Plant Physiol. (1967) 42, 1619–1632

Photosynthetic Reactions of Chloroplasts with Unusual Structures[1]

Peter H. Homann and Georg H. Schmid

Florida State University, Department of Biological Science and Institute of Molecular Biophysics, Tallahassee, Florida 32306

Received July 7, 1967.

Summary. Photosynthetic reactions of whole leaves and isolated chloroplasts from various mutants of *Nicotiana tabacum* have been correlated to the lamellar structure seen in electron micrographs of the chloroplasts. In this way it could be established that a fully active photosystem I can be associated with single unfolded thylakoids. The complete photosynthetic electron transport system including the oxygen evolving apparatus of photosystem II, on the other hand, appears to require a close packing of at least 2 thylakoids. The unusual high capacity for photosynthesis observed earlier for leaves of certain aurea mutants is reflected by a correspondingly high activity of the isolated chloroplasts in the Hill reaction. These chloroplasts contain extended areas where 2 thylakoids touch by forming simple lamellar overlappings instead of the familiar stacks of lamellar discs.

It is not surprising that the evolution of the photosynthetic apparatus from its simplest form in the green photosynthetic bacteria to the elaborate system of the green plants was accompanied by the evolution of an ever more complicated pigmented structure. The relationship, however, between the photosynthetic processes and the membrane systems on which they occur is still very little understood. A possible approach to this problem was opened by recent publications of Schmid and Gaffron (30, 31, 33). They described unusually high saturation rates of photosynthesis in leaves of certain aurea mutants of tobacco such as Su/su. The chloroplasts of these mutants do not contain high stacks of disc shaped lamellae, i.e., grana, but much simpler looking foldings and doublings of extended "thylakoids" (25) or "frets" (39). There are, however, yellow chlorophyll deficient leaf patches in a variegated tobacco mutant which showed no measurable photosynthetic oxygen evolution. The chloroplasts in this tissue contained neither grana nor foldings or doublings of thylakoids (28). We have now undertaken a comparative study of the activity of various mutant chloroplasts in photoreactions involving only system I or both photosystem I and II which permit a better insight into the relationship between structural elements and partial processes of photosynthesis.

The saturation rate of photosynthesis in normal spinach leaves corresponds closely to the maximal rates reported for the Hill reaction with isolated chloroplasts in presence of an uncoupler of photophosphorylation (14, 17). We suspected, therefore, that the rate limiting step at light saturation in normal green plants is associated with the photosynthetic electron transport system and not with the CO_2 fixing enzymes and photophosphorylation. Consequently, chloroplasts isolated from the aurea tobacco mutant Su/su should give a higher saturation rate in the Hill reaction than chloroplast preparations from any green control plants. This was indeed observed. On the other hand, chloroplasts taken from the very chlorophyll deficient leaf areas of the variegated tobacco mutant NC 95 var. were found to be inactive in the Hill reaction. The inability of these leaf patches to carry out photosynthesis (28), therefore, is reflected by a deficiency in the electron transport system of their chloroplasts.

Further experiments provided us with evidence that such chloroplast reactions which do not involve photosynthetic system II can occur in separate lamellar discs. System II activity may, as an essential structural prerequisite, require the presence of "partitions" (39) formed by lamellar overlappings.

Materials and Methods

Seeds of the tobacco plants were obtained from the Agricultural Station, Beltsville, Maryland, by the courtesy of Drs. H. Heggestad and H. Menser, and from Dr. C. Dean of the Florida Experiment Station in Quincy, Florida. The plants used for this study were the normal green tobacco varieties John Williams Broadleaf (JWB) and NC 95, the aurea mutant Su/su and the variegated variety of NC 95 (NC 95

[1] These studies were aided by grant AF-AFOSR-662-65 from the Air Force Office of Scientific Research, and contract AT-(40-1)-2687 from the United States Atomic Energy Commission.

var.). A variegated Su/su (Su/su var.) appeared in 1 of our own selfed Su/su seedlots. Only 1 plant was available which has not yet produced fertile flowers. A detailed description of all these tobacco plants will be published elsewhere (29).

The tobacco plants were grown as described earlier (31) or cultivated in a liquid nutrient culture (1, 12). Growing leaves from adult plants were used throughout. Photosynthetic oxygen evolution was measured manometrically either by floating leaf sections on 3 ml 0.1 M bicarbonate buffer (pH 8.9) or by supporting them on a wire screen above this buffer in order to prevent "drowning" (31, 32). Photosynthetic CO_2 fixation was measured using a gas tight plexiglass chamber and ^{14}C labeled CO_2 at a constant temperature (12). Pigment analyses were carried out after extraction with 80 % acetone or methanol (24, 31). Standard procedures were used for the preparation of whole chloroplasts (14, 38) and of partially purified ferredoxin (4), and for measurements of the ferricyanide Hill reaction (21), of photophosphorylation (3) and of the photoreduction of $NADP^+$ with the ascorbate-2,4-dichlorophenol indophenol (DCPIP) couple and saturating amounts of ferredoxin (36). The presence of 20 mM ascorbate pH 7.5 (3) in the homogenizing medium for the chloroplasts of tobacco was found to be advantageous. Ascorbate was omitted in the wash solution when the chloroplasts were to be checked for their Hill activity. Detailed studies of the Hill reaction were often hampered by the instability of the chloroplast preparations: chloroplasts from Su/su lost 50 % of their activity in about 4 hours. No satisfactory stabilizer for the preparation could be found. System I activity was lost much less rapidly.

To obtain maximal rates of electron transport in the ferricyanide Hill reaction, the chloroplasts were suspended in 0.4 M sucrose containing 10 mM NaCl, 50 mM tris-HCl pH 7.5, 0.2 % serum-albumin (from various sources) and 0.2 % pectinase (Nutritional Biochemicals or Sigma). The latter 2 ingredients were added because in the course of our studies we made the interesting observation that the presence of a combination of albumin and pectinase in the suspension medium of the chloroplasts gave higher rates of the Hill reactions than we had observed earlier. Neither pectinase nor albumin alone stimulated the Hill activity to a comparable extent. We have not studied the mechanism of action of these proteins, nor did we make a detailed investigation to find out which amounts of pectinase and albumin are optimal. Comparison of electron micrographs of the pectinase-albumin treated chloroplasts with those of untreated chloroplasts did not reveal any remarkable differences, although the treated chloroplasts appeared more swollen. No effect of the addition of pectinase and albumin was found on photoreactions involving only system I, i.e., the PMS-mediated photophosphorylation and the photoreduction of $NADP^+$ with the ascorbate-DCPIP couple as electron donor. On the other hand, the so-called non-cyclic photophosphorylation with ferricyanide as electron acceptor, and the Hill reaction with DCPIP were stimulated by pectinase-albumin, just like the ferricyanide Hill reaction (table I).

In our experiments, the Hill activity was determined 1 to 1½ hours after homogenization of the leaves by illuminating 0.05 to 0.20 ml of the properly diluted chloroplast suspension which was brought to 2.7 ml with 0.4 M sucrose containing 10 mM NaCl and 50 mM tricine-NaOH buffer (pH 7.5). If not otherwise stated, 18 mM methylamine (pH 7.5) were added to uncouple phosphorylation (14). Photophosphorylations were usually carried out in open testtubes which were kept in a water bath of 20° and illuminated from the side. Only for determinations of the quantum requirement did we use rectangular glass cuvettes which were illuminated from below.

Floodlights, spotlights or projection lamps served as light sources in the visible region at wavelengths below λ 700 mμ. Red light ($\lambda >$ 600 mμ) was obtained with a red plastic filter. Far-red light was separated from the light of a Sylvania sungun by passing it through a glass filter which absorbed all visible light with wavelengths lower than λ 700 mμ. Light intensity measurements were made with an Isco Spectroradiometer (Instrument Specialties Company, Lincoln, Nebraska). The intensities of the light were determined between λ 575 and 700 mμ for red and between λ 700 and 750 mμ for far-red light, but the 1000W projection bulbs used for figures 4, 5 and 6 were calibrated in the red between λ 575 and 750 mμ. For accurate measurements of absorbed light in the determination of quantum efficiencies, the experimental set-up was duplicated with the sample placed into an integration sphere which had been calibrated with a

Table I. *Effect of Pectinase + Albumin on Various Chloroplast Reactions*

Data represent rates obtained in 1 typical experiment each: μmoles electron acceptor reduced (or μmoles ATP formed respectively)/mg chl × hr + MA = 18 mM methylamine present.

Origin of chloroplast	Presence of albumin + pectinase*	Hill reaction ferricyanide —MA	Hill reaction ferricyanide +MA	Hill reaction DCPIP —MA	Hill reaction DCPIP +MA	Photophosphorylation ferricyanide	Photophosphorylation PMS	$NADP^+$ Photoreduction
Su/su	—	280	1380	175	625	260	1100	205
	+	410	2100	240	830	410	1030	170
NC 95 green	—	140	830	80	260	55	500	55
	+	200	1050	95	330	75	480	45

* See Methods.

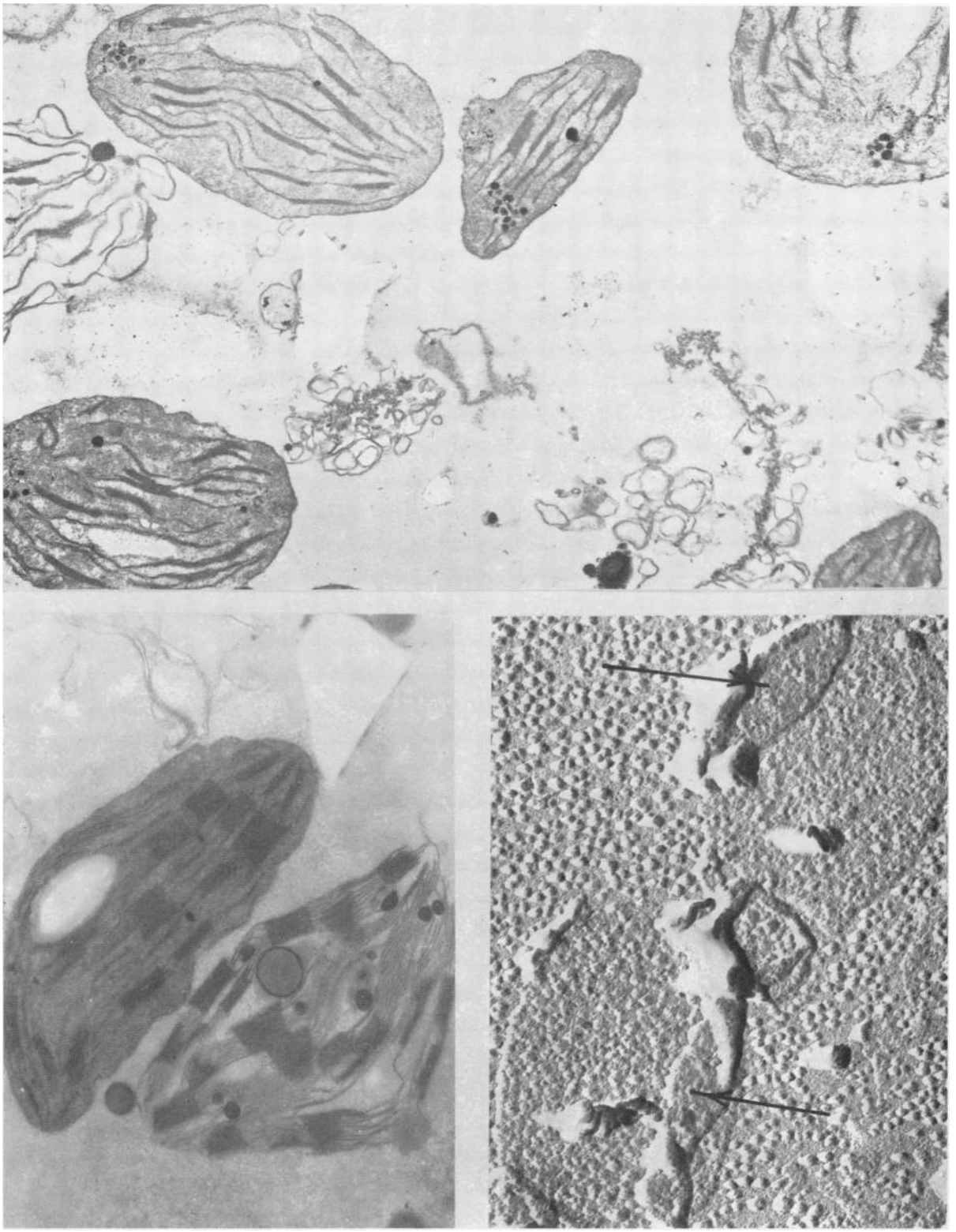

FIG. 1. Electron micrographs of chloroplasts from *Nic. tabacum* varieties Su/su and JWB. A) (top) Isolated Su/su chloroplasts, fixed in glutaraldehyde (× 11,000) (preparation reduced 2.9 mmoles ferricyanide/mg chl × hr in 250,000 ergs sec^{-1} cm^{-2} red light). B) (bottom left) Isolated JWB chloroplasts, fixed in glutaraldehyde (× 12,500) preparation reduced 0.83 mmoles ferricyanide/mg chl × hr in 250,000 ergs sec^{-1} cm^{-2} red light). C) (bottom right) Pt shadowed (<2.6°:1) lamella of a Su/su chloroplast (× 83,000).

Insert

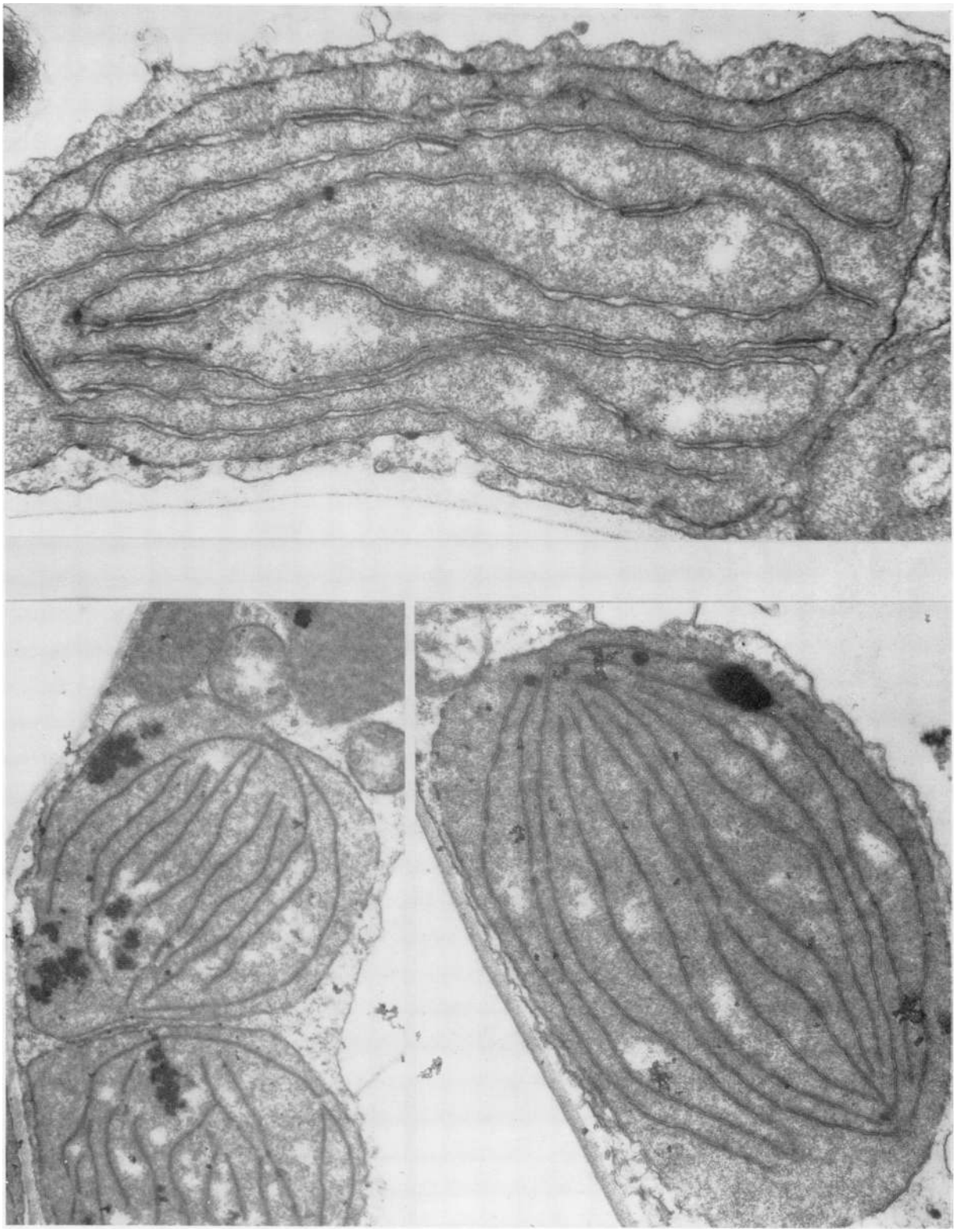

FIG. 2. Electron micrographs of chloroplasts from yellow leaf patches of *Nic. tabacum* varieties NC 95 var. and Su/su var., fixed in glutaraldehyde. A) (top) Chloroplast from Su/su var. (× 42,000). B) (bottom left) Radial section of chloroplasts from NC 95 var. (× 20,000). C) (bottom right) Axial section of chloroplasts from NC 95 var. (× 25,000).

Insert

bolometer as described elsewhere (32). Correct absorption measurements are difficult with chlorophyll-deficient chloroplasts because with equal amounts of chlorophyll present, they scatter light much more strongly than normal green ones. We therefore measured the loss of light due to scattering through its entrance hole with another chloroplast suspension which had been bleached in UV light. From the spectral distribution of the filtered light and from the absorption spectrum of the chloroplasts we calculated the average energy content to be 1.81×10^{12} ergs/einstein for the red light and 1.63×10^{12} ergs/einstein for the far-red light.

Unless otherwise noted, all data represent the average of more than 5, usually more than 10 separate measurements. The variations are given as standard deviations.

The techniques used for electron microscopy have been described (28, 33).

Results

The Structure of the Chloroplasts. It can be seen from the electron micrographs in figures 1 and 2 that the chloroplasts of our tobacco mutants are characterized by a very high degree of order of their lamellar structure. Figures 1A and 1B are pictures of typical chloroplast preparations from green tobacco and from the aurea mutant Su/su, as they were used in this study. For these 2 particular preparations, we used phosphate buffer in the suspension medium, because tris interfered with our staining procedure. The difference between the features of normal green chloroplasts and those of Su/su, which has been described in earlier papers (31, 33), is immediately apparent from a comparison of the pictures of figure 1. Another type of chloroplast was seen in the chimera Su/su var. which suddenly appeared in 1 of our Su/su seed lots. The yellow-green area of the

Table II. *Pigment Content of Different Tobacco Varieties*

Plant	Whole leaves		
	μg chl per cm²	chl a/chl b	chl/carotenoid
JWB	45 ± 15	2.9 ± 0.4	3.8 ± 0.5
Su/su	9 ± 2	5.1 ± 1.5	2.7 ± 0.6
Su/su var. yellow green sect.	6 ± 1.3	3.7 ± 0.8	2.4 ± 0.4
Su/su var. yellow section	1.5 ± 0.3	4.9 ± 0.6	1.2 ± 0.3
NC 95 green	32 ± 6	2.5 ± 0.6	3.7 ± 0.8
NC 95 var yellow	4.2 ± 1.0	3.4 ± 0.6	3.1 ± 0.1

Table III. *Saturation Rates of Photosynthesis in Intact Leaves and of the Hill Reaction with Chloroplasts from Different Tobacco Varieties and from Spinach*

The saturation rates of photosynthesis were determined at 22° in air containing 0.75 % CO_2. For reasons of comparison, the values for the ferricyanide photoreductions (25°) are given as O_2 evolution.

Plant	Photosynthesis of leaves μmoles $^{14}CO_2$ fixed/mg chl × hr	Ferricyanide Hill Reaction (μmoles O_2 evolved/mg chl × hr) No pectinase and albumin		+ pectinase and albumin		rate per mg chl a. (+MA*)
		—MA	+MA*	—MA	+MA*	
JWB	130 ± 50	35 ± 10	140 ± 40	40** ± 5	210 ± 50	290 ± 70
Su/su	550 ± 90	65 ± 10	325 ± 70	90 ± 15	505 ± 110	580 ± 120
Su/su var. yell. gr. sec.	610**	...	...	...	300**	375**
Su/su var. yellow sections	1150**	37** ± 2	180 ± 40	42** ± 8	400**	470**
NC 95 green	170 ± 50	...	...	...	210 ± 60	290 ± 70
NC 95 var. yellow sections	23 ± 18	...	9 ± 2	...	18** ± 8	...
Spinacia oleracea	170 ± 20	...	...	30 ± 5	255 ± 40	370 ± 30

* +MA = 18 mM methylamine added.
** Data represent less than 5 determinations.

leaves around their midribs is physiologically related to Su/su, whereas the outer golden yellow regions contain the type of chloroplast shown in figure 2A. No real grana are present. Instead there are single thylakoids (25) which at several places come close enough to form double layers. This lamellar arrangement resembles that found in blue-green algae. A fourth type is shown in figure 2B and 2C. These are chloroplasts from the yellow part of another variegated tobacco (NC 95 var.). In contrast to all the others the thylakoids here form no grana, are not folded, nor do they form double layers. Only about 20 % of the cells in the yellow leaf parts contain chloroplasts with a few stacked layers of thylakoids. The chloroplasts of a single cell, however, were found to be always of 1 type, and in the cells of the green leaf part they look normal. The pigment contents of the leaves of our tobacco plants are summarized in table II.

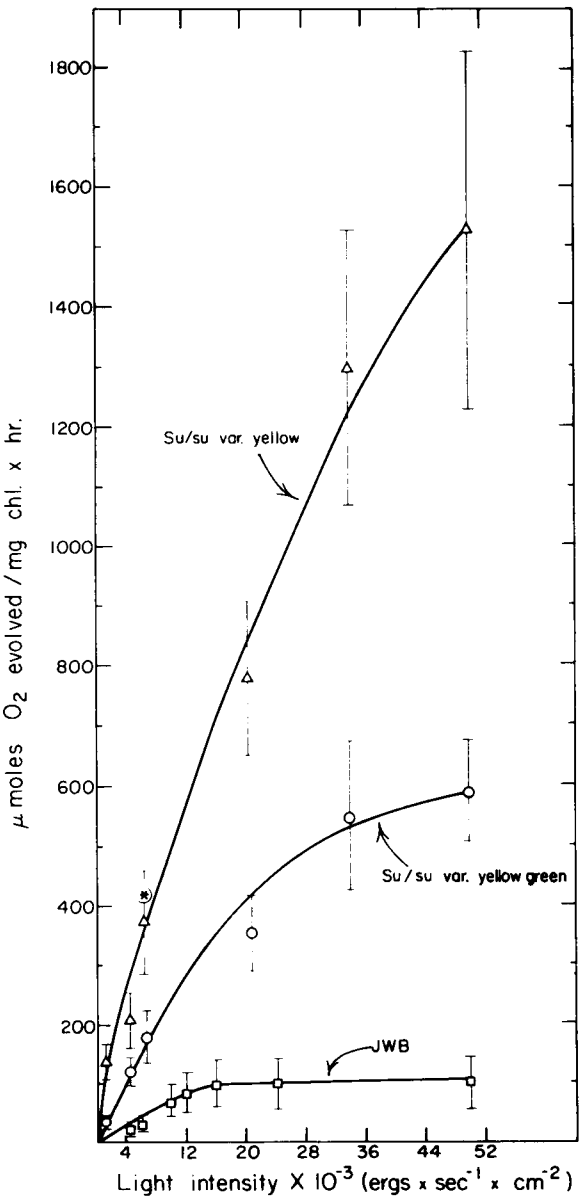

FIG. 3. Light saturation curves for photosynthesis at 22.8° measured manometrically in red light (corrected for respiration). Su/su var. 4 experiments with yellow leaf sections containing 1.2 to 1.7 μg chl/cm² : Δ — Δ; Su/su var., 4 experiments with yellow green leaf sections containing 3.6 to 4.7 μg chl/cm² : ○ — ○; JWB, experiments with 12 leaf sections containing 45 to 61 μg chl/cm² : □ — □. For a yellow leaf section of Su/su var., the quantum requirement was determined to be 8.4 $h\nu/O_2$ at the point ⊗.

Photosynthesis of Leaves. The Su/su leaves used in this study gave similar high rates of photosynthesis to those reported in recent publications (30, 31). Possibly because of the growth conditions in a new greenhouse, the chlorophyll a/chlorophyll b ratio of the leaves used in this investigation was higher than that reported in earlier studies (table II; ref. 26, 30, 33).

Table III summarizes saturation rates for photosynthetic fixation of labeled CO_2 by sections from leaves of the tobacco varieties under study. The extremes were found in the yellow leaf parts of Su/su var. with a maximal activity of about 1150 μmoles CO_2 fixed/mg chl × hr and in those of NC 95 var. which were able to fix CO_2 only at a rate of 40 μmoles/mg chl × hr. The high rates of photosynthesis in our variant of Su/su surpassed even those reported earlier for Su/su and therefore represent a new record. In other experiments we even measured rates of O_2 evolution up to 1800 μmoles/mg chl × hr. Figure 3 gives light intensity curves which were determined by manometric gas exchange. The low photosynthetic activity of the yellow leaf parts of the NC 95 var. is an example for the well-known and more frequently seen situation that a chlorophyll deficiency is not balanced by a higher photosynthetic capacity as was the case of Su/su and Su/su var. In NC 95 var. the yellow leaf areas actually do not take part in the fixation of carbon dioxide of the entire plant because respiration is never compensated. In fact, manometric determinations of the gas exchange of yellow leaf sections from NC 95 var. were not sensitive enough to detect photosynthetic O_2 evolution (28).

The Ferricyanide Hill Reaction. The high photosynthetic activity of Su/su leaves led us to expect that the maximal Hill activity of isolated chloroplasts would be higher than that of a normal green plant. In our initial experiments with Su/su chloroplasts, the Hill activity was always low. The reason turned out to be the extreme instability of the Su/su chloroplasts. Figure 4 shows that the loss of Hill activity was faster in chloroplast preparations of Su/su than in any of the other preparations and affected impartially the rates at the light saturation level as well as those below saturation, independent of the presence of pectinase and albumin.

In the following experiments, we checked the Hill activity not later than 1 hour after the preparation of

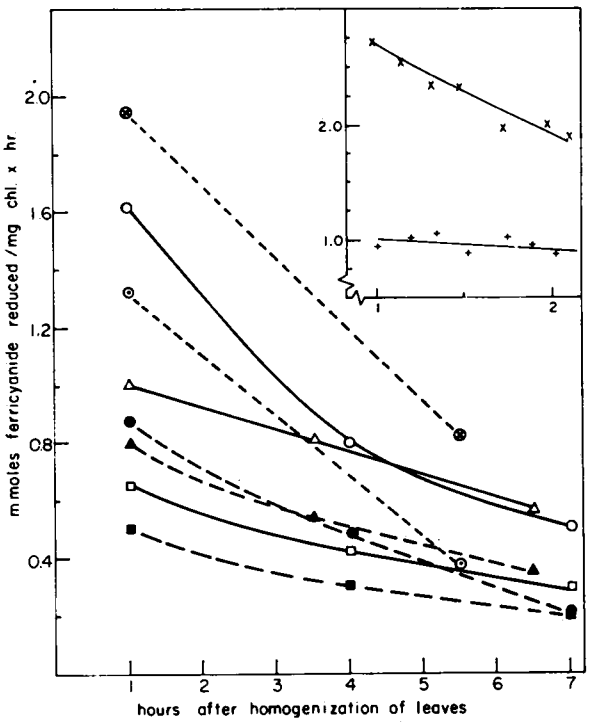

FIG. 4. Time course of deterioration of Hill activity for various chloroplast preparations at 0°. All chloroplast suspensions contained pectinase and albumin except for ⊙ - - - ⊙. Two different preparations of Su/su ⊙, ⊗, and ○, ●; JWB: □, ■; spinach: △, ▲. Illumination at 25° with red light: 250,000 ergs sec^{-1} cm^{-2} : open symbols; 160,000 ergs sec^{-1} cm^{-2} : filled symbols. Inset: decay of activity determined at 250,000 ergs sec^{-1} cm^{-2} red light to correct the data of figure 5; Su/su: x - - - x; JWB + - - - +.

the chloroplasts. With the aid of pectinase and albumin we were able to measure repeatedly saturation rates of the ferricyanide Hill reaction with chloroplasts from Su/su up to 2.9 nmoles ferricyanide reduced per mg chl per hour. This rate, which is equivalent to 700 μmoles O_2 evolved/mg chl × hr is close to the maximal rates of photosynthesis reported for Su/su leaves (31) and exceeds by far any values reported in the literature.[2] Because of the instability of the Su/su chloroplasts, all the points of a light intensity curve (fig 5) for the Hill reaction were measured within the second hour after the homogenization of the leaves. Nevertheless a slight correction for the time factor had to be applied. We checked the saturation rate periodically (inset of fig 4) and assumed that the rates at lower light intensities had decreased by the same percentage. A comparison of light intensity curves for the ferricyanide Hill reaction in various chloroplast preparations is shown in figure 5. The striking feature of the curves for Su/su chloroplasts is the nearly linear dependence of the rate on the light intensity until light saturation is approached at about 250,000 ergs of red light. The saturating rates for the ferricyanide Hill reaction of various chloroplast preparations in the presence and absence of pectinase + albumin, and in the presence and absence of methylamine are compared in table III. Because our Su/su chloroplasts had a higher chlorophyll a/chlorophyll b ratio than the green control plants, we have also included in the table the rates on the basis of chlorophyll a. It is apparent that in these figures the striking differences between the activities of the chloroplasts are somewhat reduced, but not eliminated as they are in experiments with mutant chloroplasts of barley which lack chlorophyll b completely (6). Because of a shortage of leaves of Su/su var. only 1 determination of the Hill activity of their chloroplasts was made. The saturation rate of the Hill reaction did not come out as high as was expected from the high photosynthetic activity. The agreement between photosynthesis and Hill activity, however, was good for the data obtained with the variegated NC 95. The green NC 95 had a normal activity, whereas the chlorophyll-deficient chloroplasts of the variegated leaves reduced only a very small amount of ferricyanide. It is noteworthy (see table III) that in normal green chloroplasts the capacity for oxygen evolution in an uncoupled Hill reaction exceeds the maximal rates for CO_2 fixation by intact leaves.

The quantum requirement for O_2 evolution in the ferricyanide Hill reaction of Su/su chloroplasts in red light in the presence of pectinase and albumin was the same as that measured with chloroplasts from

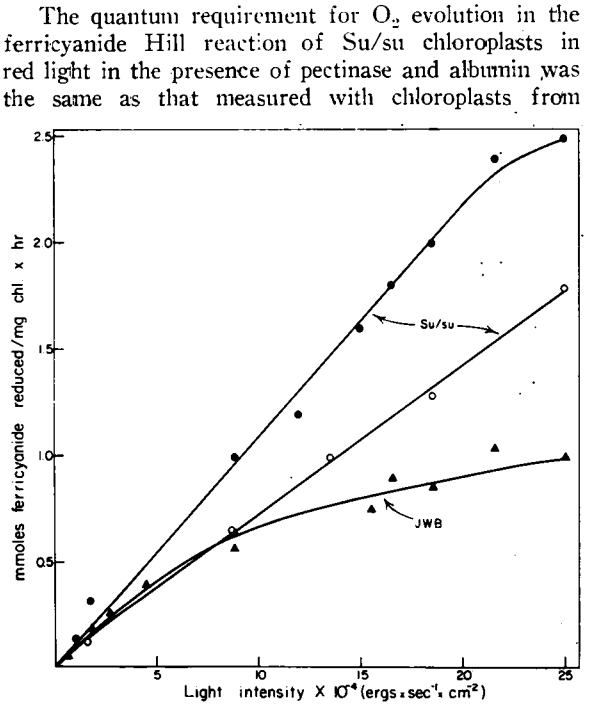

FIG. 5. Light saturation curves for the ferricyanide Hill reaction at 25°. Illumination for 1 or 2 minutes with red light; data are corrected for the decrease of the activity in the course of the experiment as described in the text (see inset of fig 4 for the decrease of the saturation rate during determination of the curves ● — ● and ▲ — ▲). Su/su, 4.5 μg chl/2.7 ml: ● — ●; different preparation of Su/su 4.9 μg chl/2.7 ml: ○ — ○; JWB, 17 μg chl/2.7 ml: ▲ —▲.

[2] A few days before this paper was submitted there appeared a note in which Highkin et al. (11) report about similar results for experiments with a chlorophyll-deficient pea mutant. No rates are given, however.

green control plants. Using 6900 ergs sec^{-1} cm^{-2} incident red light, we determined 15, 14 and 24 quanta/O_2 in experiments with Su/su chloroplasts as compared with 19, 17 and 22 quanta/O_2 found for chloroplasts of the green NC 95. The efficiency of all our preparations, therefore, was slightly lower than that of the spinach chloroplasts prepared by Sauer and Park, who measured 10 quanta/O_2 (27). Considering that the quantum requirement for CO_2 fixation by the intact tobacco leaves is around 12 (32), a somewhat higher number for O_2 evolution by chloroplasts appears to be probable because one would expect them to become damaged to some extent during the preparation.

NADP⁺ Photoreduction with Ascorbate-DCPIP. The $NADP^+$ photoreduction was measured according to Tagawa and Arnon (36) with partially purified ferredoxin and the ascorbate-DCPIP couple as electron donor. The saturation rates varied considerably, and were particularly low for chloroplasts from the green tobacco plants (table IV). These low rates were not due to a reoxidation of NADPH. On the other hand, the chloroplasts from yellow leaf sections of NC 95 var. gave rather high rates. This is in striking contrast to their low activity in Hill reaction and photosynthesis. The light intensity dependence of the $NADP^+$ photoreduction was determined for 1 chloroplast preparation of each plant and is given in figure 6. The light intensity curve for the $NADP^+$ photoreduction with Su/su chloroplasts is not as clearly linear as it was in the ferricyanide Hill reaction. Moreover, saturation is reached at much lower light intensities.

The PMS-Mediated Photophosphorylation. The PMS-mediated photophosphorylation was tested aerobically in the presence of ascorbate as described by Avron (3). Under these conditions this reaction is believed to be coupled to an electron flow mediated by photosystem I alone. As expected, it was not inhibited by 1 μM DCMU. The rates obtained with various chloroplast preparations are shown in table IV. Just as with the $NADP^+$ photoreduction, the most surprising finding was the good activity of the chlorophyll deficient chloroplasts from photosynthetically inactive yellow parts of the variegated NC 95.

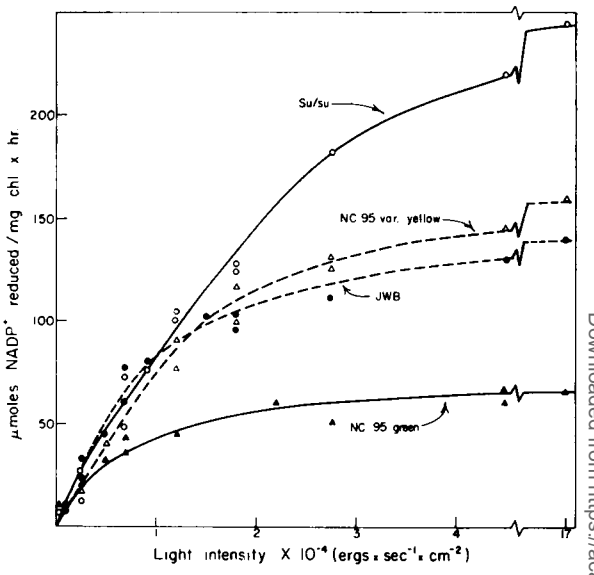

FIG. 6. Light saturation curves for the photoreduction of $NADP^+$ with ascorbate-DCPIP at 25° in the presence of saturating amounts of ferredoxin (red light). Su/su, 8 and 14 μg chl/3 ml: ○ — ○; NC 95 var., yellow leaf parts, 4.9 and 9 μg chl/3 ml: △ — △; JWB, 19 μg chl/3 ml: ● — ●; NC 95 green, 19 μg chl/3 ml: ▲ — ▲.

The absorption of photosystem I, as presently defined, extends farther into the far-red beyond λ 695 mμ than that of system II. In order to verify that we were really measuring a system I reaction, we compared the quantum requirement of ATP formation in the far-red with that in the red (see the 2 right columns of table IV).

Table IV. *Photoreduction of NADP⁺ with Ascorbate-DCPIP and PMS-Mediated Photophosphorylation by Various Tobacco Chloroplasts*

The data for the $NADP^+$ photoreduction represent saturation rates in red light at 25°. The rates of photophosphorylation were obtained by illuminating the reaction mixture for 6 to 10 minutes at 20° with 80,000 lux white light. For the determination of the quantum requirement the samples (400–500 μg chl/6 ml except for 1 preparation of NC 95 with 1100 μg chl/6 ml) were illuminated with 6900 ergs sec^{-1} cm^{-2} red or 72,000 ergs sec^{-1} cm^{-2} far-red light for 12 minutes at 20°.

Source of chloroplasts	$NADP^+$ photored. μmoles reduced/mg chl × hr	Photophosphorylation μmoles ATP formed/ mg chl × hr	einstein/mole ATP red	einstein/mole ATP far-red
JWB	75 ± 25	400 ± 100	...	...
Su/su	190 ± 55	730 ± 90	40 ± 10	10* ± 2
NC 95 green	50 ± 17	430 ± 80	20.4 ± 2.5	2.3 ± 0.4
NC 95 var. yellow sections	165 ± 40	420 ± 80	75* ± 2	13.5* ± 0.5
Spinach (one determ)	205	550	...	...

* Data represent 2 determinations on 1 preparation. The other data represent 4 determinations of quantum requirements on 2 preparations.

In the yellow parts of NC 95, the quantum efficiency was at least 4 times better in the far-red than in the red, although a low quantum requirement of close to 2 $h\nu$/ATP in the far-red was only observed with chloroplasts from the green parts of NC 95 var. or leaves of NC 95. Large quantities of leaves were needed to obtain enough chloroplasts from the chlorophyll-deficient varieties for absorption measurements in the far-red. Therefore, we do not have a sufficient number of experiments to be sure whether the chloroplasts from the aurea mutant Su/su and, in particular, from the yellow leaf areas of NC 95 var., are indeed as inefficient for ATP formation as our data suggest.

The characteristics of the PMS-mediated photophosphorylation permitted also another evaluation of the relative capacity of our chlorophyll-deficient chloroplasts from NC 95 var. for reactions involving system I or both system I and system II. Earlier Jagendorf and Margulies (15) observed that the PMS-mediated photophosphorylation is insensitive to DCMU only in the complete absence of O_2. Under aerobic conditions, this inhibition is relieved by ascorbate. Our data show that under aerobic conditions far-red light promotes photophosphorylation with good efficiency only in the presence of ascorbate, but is completely inactive in its absence. On the other hand, there is no effect of ascorbate on the rate of aerobic photophosphorylation in red light. Obviously, photosystem II has to induce an electron flow through the phosphorylation site when the electron acceptor on the reducing side of photosystem I (PMS) falls prey to oxidation by O_2. Such a switch should be impossible for our mutant chloroplasts from NC 95 var. Our experiments did indeed reveal that in the absence of ascorbate no ATP was formed in either far-red or red light (table V).

Mn Content of the Chloroplasts. Manganese is generally believed to participate directly and exclusively in the process of oxygen evolution by green plants. The active manganese is tightly bound to the protein of the chloroplast lamellae (8, 12, 35). We were interested to know whether the low system II activity in chlorophyll-deficient chloroplasts from NC 95 var. and the high system II activity of Su/su chloroplasts is reflected by their manganese content. We therefore determined the manganese content of chloroplasts from various tobacco varieties. Fragmented chloroplasts from plants grown in liquid culture containing radioactive ^{54}Mn were washed 3 times with 1 mM EDTA as described earlier (12). The removal of "unbound" manganese by washing with a chelator like EDTA or EGTA [ethylene bis (β-aminoethylether)-N,N'-tetraacetate] worked satisfactorily with fragments of normal chloroplasts because much chlorophyll was present in a relatively small amount of particles. The situation was different with chlorophyll-deficient chloroplasts, where little chlorophyll was associated with many particles. Particles isolated from whitish, essentially chlorophyll-free areas of leaves from NC 95 var. retained some manganese even after washing with 10 mM EGTA. We therefore used chloroplasts from leaf areas where the chlorophyll content was not too low. By this trick the concentration of manganese containing, chlorophyll-free particles was kept at a minimum. The purification of the chloroplasts by a glycerol-sucrose gradient centrifugation (16) could be used

Table V. *Effect of 10 mM Ascorbate and 1 μM DCMU on the PMS-Mediated Photophosphorylation under Aerobic Conditions*

The reaction mixture was kept in open testtubes and illuminated with 20,100 ergs sec^{-1} cm^{-2} red (R) or 110,000 ergs sec^{-1} cm^{-2} far-red light (FR) for 10 minutes at 20°. The data for Su/su (115 μg chl/2 ml) represent the average of 2 determinations, those for NC 95 var. (92 μg chl/1.5 ml) were obtained by single determinations.

	+ DCMU + ascorbate		— DCMU + ascorbate		+ DCMU — ascorbate		— DCMU — ascorbate	
	R	FR	R	FR	R	FR	R	FR
	μmoles ATP formed/mg chl×hr							
Su/su	20	28	23	26	nil	nil	23	nil
NC 95 var. (yellow)	14	18	12	18	nil	nil	nil	nil

Table VI. *Manganese Content of Leaves and Fragmented Chloroplasts from Different Tobacco Varieties*

The data for the leaves are averages of 2 determinations, and the data for chloroplasts represent 5 determinations (1 to 1.5 μM labeled Mn^{2+} in culture medium).

	JWB	Su/su	NC 95 var. green sections	NC 95 var. yellow sections
	molecules total chlorophyll per atom Mn			
Leaves	25	14	9	3
ChlpL fragments	70 ± 10	50 ± 15	50 ± 5 (60*)	65 ± 15

* Obtained from 2 experiments after sucrose-glycerol gradient purification of whole chloroplasts.

for normal chloroplasts, but even modifications of this procedure did not work well with chlorophyll-deficient chloroplasts from NC 95 var. The results of our Mn determinations (table VI) show no significant difference in the manganese content of our chloroplast preparations on the basis of chlorophyll. Obviously, the manganese content of the chloroplasts was not responsible for the differences observed in their system II activity.

Discussion

To combine the diversity of our observations within a more general concept, we shall discuss 3 main results. 1) The photosynthetic capacity of intact leaves is paralleled by the activity of isolated chloroplasts in the Hill reaction. 2) Unfolded, single thylakoids (25) or frets (39) are capable of carrying out those photoreactions which are generally ascribed to the so-called photosystem I. 3) No system II activity was detected in mutant chloroplasts without any grana or overlappings of thylakoids, although a normal amount of manganese was found to be bound to the lamellae.

The unusual high photosynthetic activity of Su/su leaves was reflected in an abnormally high saturation rate of the ferricyanide Hill reaction by their chloroplasts. It is known that the process of water oxidation is the slowest reaction in the light-induced transport of an electron from water to an acceptor on the reducing side of system I (7). Kok and Cheniae (20) have based a calculation of the saturation rate in continuous light on the findings that the rate limiting dark enzymes have a turnover time of about 10 msec ($K = 100\ sec^{-1}$), and that the concentration C of the dark enzymes is 1/2500 chlorophyll according to a photosynthetic unit of 2500 molecules of chlorophyll for the oxygen evolving system (9). A typical saturation rate would be $KC = 0.04\ O_2\ sec^{-1}\ chl^{-1} = 150\ O_2\ hr^{-1}\ chl^{-1}$ (20). With the maximal rate of about 700 μmoles O_2 evolved/mg chl $\times$ hr found with Su/su chloroplasts, one would have to postulate either a higher concentration—C (a smaller unit) or a shorter turnover time by a factor of about 5 (this factor would be about 12 for Su/su var. considering a maximal saturation rate of 1800 O_2 evolved per chl per hr).

Future experiments in continuation of those described earlier (30) may give an answer to the question whether the photosynthetic unit of Su/su is smaller than that of normal green plants (see ref. 13). The manganese content of the chloroplasts, however, gives no hint that the units may be different (table VI). The negligible capacity for oxygen evolution in the yellow leaf patches of NC 95 var. and their chloroplasts is in remarkable contrast to the high activity of Su/su. Surprisingly, the chloroplasts of NC 95 var. gave the usual rates for $NADP^+$ reduction with ascorbate-DCPIP and for PMS mediated photophosphorylation, i.e., they were normal except for reactions involving photosystem II. One might say that the chlorophyll deficient chloroplasts of NC 95 var. are similar to Bishop's *Scenedesmus* mutant No. 11 (5) with 1 fundamental difference: the *Scenedesmus* mutant No. 11 has no easily detectable structural deficiencies while the lamellar structure of our chlorophyll deficient chloroplasts is distinctly different from that of normal green chloroplasts. Their main feature is the nearly complete lack of any grana or simple thylakoid doublings which Weier et al. (40) believe to be essential for photosynthesis. Our data prove that an excellent system I activity can be associated with single, unfolded thylakoids (frets). An interesting difference between the $NADP^+$ photoreduction and the PMS-mediated photophosphorylation emerges when the structural order in the chloroplasts is strongly disturbed by manganese deficiency. The $NADP^+$ photoreluction with ascorbate-DCPIP is still carried out by these chloroplasts, but their photophosphorylating activity is lost (12).

The data of table IV on the $NADP^+$ photoreduction certainly do not represent the highest possible saturation rates. Katoh and San Pietro (18) have observed that the rates are about 8 times higher in small chloroplast fragments than in whole chloroplasts. Vernon et al. (37) achieved a further increase of the rate with detergent treated chloroplasts. In small chloroplast fragments the substrate might reach the reaction site on the lamellae more easily (18). For a better comparison of the activity of our chloroplasts we probably should have used small particles instead of whole chloroplasts.

The low capacity for O_2 evolution in the chlorophyll-deficient chloroplasts of NC 95 var. could be due to a complete absence of the pigments of system II. According to the present dogma of the 2 photosystems, this would mean that the extended frets (39) of the chloroplasts contain only pigments associated with photosystem I. Consequently the PMS-mediated photophosphorylation in the presence of ascorbate should occur with an equal efficiency in red and in far-red light. Our table IV shows that this was not the case. This finding, together with unpublished fluorescence data and the demonstration of a normal manganese content of the chloroplasts, indicate the presence of an inactive photosystem II in the isolated thylakoids of the chloroplasts of NC 95 var. The PMS-mediated, DCMU-insensitive, photophosphorylation with chloroplast preparations from green NC 95 had a 7 times higher quantum efficiency in far-red light than in red light. This finding supports the view that the 2 photosystem concept does not cover all possibilities. As pointed out by Gaffron et al. (10) the presence of a third photosystem specializing in photophosphorylation would help to explain the observations of Wiessner (41) with the acetate assimilating alga *Chlamydobotrys*. It would also fit the data of Schmid and Gaffron (32) on an unusually high Emerson enhancement effect, and the recent experiments by Arnon et al. (2) on the efficiency of the ferredoxin-mediated photophosphorylation in red and far-red light. None of the present hypotheses

can explain quantum yields of 0.5 for the PMS-mediated ATP formation at λ 640 mμ as determined by Lynn and Brown (23) with spinach chloroplasts. At present we cannot accept their finding, however. For example, in the set up for light measurements used by these authors, the light beam reaching the photometer in absence of a chloroplast suspension can certainly not be compared with the light monitored by the photometer in presence of a light-scattering sample. Lynn and Brown do not give any information about the corrections they applied to take care of this difficulty.

The possible intimate connection between the photophosphorylation in far-red light and the Emerson effect is supported by our results. It may be that the functioning of the far-red mediated phosphorylation depends also in vivo (34) on reducing conditions just as in the in vitro system with PMS. In vivo, this situation may occur when the utilization of NADPH is limited by the supply of ATP. It is perhaps significant that our quantum requirements of 2.3 and 10 for photophosphorylation in the far-red with chloroplasts from green NC 95 and the aurea mutant Su/su respectively agree well with the quantum requirements of 3 and 7 calculated for the far-red induced increment of O_2 evolution by intact leaves in blue light (32).

Finally we have to look for a reason why the yellow leaf areas of NC 95 var. lack system II activity. Their main characteristic was that nearly all their chloroplasts did not contain any grana or thylakoid doublings. The process of water oxidation may occur only when a close packing of thylakoids provides a protected niche in which the 4 oxidizing equivalents needed for the evolution of an oxygen molecule can accumulate.[3] The presence of an enclosed reaction complex is also indicated by the finding that water molecules are tightly bound to their oxidation site in the chloroplast (22). Manganese in a yet unknown oxidation state may be the primary acceptor for the electrons originating from water. Chloroplasts contain, however, more than 4 tightly bound manganese ions per oxygen evolving photosynthetic unit (12, 20; table IV). It is possible that the excess manganese serves as a pool of electron carriers on the oxidizing side of photosystem II to make the oxidation of water by a concerted 4 electron transfer process more probable. The well-known burst of O_2 at the onset of a strong illumination may be a discharge of this pool. An appropriate description of our concept of the structural requirements of photosystem II can easily be obtained by a slight modification of the model proposed by Kelly and Sauer (19) for the spatial separation of the 2 photosystems in the lamellar structure of the chloroplasts.

The unusually high capacity for oxygen evolution in 2 of our tobacco mutants can be explained only after we know more about the organization of photosystem II. In respect to the overall structural characteristics, it may be that the total area of the partitions (39) is more important than the height of the grana. For example, the thylakoid doublings in Su/su chloroplasts appear to represent cross sections of rather large areas of overlappings (fig 1C) which are different from the stacks of disc-shaped lamellae found in normal grana.

[3] It is unfortunate that we became aware of the interesting observations by Izawa and Good (Plant Physiol. 41: 533–43) on the influence of low-salt environments on the chloroplast structure only after it was too late to include their results in the discussion of our findings. The lack of any distinct structural difference between the frets and the membranes of the partitions suggested by our experience with NC 95 var., is also evident from the electron micrographs published by Izawa and Good. Moreover, their pictures show that system II dependent electron transport induced a shrinkage of the thylakoids with concomitant accentuations and possibly reconstructions of lamellar overlappings in artificially disorganized lamellar systems. In respect to our hypothesis on the structural requirements of photosystem II, it should be worthwhile to compare the structural and functional details of our mutant chloroplasts with those of the artificial chloroplast modifications investigated by Izawa and Good.

Acknowledgments

The authors thank Dr. H. Gaffron, in whose laboratory this work was carried out, for helpful discussions during this study. They also acknowledge the skillful technical assistance of Mrs. Mary C. Fray and Mrs. Soheir El Bayoumi.

Literature Cited

1. Arnon, D. I. and P. R. Stout. 1939. Experimental methods for the study of the role of copper, manganese and zinc in the nutrition of higher plants. Am. J. Botany 36: 144–49.
2. Arnon, D. I., H. Y. Tsujimoto, and B. D. McSwain. 1967. Ferredoxin and photosynthetic phosphorylation. Nature 214: 562–66.
3. Avron, M. 1960. Photophosphorylation by Swiss chard chloroplasts. Biochim. Biophys. Acta 40: 257–72.
4. Bendall, D. S., R. D. F. Gregory, and R. Hill. 1960. Chloroplast ferredoxin from parsley. Nature 187: 417–18.
5. Bishop, N. I. 1962. Separation of the oxygen evolving system of photosynthesis from the photochemistry in a mutant of *Scenedesmus*. Nature 195: 55–57.
6. Boardman, N. K. and H. R. Highkin. 1966. Studies on a barley mutant lacking chlorophyll b; I. Photochemical activity of isolated chloroplasts. Biochim. Biophys. Acta 126: 189–99.
7. Bültemann, V., H. Rüppel, and H. T. Witt. 1964. Intermediary reactions in the water splitting part of photosynthesis. Nature 204: 646–48.
8. Cheniae, G. M. and I. Martin. 1966. Studies on the function of manganese in photosynthesis. In: Brookhaven Symposia in Biology No. 19, p 406–17.
9. Gaffron, H. and K. Wohl. 1936. Zur Theorie der Assimilation. Naturwissenschaften 24: 81–90; 103–07.

10. GAFFRON, H., W. WIESSNER, AND P. H. HOMANN. 1963. Utilization of far-red light by green algae and the problem of oxygen evolution. In: Photosynthetic Mechanisms in Green Plants. Publ. 1145 of the N.A.S.-N.R.C, p 436–40.
11. HIGHKIN, H. R., N. K. BOARDMAN, AND D. J. GOODCHILD. 1967. Photosynthetic studies on a pea mutant deficient in chlorophyll b. Plant Physiol. 42: S-35.
12. HOMANN, P. H. 1967. Studies on the manganese of the chloroplast. Plant Physiol. 42: 997–1007.
13. HOMANN, P. H., G. H. SCHMID, AND H. GAFFRON. 1967. Structure and photochemistry in tobacco chloroplasts. Proc. Symp. Comp. Biochem. Biophys. Photosynthesis, Hakone, Japan, August, 1967. In press.
14. IZAWA, S. AND N. E. GOOD. 1965. Hill reaction and chloroplast fragment size. Biochim. Biophys. Acta 109: 372–81.
15. JAGENDORF, A. T. AND M. MARGULIES. 1960. Inhibition of spinach chloroplast photosynthetic reactions by p-chlorophenyl-1, 1-dimethylurea. Arch. Biochem. Biophys. 90: 184–95.
16. JAMES, W. O. AND V. S. R. DAS. 1957. The organization of respiration in chlorophyllous cells. New Phytologist 56: 325–46.
17. JENSEN, R. G. AND J. A. BASSHAM. 1966. Photosynthesis by isolated chloroplasts. Proc. Natl. Acad. Sci. 56: 1095–1100.
18. KATOH, S. AND A. SAN PIETRO. 1966. Activities of chloroplast fragments. J. Biol. Chem. 241: 3575–81.
19. KELLY, J. AND K. SAUER. 1965. Action spectrum and quantum requirements for the photoreduction of cytochrome c with spinach chloroplasts. Biochemistry 4: 2798–802.
20. KOK, B. AND G. M. CHENIAE. 1966. Kinetics and intermediates of the oxygen evolution step in photosynthesis. In: Current Topics in Bioenergetics, Vol. I. D. R. Sanadi, ed. Academic Press, New York. p 1–47.
21. KROGMANN, D. W. AND A. T. JAGENDORF. 1957. A spectrophotometric assay of the Hill reaction with ferricyanide. Plant Physiol. 32: 373–74.
22. KUTIURIN, W. M., N. M. NAZAROV, AND K. G. SEMENIUK. 1966. The absence of oxygen exchange between the water of chloroplasts and the oxygen evolving complex of photosynthesis (in Russian). Dokl. Akad. Nauk SSSR 171: 215–17.
23. LYNN, W. S. AND R. H. BROWN. 1967. P : O and ADP : O ratios and quantum yields in chloroplasts with the use of chloranil as electron acceptor. J. Biol. Chem. 242: 418–25.
24. MCKINNEY, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140: 315–22.
25. MENKE, W. 1962. Structure and chemistry of plastids. Ann. Rev. Plant Physiol. 13: 27–44.
26. MENSER, H. A., T. SOROKIN, AND M. E. ENGELHAUPT. 1965. The pigments, amino acids, alkaloids, growth rate and response to ozone of a chlorophyll-deficient mutation of tobacco. Tobacco Sci. 9: 21–25.
27. SAUER, K. AND R. B. PARK. 1965. The Hill reaction of chloroplasts. Action spectra and quantum requirements. Biochemistry 4: 2791–98.
28. SCHMID, G. H. 1967. Photosynthetic capacity and lamellar structure in various chlorophyll deficient plants. J. Microscopie: 6: 485–98.
29. SCHMID, G. H. 1967. The influence of different light intensities on the growth of the tobacco aurea mutant Su/su. Planta 77: 77–94.
30. SCHMID, G. H. AND H. GAFFRON. 1966. Chloroplast structure and the photosynthetic unit. In: Brookhaven Symp. Biol. 19: 380–92.
31. SCHMID, G. H. AND H. GAFFRON. 1967. Light metabolism and chloroplast structure in chlorophyll deficient tobacco mutants. J. Gen. Physiol. 50: 563–82.
32. SCHMID, G. H. AND H. GAFFRON. 1967. Quantum requirement for photosynthesis in chlorophyll-deficient plants with unusual lamellar structures. J. Gen. Physiol. 50: 2131–44.
33. SCHMID, G. H., J. M. PRICE, AND H. GAFFRON. 1966. Lamellar structure in chlorophyll deficient but normally active chloroplasts. J. Microscopie 5: 205–12.
34. SIMONIS, W. AND W. URBACH. 1964. Untersuchungen zur Photosynthese Phosphorylierung an intakten Algenzellen (*Ankistrodesmus braunii*) Ber. Deut. Botan. Ges. 77: 5–13.
35. SPENCER, D. AND J. V. POSSINGHAM. 1962. Manganese as functional component of chloroplasts. Australian J. Biol. Sci. 15: 58–68.
36. TAGAWA, K. AND D. I. ARNON. 1962. Ferredoxin as electron carrier in photosynthesis and in the biological production and consumption of hydrogen gas. Nature 195: 537–43.
37. VERNON, L. P., E. R. SHAW, AND B. KE. 1966. A photochemically active particle derived from chloroplasts by the action of the detergent Triton X-100. J. Biol. Chem. 241: 4101–09.
38. WALKER, D. A. 1965. Correlation between photosynthetic activity and membrane integrity in isolated pea chloroplasts. Plant Physiol. 40: 1157–61.
39. WEIER, T. E. AND A. W. BENSON. 1967. The molecular organization of chloroplast membranes. Am. J. Botany 54: 389–402.
40. WEIER, T. E., C. R. STOCKING, AND L. K. SHUMWAY. 1966. The photosynthetic apparatus in chloroplasts of higher plants. In: Brookhaven Symp. Biol. 19: 353–73.
41. WIESSNER, W. 1965. Quantum requirement for acetate assimilation and its significance for quantum measurements in photophosphorylation. Nature 205: 56–57.