the case of Table II where the stimulation is observed in the system $H_2O \rightarrow$ methylviologen inhibition of accessible plastocyanin might indeed result in a stimulation effect on an electron transport between inaccessible plastocyanin and methylviologen across the thylakoid membrane *. This would only be possible if the accessible plastocyanin was merely involved in linear electron transport or coupled to a lesser extent to photophosphorylation than the inaccessible plastocyanin. Eventually, this would speak in favor of two types of plastocyanin namely one type in an accessible location on the outer surface of the thylakoid membrane (in the partitions), the other type in an inaccessible location inside the thylakoid membrane [11]. We cannot exclude this possibility. However, the amount of plastocyanin as determined by the number of antibody molecules bound which we find to be accessible in *Antirrhinum* chloroplasts is one molecule per 500 chlorophyll molecules, in spinach chloroplasts the amount is 1 molecule plastocyanin per approximately 300 chlorophyll molecules. (Radunz unpublished results.) According to the literature this is the usual plastocyanin content.

Summarizing our results we favor the view that in the lamellar system of chloroplasts from tobacco, *Antirrhinum*, and spinach either all the plastocyanin or an appreciable amount of it is located in the outer surface of the thylakoid membrane, that is in the grana and intergrana regions of the lamellar system. This plastocyanin is involved in linear photosynthetic electron transport and the accompanying photophosphorylation (Table VIII). The sigmoid shape of the inhibition curves hints at a co-operative effect. On the other hand, some of our experiments, namely the stimulation of ATP-formation in the system $H_2O \rightarrow$ methylviologen (Tables II and III) can be interpreted that in addition part of the plastocyanin might be located in the interior of the thylakoid membrane.

The authors would like to thank Professor A. Trebst for helpful suggestions and for putting at the time an unpublished manuscript at their disposition. The authors also thank Mrs. M. Russo, Mrs. C. Essmann and Miss E. Schölzel for skillfull technical assistance.

* This has been suggested by Prof. Trebst in the course of a discussion.

[1] S. Katoh, Nature **186**, 533 [1960].

[2] S. Katoh, J. Shiratori, and A. Takamiya, J. Biochem. (Tokyo) **51**, 31 [1962].

[3] A. M. Ramshaw, R. H. Brown, M. D. Scawen, and D. Boulter, Biochim. Biophys. Acta **303**, 269 [1973].

[4] S. Katoh, J. Suga, J. Shiratori, and A. Takamiya, Arch. Biochem. Biophys. **94**, 136 [1961].
[5] D. S. Gorman and R. P. Levine, Plant Physiol. **41**, 1637 [1966].
[6] S. Katoh and A. San Pietro, Proc. Intern. Symp. on the Biochemistry of Copper, p. 407, Acad. Press, New York 1966.
[7] B. Kok, H. J. Rurainski, and E. A. Harmon, Plant Physiol. **39**, 513 [1964].
[8] A. Trebst and E. Elstner, Z. Naturforsch. **20 b**, 925 [1965].
[9] J. S. C. Wessels, Biochim. Biophys. Acta **109**, 614 [1965].
[10] L. P. Vernon, E. R. Shaw, and B. Ke, J. Biol. Chem. **241**, 4101 [1966].
[11] G. A. Hauska, R. E. McCarty, R. J. Berzborn, and E. Racker, J. Biol. Chem. **246**, 3524 [1971].
[12] P. H. Homann and G. H. Schmid, Plant Physiol. **42**, 1619 [1967].
[13] W. Kreutz and W. Menke, Z. Naturforsch. **15 b**, 402 [1960].
[14] G. H. Schmid, J. Microscopie (Paris) **6**, 485 [1967].
[15] G. H. Schmid, Planta **77**, 77 [1967].
[16] L. Burk and H. A. Menser, Tobacco Sci. **8**, 101 [1964].
[17] G. L. Ellmann, Arch. Biochem. Biophys. **82**, 70 [1959].
[18] G. H. Schmid and A. Radunz, Z. Naturforsch. **29 c**, 384 [1974].
[19] M. Avron, Biochim. Biophys. Acta **40**, 257 [1960].
[20] J. Goffer and J. Neumann, FEBS Letters **36**, 61 [1973].
[21] A. T. Jagendorf and M. Avron, J. Biol. Chem. **231**, 277 [1958].
[22] A. Radunz, G. H. Schmid, and W. Menke, Z. Naturforsch. **26 b**, 435 [1971].
[23] O. H. Lowry, N. J. Rosebrough, A. L. Farrand, and R. J. Randall, J. Biol. Chem. **193**, 265 [1951].
[24] F. J. Viles, Chem. Zentralblatt **112**, II 641 [1941].
[25] K. Schmid and G. Schmid, Der Deutsche Tabakbau **5**, 48 [1970].
[26] W. Menke and E. Schölzel, Z. Naturforsch. **26 b**, 378 [1971].
[27] V. K. Vambutas and E. Racker, J. Biol. Chem. **240**, 2660 [1965].
[28] M. M. Anderson and R. E. McCarty, Biochim. Biophys. Acta **189**, 193 [1969].
[29] M. B. Allen, F. R. Whatley, and D. J. Arnon, Biochim. Biophys. Acta **32**, 32 [1959].
[30] A. Radunz, Z. Naturforsch. **27 b**, 822 [1972].
[31] R. R. A. Coombs, M. H. Gleeson-White, and J. G. Hall, Brit. J. Exp. Pathol. **32**, 195 [1951].
[32] G. Uhlenbruck, Med. Welt **17**, 906 [1965].
[33] C. G. Kannangara, D. van Wyk, and W. Menke, Z. Naturforsch. **25 b**, 613 [1970].
[34] S. Izawa and N. E. Good, Plant Physiol. **41**, 544 [1966].
[35] G. H. Schmid, Methods in Enzymology (ed. A. San Pietro), **Vol. 23**, p. 171, Acad. Press, New York 1971.
[36] H. Strotmann and C. von Gösseln, Z. Naturforsch. **27 b**, 454 [1972].
[37] G. Hauska, S. Reimer, and A. Trebst, Biochim. Biophys. Acta **357**, 1 [1974].
[38] B. R. Selman, G. L. Johnson, R. A. Dilley, and K. K. Voegeli, Proceedings of the 3rd International Congress on Photosynthesis Research, Rehovoth, Israel.
[39] J. Brand, A. San Pietro, and B. C. Mayne, Arch. Biochem. Biophys. **152**, 426 [1972].
[40] H. T. Witt, B. Rumberg, and W. Junge, Biochemie des Sauerstoffs (eds. B. Hess and H. J. Staudinger), p. 262, Springer-Verlag, 1968.
[41] A. Trebst, Ann. Rev. Plant Physiol. **25**, 423 [1974].
[42] S. Izawa and N. E. Good, Biochim. Biophys. Acta **162**, 380 [1968].
[43] D. B. Knaff and D. I. Arnon, Proc. Nat. Acad. Sci. U.S. **64**, 715 [1969].
[44] Y. Fujita and F. Murano, Plant and Cell Physiol. **8**, 269 [1967].