Quantitation of the Rapid Electron Donors to $P_{700}$, the Functional Plastoquinone Pool, and the Ratio of the Photosystems in Spinach Chloroplasts

(Received for publication, May 11, 1984)

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Recent studies of chloroplast architecture have emphasized the segregation of photosystem I and photosystem II in different regions of the lamellar membrane. The apparent localization of photosystem II reaction centers in regions of membrane appression and of photosystem I reaction centers in regions exposed to the chloroplast stroma has focused attention on the intervening electron carriers, carriers which must be present to catalyze electron transfer between such spatially separated reaction sites. Information regarding the stoichiometries of these intermediate carriers is essential to an understanding of the processes that work together to establish the mechanism and to determine the rate of the overall process. We have reinvestigated the numbers of photosystem I and photosystem II reaction centers, the numbers of intervening cytochrome $b_6/f$ complexes, and the numbers of molecules of the relatively mobile electron carriers plastoquinone and plastocyanin that are actively involved in electron transfer. Our investigations were based on a new experimental technique made possible by the use of a modified indophenol dye, methyl purple, the reduction of which provides a particularly sensitive and accurate measure of electron transfer. Using this dye, which accepts electrons exclusively from photosystem I, it was possible to drain electrons from each of the carriers. Thus, by manipulation of the redox condition of the various carriers and through the use of specific inhibitors we could measure the electron storage capacity of each carrier in turn. We conclude that the ratio of photosystem I reaction centers to cytochrome $b_6/f$ complexes to photosystem II reaction centers is very nearly 1:1:1. The pool of rapid donors of electrons to $P_{700}$ includes not only the 2 reducing equivalents stored in the cytochrome $b_6/f$ complex but also those stored in slightly more than 2 molecules of plastocyanin per $P_{700}$. More slowly available are the electrons from about 6 plastoquinol molecules per $P_{700}$.

A minimal description of photosynthetic electron transfer in eucaryotes must accommodate kinetic features of the individual electron transfer reactions as well as the structural features of orientation and distribution of electron transfer components in the lamellar membrane. The sequence of reactions in electron transfer, insofar as it is known, has been established for many years with only relatively few recent embellishments (1). However, our knowledge of the sequential order of the charge transfer reactions may give a falsely optimistic view of our understanding of the mechanism of photosynthetic electron transfer if our information of stoichiometries and kinetic factors remains inadequate.

Recent progress in elucidating lamellar membrane architecture has appropriately focused attention on communication of the PS I and PS II reaction center complexes with the intervening cytochrome $b_6/f$ complex. There are many independent lines of evidence which demonstrate that photosystem I and photosystem II are segregated in different regions of the membrane; this is in spite of the fact that the glycerolipid phase of the membrane has an unusually low viscosity (2). The majority of PS II reaction centers are presently thought to be located in regions of membrane appression, their lateral mobility apparently arrested by membrane surface-to-surface interactions (3). PS I reaction centers are located predominantly in stroma-exposed portions of the membrane (3) excluded from membrane-appressed regions by as yet undefined factors. The cytochrome $b_6/f$ complex appears to be fairly uniformly distributed (3) and appears to retain a translational mobility which allows it to diffuse to and from appressed membrane regions. The different placements of PS I and PS II reaction centers in the lamellar membrane require that efficient intersystem electron transfer occur over relatively large average distances. Plastoquinol is known to mediate electron transfer between the PS II reaction center (4) and the cytochrome $b_6/f$ complex and plastocyanin between the cytochrome $b_6/f$ complex and the PS I reaction center (5, 6). However, there is little hope of understanding the relative contributions of the mobilities of plastoquinol, plastocyanin, and the cytochrome $b_6/f$ complex in catalyzing efficient electron transfer over the necessarily large distances without accurate information about the stoichiometry of the involved components. In addition, this same quantitative information is essential to an understanding of rate limitation in photosynthetic electron transfer.

This paper presents the results of experiments designed to measure the stoichiometries of the known carriers relative to the stoichiometries of the PS I and PS II reaction centers. The method employed involved the use of a modified indophenol dye, methyl purple, as a uniquely sensitive and reliable measure of electron transfer through PS I. Since this dye,

* This work was supported in part by National Science Foundation Grant PCM-8314461. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: PS I and PS II, photosystem I and photosystem II; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $P_{00}$, primary electron donor of the photosystem I reaction center; $Q_a$, primary quinone electron acceptor of the photosystem II reaction center.
unlike the more commonly used indophenols, accepts electrons exclusively through photosystem I (18), its reduction can be used to measure quite precisely the number of electrons delivered to P700 from the intermediate carriers. Furthermore, the redox state of the different carriers can be manipulated by the experimental protocol, and the accessibility of the different carriers to PS I can be varied by inhibitors. Thus the electrons can be drained from various carriers at will and their electron storage capacity determined by measuring the amount of methyl purple reduced. Our findings corroborate those of Whittmarsh and Ort (7) arrived at by an entirely different approach, namely that there is a 1:1 relationship among the three electron transport complexes in spinach chloroplasts: photosystem II reaction centers, cytochrome b6/f complexes, and photosystem I reaction centers. Furthermore, we find that the photochemically active plastocyanine pool amounts to nearly 6 molecules/PS I, that the number of reducing equivalents stored in the cytochrome b6/f complex is slightly more than 2 electrons/P700, and that slightly more than 2 molecules of plastocyanin are present for each PS I reaction center.

**MATERIALS AND METHODS**

Chloroplast Isolation—Chloroplast lamellar membranes were isolated from commercial spinach (Spinacia oleracea L.) as described previously (8).

Chlorophyll Determination—Chlorophyll was measured spectrophotometrically in 80% acetone using the specific absorption coefficients for chlorophyll c at 664 nm and chlorophyll b at 647 nm determined by Ziegler and Egle (12). These two wavelengths correspond well with the absorption peaks that we measured (0.3-nm half-band width) for chlorophyll a and chlorophyll b determined by Ziegler and Egle (12).

Absorption changes at 591 nm were measured in a thermostatted (20 ± 0.2°C) cuvette (10 × 10 mm). The intensity of the measuring beam (spectral band width = 4 nm) was 6 × 10-17 J m-2 s-1. In order to minimize actinic effects of the measuring beam a shutter was placed between the sample and the measuring beam lamp. The sample was illuminated by the measuring beam for 1 s during each measurement. It was verified that neither lower measuring beam intensity nor shorter exposure of the sample to the measuring beam resulted in any change in the state of reduction of the electron transport chain. The photomultiplier was protected from actinic light by two 590-nm filters that both the laser and xenon flashes were more than 98% saturating, i.e., that more than 98% of reaction centers were oxidized by the unattenuated flashes.

Assuming the predicted exponential saturation of the reaction centers as a function of increasing flash intensity, it was calculated from light attenuation experiments with calibrated neutral density filters that both the laser and xenon flashes were more than 98% saturating, i.e., that more than 98% of reaction centers were oxidized by the unattenuated flashes.

### RESULTS

An understanding of the rationale of the following experiments is aided by reference to Fig. 1. This figure together with its legend depicts the effect of the various experimental protocols described below on the redox state of the individual electron carriers which in turn dictates whether or not a particular carrier is able to donate electrons to methyl purple.

**PS I Reaction Center Content**

The PS I reaction center content of the lamellar membranes was determined by measuring the amount of methyl purple reduced in response to a saturating laser flash (Fig. 2A and Table I). In this experiment poly-L-lysine (290,000 average Mw) was present to inactivate plastocyanin (16) and thereby remove altogether any possibility of rapid reduction of oxidized plastocyanin with calibrated ascorbate solutions.2

![FIG. 1. Schematic depicting the experimental rationale for quantitation of the photosynthetic electron transport components.](image)

Full reduction of plastocyanine (PQ) and the intersystem carriers can be achieved by illuminating the chloroplast lamellar membranes in the absence of DCMU or any exogenous electron acceptor whereas full oxidation of the carriers can be achieved by illumination in the presence of both methyl purple and DCMU. Thus, by manipulation of the redox condition of the various carriers and through the use of specific inhibitors the electrons can be drained from the reduced carriers via photosystem I and their electron content determined from the amount of methyl purple reduced. The number of photosystem I reaction centers (RC I) can be determined by the yield of reduced methyl purple from a laser flash, and the relative abundances of the reaction centers of the two photosystems can be determined by the effect of a series of single turnover flashes on the reduction level of the plastocyanine pool PC, plastocyanin.
dized PS I reaction centers and, therefore, any possibility of double turnovers of the reaction centers. Inactivation of plastocyanin by FCN (17) rather than by polylysine gave identical transfer of electrons originating from water. Trace systems followed by a slower steady-state rate associated with the transfer of electrons stored in the high-potential carriers between the photo-systems.

**Table I**

| Treatment | PS I reaction center component | meq-mol chlorophyll-1 |
|-----------|-------------------------------|-----------------------|
| Control   |                               | 1.70                  |
| Heat treated |                             | 1.67                  |
| Heat treated, disrupted |                     | 1.71                  |
| Heat treated, disrupted + 0.8 μM DBMIB |           | 1.68                  |

**Table II**

| Component                             | meq-mol chlorophyll-1 | mol-mol P7w | N  |
|---------------------------------------|-----------------------|-------------|----|
| Rapid donors plus P7w                 | 8.66 ± 0.33           | 5.5+        | 16 |
| P7w                                   | 1.57 ± 0.10           | 1           | 16 |
| Plastocyanin                          | 3.27 ± 0.32           | 2.1         | 16 |
| Cytochrome b/f complex                 | 1.81 ± 0.12           | 1.2+        | 16 |
| Plastocyanin+                         | 16.67 ± 1.41          | 5.3         | 16 |
| Photosystem II+                       | 1.67 ± 0.13           | 1.1         | 8  |

*a Two equivalents of electrons per cytochrome b/f complex contribute 2.4 mol of electron of P7w to the total rapid donor pool.
*b The photochemically active plastocyanine pool is about 10% larger than this value due to the fast and, therefore, undetected loss of about 1 electron/P7w from the pool to the rapid donor chain. The reduction of the partially oxidized rapid donor chain by the plastoquinol occurs within a few milliseconds following the end of the preillumination (see text).

For the purposes of this table the term Photosystem II is defined as PS II reaction centers that are able to oxidize water and reduce plastoquinone.

By such experiments it was shown that the PS I reaction center content of lamellar membranes from spinach was 1.57 ± 0.10 meq-mol chlorophyll-1 (Table II).

**Interphotosystem Electron Carriers**

**Carriers Which Donate Electrons Rapidly to PS I Reaction Centers**

Electrons in the higher potential electron carriers on the PS I side of the rate-limiting step of electron transfer can be measured in chloroplast lamellae in which plastoquinone is fully oxidized and PS II turnover is prevented by the presence of DCMU or by heat inactivation of water oxidation (see Fig. 1).

We have reported previously (18) see also Ref. 19) that the light-induced reduction of methyl purple indicates an initial period of rapid electron transfer. This initial period persists for about 20 ms (Fig. 2, trace B) and is insensitive to DCMU (Fig. 2, trace C). Subsequent to the period of rapid electron transfer, inhibitors of PS II turnover abolish any further absorption changes at 591 nm (Fig. 2, trace C) which indicates that methyl purple is neither further reduced nor reoxidized. The DCMU-insensitive electron transfer amount to 8.66 ± 0.33 meq-mol chlorophyll-1 (Table II) and represents the flow of electrons to methyl purple from the already reduced elec-
electron carriers in the chain of dark-adapted membranes, carriers which function on the PS I side of the site of DCMU inhibition. This pool of rapid electron donors was thus 4.5 times larger than the number of PS I reaction centers \( (F_{700}) \) itself (compare traces B and C of Fig. 2) (i.e. \((8.7 - 1.6)/1.6 \) = 4.4).

Quantitation of the rapid donors to \( P_{700} \) presupposes that the plastoquinone pool is fully oxidized and that the pool of rapid donors (carriers of the cytochrome \( b_6/f \) complex and plastocyanin) is fully reduced. In order to establish the fact that the higher potential rapid donors are indeed all reduced the sample can either be preilluminated in the absence of both DCMU and any exogenous acceptor, or ascorbate can be added until there is no further increase in the DCMU-sensitive methyl purple reduction (Fig. 3). The effects of preillumination on the reduction state of the electron transfer chain warrant further comment. Preillumination in the absence of an electron acceptor promotes not only the reduction of the rapid donor pool of \( P_{700} \) but also of the plastoquinone pool that is normally fully oxidized at ambient redox potential in dark-adapted lamellar membranes. Indeed, the light-induced reduction of plastoquinone by PS II turnover is exploited in experiments described in Fig. 7 to quantitate the size of the donor pool which occurred upon addition of DCMU and heat-treated chloroplast lamellar membranes (3-min incubation at 50 °C). DBMIB was chemically reduced with \( \text{NaBH}_4 \), removed by \( \text{HCl} \) addition) prior to addition to the sample. All other conditions were as in Fig. 2 with full reduction of the pool of rapid donors to \( P_{700} \) ensured by the presence of 10 \( \mu \text{M} \) ascorbic acid. The lower trace \((\Delta -- \Delta)\) shows the effect of the removal of the Rieske FeS center from the rapid donor pool for membranes partially depleted of their plastocyanin (see text). The \( P_{700} \) content of this membrane preparation was 1.57 meq-mol chlorophyll⁻¹.

![Fig. 3. The effect of ascorbate on the reduction state of the rapid donors to \( P_{700} \). The state of reduction of the rapid donors to \( P_{700} \) was determined from the amount of DCMU-insensitive methyl purple reduction as shown in Fig. 2C. When the rapid donor chain was reduced by preillumination in the absence of DCMU and methyl purple (O --- O) exogenous ascorbate had no effect on the amount of DCMU-insensitive methyl purple reduction. The rapid donor pool of dark-adapted lamellar membranes \((\Delta -- \Delta)\) or heat-treated membranes \((\Delta -- \Delta)\) was generally partially oxidized, and in these cases exogenous ascorbate restored the fully reduced state. The \( P_{700} \) content of this chloroplast lamellar membrane preparation was 1.51 meq-mol chlorophyll⁻¹.](image1)

![Fig. 4. Removal of the Rieske FeS center from the rapid donor pool by titration with DBMIB. All samples contained 15 \( \mu \text{M} \) DCMU and heat-treated chloroplast lamellar membranes (3-min incubation at 50 °C). DBMIB was chemically reduced with \( \text{NaBH}_4 \), (excess \( \text{NaBH}_4 \) removed by \( \text{HCl} \) addition) prior to addition to the sample. All other conditions were as in Fig. 2 with full reduction of the pool of rapid donors to \( P_{700} \) ensured by the presence of 10 \( \mu \text{M} \) ascorbic acid. The lower trace \((\Delta -- \Delta)\) shows the effect of the removal of the Rieske FeS center from the rapid donor pool for membranes partially depleted of their plastocyanin (see text). The \( P_{700} \) content of this membrane preparation was 1.57 meq-mol chlorophyll⁻¹.](image2)
electrons from the cytochrome b₆/f complex to P₇₀₀. Mild physical disruption of the heat-treated membranes caused by rapid passage through a narrow bore (150-μm) needle resulted in the loss of a component from the rapid donor pool (see Fig. 4). The amount of this component that was lost was often almost equivalent to the P₇₀₀ content of these membranes. Yet, based on the amount of methyl purple reduced in the absence of an exogenous electron acceptor and the reduction state of the pool monitored subsequently by measurement of light-induced methyl purple reduction in the presence of DCMU that is added to prevent any further flow of electrons from PS II to plastoquinone. Fig. 6 shows the amount of methyl purple reduced by electrons from the intersystem carriers under a variety of conditions. In trace a, all of the carriers had been reduced by preillumination as described above, whereas during a second illumination given to the same sample (trace d) only the higher potential rapid donors remained reduced (by s slow back reaction with the photosynthetically reduced methyl purple). Thus the number of electrons stored in the plastoquinol pool is given by the difference in methyl purple reduction shown in traces a and d of Fig. 6.

Following the initial rapid phase of electron transfer to methyl purple, assigned to oxidation of the cytochrome b₆/f complex, plastocyanin, and P₇₀₀ itself, the rate of further methyl purple reduction is sensitive to the inhibitors of plastocyanin oxidation 2,4-dinitrophenylether of iodonitrothymol (Fig. 6, traces b and c) and DCMU (data not shown).

Carriers Which Donate Electrons Slowly to P₇₀₀ (Plastoquinol)—Electrons move more slowly from the lower potential plastoquinol pool to the higher potential rapid donors in a reaction which is both rate determining and energy conserving. As pointed out earlier, the reduction of plastoquinone can be achieved by preillumination of the membranes in the absence of an exogenous electron acceptor and the reduction state of the pool monitored subsequently by measurement of light-induced methyl purple reduction in the presence of DCMU that is added to prevent any further flow of electrons from PS II to plastoquinone. Fig. 6 shows the amount of methyl purple reduced by electrons from the intersystem carriers under a variety of conditions. In trace a, all of the carriers had been reduced by preillumination as described above, whereas during a second illumination given to the same sample (trace d) only the higher potential rapid donors remained reduced (by s slow back reaction with the photosynthetically reduced methyl purple). Thus the number of electrons stored in the plastoquinol pool is given by the difference in methyl purple reduction shown in traces a and d of Fig. 6.

Following the initial rapid phase of electron transfer to methyl purple, assigned to oxidation of the cytochrome b₆/f complex, plastocyanin, and P₇₀₀ itself, the rate of further methyl purple reduction is sensitive to the inhibitors of plastocyanin oxidation 2,4-dinitrophenylether of iodonitrothymol (Fig. 6, traces b and c) and DCMU (data not shown).

FIG. 5. Oxidized minus reduced difference spectrum of plastocyanin released from heat-treated chloroplast lamellar membranes by mild physical disruption. A 2-ml suspension of lamellar membranes (2.3 mM chlorophyll) was heat treated and then physically disrupted by rapid passage through a narrow bore (150-μm) syringe needle. The sample was centrifuged at 135,000 × g for 90 min in order to remove the membranes. Further purification was accomplished by passing the sample through a 0.2-μm Millipore filter. Absorption spectra were recorded with a Cary 219 spectrophotometer before and after the addition of 0.5 mM potassium ferricyanide.

FIG. 6. Enhanced DCMU-insensitive methyl purple reduction due to reduction of plastoquinone by preillumination. The chloroplast lamellar membranes were preilluminated for 460 ms in the absence of DCMU and any exogenous electron acceptor. The preillumination was followed by the addition of DCMU (15 μM) and methyl purple (20 μM) and then, after 30 s had elapsed, by a second 460-ms illumination period (trace a). The DCMU-insensitive methyl purple reduction in trace a is the result of the electrons stored in the rapid donor pool as well as electron stored as plastoquinol. Trace d represents the DCMU-insensitive methyl purple reduction observed in the third and all subsequent flashes spaced at 60-s intervals. This methyl purple reduction results from electrons stored in the rapid donor pool, electrons which are replenished in the dark intervals by the photosynthetically reduced methyl purple. Thus the difference between traces a and d is a quantitative measure of the number of electrons stored as plastoquinol. The effect of 2,4-dinitrophenylether of iodonitrothymol on light-induced plastoquinol oxidation is shown in traces b (10 μM) and c (20 μM). The traces are the averages of one flash to each of two samples.
Plastoquinol undergoes an aerobic oxidation, and this loss of electrons from the pool to oxygen must be accounted for in determining the maximum capacity of the pool to store electrons. Under the conditions of our measurements, the aerobic oxidation of plastoquinol appears first order with a half-time of approximately 80 s (Fig. 7). Extrapolation of the linear semi-log plot back to end of the preillumination period used to reduce plastoquione shows a pool size of 16.57 ± 1.41 meq-mol chlorophyll⁻¹ (Table II) or 10 to 11 electrons per P₇₀₀.

This extrapolation does not take into account any rapid oxidation of plastoquinol which would occur due to the reduction of any of the higher potential PS I components that were in the oxidized state when the preillumination ended. However, the conditions of the preillumination, that is saturating light, the absence of an electron acceptor, and the presence of an uncoupler favor reduction of the entire chain. The lower set of points in Fig. 7 depicts the results obtained when methyl purple was present during the preillumination, a condition which would favor oxidation of the rapid donors to P₇₀₀. The fact that the extrapolated values (in the presence of an exogenous electron acceptor and its absence) differ by an amount close to the value obtained for the rapid donor pool (7.2 versus 8.7 meq-mol chlorophyll⁻¹) indicates that the rapid donors to P₇₀₀ shared no more than about one oxidizing equivalent per Psq when the preillumination period in the absence of an exogenous electron acceptor ended. This conclusion is corroborated by measurement of the oxidation state of cytochrome f a few milliseconds after the end of the preillumination. These measurements (data not shown) indicated that with no exogenous acceptor present from 1 to 1.5 oxidizing equivalents were distributed among the rapid donors, predominantly between cytochrome f and the Rieske FeS center, at the end of the preillumination period. The levels of oxidation of the Rieske center and of plastocyanin were calculated (24) based on their equilibrium midpoint potentials, the equilibrium midpoint potential of cytochrome f, and the oxidation state of cytochrome f measured at the end of the preillumination period. Thus a maximum correction of about 10% should be applied to the value for size of the active plastoquinone pool obtained by the extrapolation procedure depicted in Fig. 7 giving a corrected value very close to 6 molecules (11 to 12 electrons) per P₇₀₀.

The Relative Abundances of PS I and PS II Reaction Centers

The ability to monitor changes in the reduction level of the plastoquinone pool gave us an opportunity to measure the balance between PS I and PS II in spinach chloroplast lamellae. Any excess of water-oxidizing PS II centers over plastoquinol-oxidizing PS I centers will result in the accumulation of electrons as plastoquinol when all reaction centers are turned over in unison by saturating single turnover flashes. Any accumulation of plastoquinol will be exactly in proportion to the imbalance of PS II over PS I. When the chloroplast lamellar membranes were illuminated by single turnover flashes delivered at 5 Hz in the presence of an electron acceptor and an uncoupler, only a very small net reduction of the plastoquinone pool was observed (Fig. 8). The data in Fig. 8 and Table II show only a 5 to 10% excess of PS II centers over PS I centers. A doubling of the flash frequency had no effect on these results. The fact that substitution of the very low redox potential acceptor methyl viologen for methyl purple during the preilluminating flash series resulted in no appreciable difference in the measured reduction state of the plastoquinone pool (3) confirms our belief that methyl purple does not oxidize plastoquinol or otherwise intercept electrons prior to photosystem I. Substantial reduction of the plastoquinone pool can be achieved by single turnover flashes if P₇₀₀ oxidation is impaired by the absence of an electron acceptor (Fig. 8, upper curve). The

![Fig. 7. The dark oxidation kinetics of the photochemically active plastoquinol pool. The maximum size of the photochemically active plastoquinone pool was determined by correcting for the oxidation of plastoquinol which occurred before the first data point could be taken (i.e. during the first 30 s after the end of the preillumination period). Experimental conditions were as in Fig. 5 except that the time between the preillumination and the first measuring flash was varied and in the lower curve (O—O) methyl purple was present during the preillumination.](image)

![Fig. 8. Determination of the balance between PS II reaction center and PS I reaction center content in spinach lamellar membranes. Any excess of PS II reaction center content was quantitated based on the net reduction of plastoquinone which must occur when all reaction centers of both photosystems are turned over in unison by saturating xenon flashes. The samples were illuminated by xenon flashes at 5 Hz in the presence of 10 μM ascorbate and 5 μM gramicidin D. The concentration of methyl purple was 20 μM when present (O). Immediately after the last xenon flash 18 μM DCMU was added (and 20 μM methyl purple if absent during preilluminating flashes) to the sample. Methyl viologen replaced methyl purple during the preilluminating flashes for the data shown as □. Reducing equivalents in the plastoquinone pool were assayed by the procedures described in Fig. 6.](image)
initially gentler slope of the no-acceptor condition ($\Delta - \Delta$) probably results from the presence of the endogenous PS I acceptors. Thereafter (after about 5 turnovers) approximately 0.75 electron/flash accumulates as plastoquinol in the absence of an electron acceptor. An increase in plastoquinol formation from saturating single turnover flashes may also occur when P$_{700}$ re-reduction is impaired by the incomplete reduction of the rapid donor pool as when the absence of an uncoupler limits the oxidation of plastoquinol by the cytochrome $b_6/f$ complex (data not shown).

**DISCUSSION**

The data presented in this paper demonstrate that the pool of high-potential electron carriers which serve as the rapid donors to P$_{700}$ in spinach lamellar membranes is about 4.5 times larger than the content of P$_{700}$ itself. This value is substantially larger than earlier reports have suggested (e.g. Refs. 25–28). In view of the narrow range of values that we observed over the course of this study for commercial spinach obtained in different seasons and from different geographical locations, we do not believe that the origin of the difference lies with inherent differences in plant material. We observed in our own experiments several ways in which misleadingly low estimates of the rapid donor pool size may occur. 1) In chloroplasts incapable of water oxidation (due, for instance, to heat treatment or the addition of DCMU) an anticipated oxidation of the electron carriers can result from actinic effects of the measuring beam. In the experiments reported here the sample was exposed to a weak measuring beam ($6 \times 10^{-5}$ J/cm$^2$ s$^{-1}$, most of which was absorbed by the methyl purple present in the sample) for only 600 ms prior to the actinic light flash. 2) In many preparations of dark-adapted chloroplast lamellar membranes these high-potential electron carriers were, at ambient redox potential, in a partially oxidized state. The fully reduced state can be ensured by preillumination or by the addition of low concentrations of ascorbate as shown by the data of Fig. 3. 3) A decrease in the number of stored reducing equivalents might also result from isolation or from measurement procedures in which a portion of the plastocyanin is lost. These and perhaps other factors could contribute to decrease the apparent size of the rapid donor pool of P$_{700}$.

We measured the cytochrome $b_6/f$ complex content of the lamellar membrane based on the effect of DBMIB on the DCMU-insensitive methyl purple reduction. The effect was to decrease the amount of methyl purple by an amount just slightly greater than the P$_{700}$ content of the membrane (Fig. 4 and Table II). A strong interaction between DBMIB and the Rieske FeS center of the cytochrome $b_6/f$ complex is implicit from the observation by Malkin (20, 28, 30) that DBMIB caused a displacement of the characteristic low temperature EPR signal at $g = 1.98$ to $g = 1.94$. The notion that DBMIB prevents the oxidation of the Rieske FeS center by PS I is consistent with an increase in cytochrome $f$ oxidation induced by DBMIB following a single turnover flash (21). This observation indicates that, in the presence of DBMIB, the Rieske FeS center can no longer share the flash-induced oxidizing equivalent with cytochrome $f$. The number of electrons stored by the cytochrome $b_6/f$ complex was taken to be 2-fold greater than the number deduced from the effect of DBMIB on DCMU-insensitive methyl purple reduction based on the relationship of unity shown for the Rieske center and cytochrome $f$ in the complex (22). The value of 1.91 ± 0.12 meq-mol chlorophyll$^{-1}$ (Table II) obtained for the membrane's content of the cytochrome $b_6/f$ complex is in close agreement with the value of 1.79 meq-mol chlorophyll$^{-1}$ that we measured for photooxidation of cytochrome $f$ (data not shown). In the latter experiments methyl viologen replaced methyl purple as the electron acceptor in order to avoid a large interfering light-induced absorption change overlapping with the cytochrome absorption spectrum.

In view of the necessity for a plastoquinol oxidase-binding site on the cytochrome $b_6/f$ complex (4) it is worthwhile to consider whether any of the electrons which we assign to the rapid donor pool of P$_{700}$ might originate from some sort of bound plastoquinol. Both linear and Q-cycle models for the oxidation of quinol involve the Rieske FeS center as a direct oxidant in the reaction mechanism. Thus, inhibition of the Rieske center by DBMIB would remove not only this component from the rapid donor pool but any contributing bound quinol as well. The data indicate the removal of a single component by DBMIB which is stoichiometric with P$_{700}$ (Fig. 3) and thus argue strongly against any contribution by a bound quinol in the measurements reported here.

We have assigned the remaining 2.1 (Table II) electrons in the rapid donor pool to plastocyanin. As much as one-half this amount can be released from the membrane vesicles by heat treatment followed by mild physical disruption. The reducing equivalents lost from the rapid donor pool by this treatment can be nearly quantitatively accounted for by the plastocyanin detected in the supernatant (Fig. 5). The plastocyanin which is resistant to release from within the vesicles is approximately equal to the P$_{700}$ content and must serve as a mobile electron carrier in subsequent illuminations, since the membranes' entire complement of cytochrome $b_6/f$ complex can still be rapidly photooxidized. Inasmuch as plastocyanin is fully reduced under the conditions in which a maximum of one-half of the total can be released from the membranes, it is likely that much of the part retained is bound to the PS I reaction center. Subsequent photooxidation would result in release of the plastocyanin from the reaction center binding site and allow it to serve as a mobile electron shuttle.

The method described in Figs. 6 and 7 of this paper introduces a reliable and accurate procedure for the quantitation of the photochemically active plastiquinone pool. The reliability of the method results from the fact that a relatively large absorbance change is measured upon reduction of a dye present in aqueous solution. The molar absorptivity of this exogenous electron acceptor is known with considerable precision, and interpretation of its light-induced absorbance change is straightforward. Our value of nearly 6 active plastiquinones per P$_{700}$ (i.e. 11 to 12 electrons to P$_{700}$) is in close agreement to the value reported for spectroscopic measurements in the ultraviolet by Stiehl and Witt (25). These determinations compared the absorbance change at 265 nm induced by a single turnover of PS II with the maximum change at 265 nm associated with complete conversion of plastiquinone to plastocyanin. Direct calculation of the plastiquinone pool size based on a differential extinction coefficient for plastiquinone/plastoquinol is complicated by the uncertainties of absorption flattening. When the seemingly appropriate correction is applied (a multiplicative factor of about 1.5 at 265 nm) much larger plastiquinone pool sizes are suggested (e.g. Ref. 31). The pool of PS II electron acceptors has also been estimated from the area under the fluorescence induction curve, and values of 10 to 20 times larger than PS II reaction center content are generally reported for spinach thylakoids (32).

There has been a controversy regarding the relative abundance of PS II and PS I in spinach membranes. Recently, Whitmarsh and Ort (7) have challenged the reports of a 2-
fold excess of PS II centers over PS I centers (33), reports that were based on UV absorption measurements of Qo reduction. Whitmarsh and Ort found that the PS II content of spinach lamellar membranes estimated by the UV absorption measurement was twice the value given by direct measurement of water oxidation. In this paper we have taken an entirely new approach which confirms the conclusion by Whitmarsh and Ort (7) of a one-to-one relationship between PS I and PS II in spinach chloroplast membranes isolated from normally developed plants. We have measured the change in reduction state of the plastoquinone as a function of the number of synchronous turnovers of all reaction centers. Any excess of PS II centers over PS I centers would lead to net reduction of the plastoquinone pool whereas the pool would remain fully oxidized if the two photosystems were present in the membranes in equivalent amounts or if PS I was present in excess. Our results indicate only a very slight excess of PS II centers (5 to 10%, Table II) since only 0.05 to 0.1 meq mol chlorophyll^{-1} flash^{-1} was observed to accumulate in the plastoquinone pool when all PS I and PS II reaction centers operated in unison. Melis and Anderson (33) cite the net reduction of the plastoquinone pool that they observe under weak green light illumination as qualitative support for a substantial excess of PS II reaction centers. Their interpretation of these measurements depends upon optimistic assumptions about the absorption characteristics of the photosystem for the incident green photons, and the uncertainty introduced by these assumptions is difficult to evaluate. In addition the measurements were conducted in the absence of any uncoupler. Our observation that even with low frequency flashes (5 Hz) PS I turnover can be somewhat impaired by incomplete P_{700} rereduction when an uncoupler was absent leaves little doubt that an uncoupler is necessary to ensure rereduction of P_{700} in low light experiments as well.

The data presented in this paper demonstrate a remarkable constancy in the stoichiometric relationships of electron transfer components as well as a constancy in the ratio of each component to the total chlorophyll content of the membrane (Table II). Extreme values were never encountered even though the spinach was obtained from commercial sources originating from widely different places during all seasons of the year. A survey of the literature of the past decade does not reveal the constancy demonstrated in our studies even for this single species. Indeed, the wide range of literature values has been viewed as support for plasticity in electron transfer component ratios and chlorophyll antenna sizes. There is considerable current interest in the extent to which a species of plant can tailor its photosynthetic apparatus to a particular set of environmental conditions and thereby optimize its photosynthetic performance. There is also much current interest in the different strategies adopted by those plant species evolutionarily adapted for extremes in light intensity and spectral distribution. Our study presents a reliable and highly versatile set of techniques ideally suited for such studies. In addition, our results as well as those of Whitmarsh and Ort (7) show the need to re-evaluate some of the conclusions regarding capacity of plants to manipulate their photosynthetic apparatus, particularly those conclusions which are based on the wide range of numbers and component ratios in the literature.

Acknowledgments—We thank Dr. J. Whitmarsh for conducting the measurements of cytochrome f photooxidation and for valuable discussions during the course of this study. We also thank Professor N. E. Good for critical evaluation of the manuscript.

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