Reconciliation of the Absorption Change at 325 nm and other Flash-Yield Determinations of Concentrations of Active Photosystem II Centers

PAUL JURSINIC and RONALD DENNENBERG

Northern Regional Research Center, Agricultural Research Service,
U. S. Department of Agriculture, Peoria, Illinois 61604

Received January 8, 1985, and in revised form May 20, 1985

The concentration of photosystem II was determined in thylakoids of dwarf peas by the use of the following methods: absorption change at 325 nm; atrazine binding; and flash yields of oxygen evolution (Emerson-Arnold method), of protons from oxidation of water, and of reduction of DCIP. For the first time all of the flash-yield measurements have been done on the same sample and give equivalent values for the concentration of photosystem II. Agreement of the absorption change measurement at 325 nm with the other measurements was accomplished by the introduction of important improvements to the methods of Melis and co-workers [Proc. Natl. Acad. Sci. USA (1980) 77, 4712-4716]. The atrazine-binding method gave photosystem II values that were twice as large as any of the other photosystem II measurements. Possible reasons are discussed for this discrepancy in terms of the secondary acceptor (Q400) of Ikegami and Katoh [Plant Cell Physiol. (1973) 14, 829-836]. The concentration of photosystem I was measured by absorption change at 705 nm. From the concentration values of photosystem II and I the system II/I stoichiometry was calculated.

Green plant photosynthesis takes place through the cooperation of two photo-reactions. Photosystem I drives the reduction of carbon dioxide and photosystem II drives the oxidation of water, which results in the production of oxygen. These two photosystems are connected in series by a chain of electron carriers. It seems reasonable that the best efficiency would occur if the two photosystems had a stoichiometric relationship close to unity. However, recent reports have indicated photosystem II/I ratios greater than 1 (1–5). Values of these ratios have been criticized due to possible uncertainties in the determination of the photosystem II reaction center (RCII) concentration.

The photosystem I reaction center (RCI) concentration has been determined by the photoinduced absorption change at 705 nm, which is due to the photooxidation of the reaction center chlorophyll (P700). Most workers agree that this type of measurement is being carried out correctly.

In previous work the RCII concentration has been determined by several methods. One is a determination of oxygen yield

---

1 The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

2 To whom correspondence should be addressed.

3 Abbreviations used: Chl, chlorophyll; DCBQ, 2,6-dichloro-p-benzoquinone; DCIP, 2,6-dichloroindophenol; PPO, 2,5-diphenyloxazole; Qa, primary quinone acceptor of photosystem II; RCI, photosystem I reaction center; RCII, photosystem II reaction center; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino} ethanesulfonic acid.
per flash (6) measured with a concentration electrode illuminated with microsecond-long flashes of saturating light. This determination can be in error due to the choice of number of quanta required per oxygen molecule evolved and to possible destruction of oxygen-evolution capacity during sample preparation. Another method is a determination of the concentration of Qa, the primary quinone acceptor, by its absorption change at 325 nm (7). This determination has been criticized due to assumptions about the extinction coefficient of Qa, the correction for absorption of strongly absorbing particles (flattening factor), and the possible existence of other contributors to the 325-nm absorption change besides Qa (8).

In a recent report Whitmarsh and Ort (8) determined the concentration of RCII by the use of proton evolution from the breakdown of water and the absorption change at 325 nm due to quinones, and the absorption change due to Cyt b-559. The proton and Cyt b-559 measurements gave a RCII concentration of 1 per 600 Chl, and the 325-nm absorption change gave a concentration of 1 per 300 Chl. This latter value was in good agreement with measurements reported by Melis and co-workers (3, 9–11). Whitmarsh and Ort (8) were unable to reconcile these different values and concluded the absorption-change measurements were in error or misinterpreted.

In this work we were able to get agreement between the values for the concentration of RCII obtained from the absorption change at 325 nm and other flash-yield measurements. This was accomplished by corrections to the methods of Melis and co-workers (3, 9–11). Essentially, we were able to identify and eliminate contributions to the absorption change at 325 nm by oxygen S-state change, photosystem I components, and quinones other than Qa. In this way the absorption change at 325 nm due solely to Qa was isolated and quantified. In addition to the water-proton measurements of Whitmarsh and Ort (8), the flash yields of oxygen evolution and DCIP reduction were used to determine the RCII concentration. An additional method for determination of RCII concentration was also applied. Binding studies with [14C]-atrazine were carried out, and parameters obtained from these studies allowed an estimate of the RCII concentration.

All the methods gave RCII concentrations that agree within experimental error except for atrazine. Since four of these methods agree, it is concluded that our new 325-nm absorbance change measurement is valid. Based on these methods, a RCII/RCI ratio of 1.39 ± 10% was found for peas grown under 45 W/m² light. The RCII concentration based on atrazine binding is larger by a factor of two. These different results are believed to be due to the presence of a secondary quinone acceptor, which has a herbicide binding site.

MATERIALS AND METHODS

Broken chloroplasts were isolated from leaves of dwarf pea seedlings (*Pisum sativum* L. var. Progress 9) grown in a laboratory growth chamber (16-h day; 25/20°C; 45 W/m² light intensity from a combination of Gro-lux fluorescent lamps and incandescent lamps). The plants were harvested 18 to 21 days after germination. The isolation procedure has been previously described (12). The following isotonic reaction medium was used: 400 mM saccharose, 50 mM Tes (pH 7.5), 10 mM NaCl, 5 mM MgCl₂. *Chlamydomonas reinhardtii* (Strain CC-125, Mt+) was grown phototrophically at 25°C in HS medium (13) bubbled with 5% CO₂.

*Chlorella pyrenoidosa* (University of Texas, UTEX no. 251) were grown in Emerson medium (14) bubbled with 5% CO₂. Cells were harvested in the logarithmic phase of growth by centrifugation at 5000g for 3 min. The cells were then resuspended in the following medium: 10 mM phosphate (pH 7.0), 20 mM KCl, and 2.5 mM MgCl₂.

Tris washing was done by a method similar to that of Yamashita and Butler (15). Thylakoids were suspended in 0.3 M Tris (pH 8.2) for 10 min at 0°C in room light at a chlorophyll concentration of 200 μg/ml. After this treatment, the sample was diluted with 0°C reaction medium and centrifuged at 5000g for 5 min. The pellet was resuspended in reaction medium. NH₂OH treatment was done in a manner similar to that of Cheniae and Martin (16). Thylakoids were suspended in 5 mM NH₂OH for 10 min at 20°C in the dark at a chlorophyll concentration of 25 μg/ml.

Light-induced absorption changes at 705 and 325 nm were used to calculate concentrations of P700,
the primary electron donor of photosystem I, and QA, the primary electron acceptor of photosystem II, respectively. The extinction coefficient used at 705 nm for P700 was 64 mM\(^{-1}\) cm\(^{-1}\) (17), and at 325 nm for QA was 12 mM\(^{-1}\) cm\(^{-1}\) (18). The photochemically induced absorption changes at 705 and 325 nm were measured with a laboratory built dual-beam spectrophotometer. The sample was at a chlorophyll concentration of 25 \(\mu\)g/ml and was held in a 1-cm-square quartz cuvette. Single saturating flashes were provided by a xenon flash-lamp of 3 \(\mu\)s width at half height. The actinic flashes were filtered through a combination of CS 3-72 and CS 4-96 Corning glass filters. Analytic light was provided by a Bausch and Lomb high-intensity monochromator with a 150-W tungsten-halogen lamp powered by a regulated dc supply. An Oriel monochromator was placed in front of the sample-beam photomultiplier. A shutter positioned in the analytic beam was opened just prior to the start of the measurement to avoid any large amount of excitation by the analytic light. Analog data were digitized by a Biomation Model 805 transient recorder and improvement in signal-to-noise ratio was accomplished by averaging a number of data traces with a Classic 7870 computer from Modular Computer Systems, Inc.

Corrections were made for particle flattening (19) to allow light-induced absorption changes to be related to changes in concentration of the photochemically altered component absorbing at a particular wavelength. Correction factors for the measurements reported here were determined by the pigment extraction method of Pulles et al. (20).

Herbicide binding analysis was carried out with methods similar to those of Pfister et al. (21) and Tischer and Strotmann (22). Illumination conditions were found to alter herbicide binding characteristics (23), so all binding studies were done on samples dark-adapted for 10 min or longer. Binding reactions were initiated by vortex-mixing 1-ml suspensions of 50 \(\mu\)g Chl/ml with various amounts (5 to 30 \(\mu\)l) of \(^{14}\)C]atrazine (14.6Ci/mol) dissolved in ethanol. After 1 min or longer incubation, the samples were centrifuged for 2 min at 15,600g in an Eppendorf 5414 centrifuge. Aliquots (0.5 ml) of the supernatants were removed and added to 4 ml of scintillation fluid (8 g PPO, 1 liter Triton X-100, 2 liters toluene, 400 ml H\(_2\)O). Radioactivity of the samples was measured by a liquid scintillation counter. The amount of bound inhibitor was calculated from the difference between the total inhibitor added to the thylakoids and the amount of free inhibitor found in the supernatant after centrifugation. Data presentation and calculations were carried out as previously described by Tischer and Strotmann (22). The amount of RCII was equated to the number of atrazine binding sites, assuming that there was only one site per RCII.

Oxygen yield per flash was determined with a Clark-type electrode. Excitation was provided by two xenon flash lamps that were positioned on opposite sides of the measuring vessel. The flashes were triggered simultaneously and were sufficiently intense to saturate oxygen evolution. Flashes were given at a rate of 5 Hz. From the oxygen yield per flash, the photosynthetic unit size for oxygen production was calculated. This was converted to the RCII concentration by dividing by 4, since on a particular flash only one-fourth of the reaction centers are evolving an oxygen molecule (24).

Flash yields of proton evolution from breakdown of the water molecule were measured with a pH electrode (Cole-Parmer, C-5992-12). A Cole-Parmer Model 5982-50 pH meter in combination with a laboratory-built amplifier and offset circuit were used to record the signals. A 3-ml reaction vessel was vigorously stirred and illuminated by two xenon flash lamps that were triggered simultaneously. Flashes were given at 5 Hz. Chl concentration was 35 \(\mu\)g/ml. The reaction medium contained 10 mM NaCl and 5 mM MgCl\(_2\). This hyposmotic medium was essential for a rapid release of protons from the thylakoid membrane to the bulk solution (25). In this way a significant pH drift, due to a slow equilibrium of sequestered protons with the bulk solution, was eliminated. Other additions to the reaction medium were 0.5 \(\mu\)M gramicidin D as an uncoupler to allow rapid movement of protons across the thylakoid membrane (26), 200 \(\mu\)M DCBQ, an electron acceptor that accepts electron near photosystem II (27), 1 mM ferricyanide to keep the DCBQ oxidized, and small amounts of HCl or NaOH to adjust the pH to 7.5 prior to illumination. The pH change was calibrated by injecting known amounts of HCl. The RCII concentration was calculated by assuming that under steady-state conditions one proton was released per flash per RCII.

Flash yields of DCIP reduction were measured by the absorption change at 600 nm. Thylakoids were suspended in the isotonic reaction medium at a concentration of 25 \(\mu\)g Chl/ml and 50 \(\mu\)M DCIP. The extinction coefficient used was 2.1 \(\times\) 10\(^4\) cm\(^{-1}\) M\(^{-1}\) (28). Flashes of saturating intensity were given at 5 Hz. The reduction of DCIP was transformed into RCII concentration by assuming that one-half molecule of DCIP was reduced per flash per RCII. DCIP is a two-electron acceptor, and under flash conditions accepts electrons from the plastocyanine pool before plastocyanin (29).

RESULTS

In a dark-adapted sample with DCMU present a single flash will cause a charge separation to occur at photosystem I and II. At photosystem II oxygen evolution
S-states will turnover and Qa will be reduced. At photosystem I P700 will be oxidized and then reduced as the inter-system charge carriers (plastoquinone, cytochrome, and plastocyanin) become oxidized. Figure 1 (control) shows the absorption change at 325 nm observed after one excitation flash. For this measurement the sample was dark adapted at least 10 min at 20°C prior to addition of DCMU. This gave a stable and maximal signal. Apparently it took this amount of time for charge carriers of the electron transport chain to come to a redox equilibrium. Included in the reaction medium were 4 μM DCMU and 0.4 μM gramicidin D. The DCMU was present to inhibit the rapid reoxidation of Qa, and the gramicidin eliminated the membrane potential and its associated absorption changes. The shutter that blocked the measuring beam was opened 200 ms prior to the sample excitation flash. Under these conditions the analytic beam did not cause a significant amount of charge separation.

This absorption change at 325 nm is a composite of absorption changes associated with all the charge carriers mentioned above. Based on the difference spectrum of VanGorkom (18), it is believed that Qa is the major contributor to the absorption change at 325 nm. In order to use this absorption change to quantitate Qa in our sample its proportionality had to be determined with respect to the other contributors.

Thylakoids that are treated with NH₂OH and illuminated in the presence of DCMU have Qa irreversibly reduced and turnover of oxygen S-states stopped (16, 30). In this way, contributions to the absorption change at 325 nm only due to photosystem I and associated charge carriers can be measured. Figure 1 shows that the contribution due to photosystem I and associated charge carriers is about 18% of the control absorption change and in the opposite direction. In earlier work (3, 9) ferricyanide was added to the reaction medium to chemically oxidize all photosystem I charge carriers and so inhibit their photoreaction and contribution to the absorption change at 325 nm. We found this to be an unacceptable procedure since small amounts of ferricyanide oxidized the secondary acceptor, Q400 (31), which has a large absorption change at 325 nm (32) and thus obscures detection and quantitation of Qa.

In order to determine if our correction for photosystem I charge carriers is reasonable, we calculated the contribution of cyt f to the absorption change at 325 nm. The extinction of the cyt f band near 325 nm is -11 mM⁻¹ cm⁻¹ (33, 34) or about the same magnitude as Qa. We measured the cyt f absorption change at 554 minus 541 nm in the presence of DCMU and gramicidin with one saturating flash (i.e., the same conditions used in our Qa measurement) in a number of pea thylakoid preparations. The amount of cyt f that was reduced under these conditions was equivalent to 2200–2600 Chl/cyt f using ε = -20 mM⁻¹ cm⁻¹ (35). These preparations typically have Chl/photosystem II ratios of 550–600 based on both the absorption change at 325 nm and oxygen flash yield. From these numbers the cyt f contribution would be expected to be about -24% of

![Fig. 1](image-url). Absorption change at 325 nm in pea thylakoids after a single saturating flash. An upward deflection is an increase in absorption, which corresponds to Qa reduction. The samples were measured at a chlorophyll concentration of 25 μg/ml with 0.4 μM gramicidin D present, and were dark-adapted for 10 min at 20°C before the addition of 4 μM DCMU. These signals were an average of 49 measurements. The electronic rise time was 20 ms. The upper trace shows control thylakoids and the bottom trace shows NH₂OH-treated thylakoids after preillumination in the presence of DCMU.
the total 325-nm absorption change. The difference between the measured \(-18\%\) and the calculated \(-24\%\) may be offset since P700 would be expected to have a small positive contribution at 325 nm (36).

A dark-adapted sample given one flash will have oxygen evolution S-state transitions of \(S_0 \rightarrow S_1\) in 25% of the centers and \(S_1 \rightarrow S_2\) in 75% of the centers (24). These S-state transitions have been reported (37-39) to be associated with absorption changes at 325 nm. To estimate the extent of these contributions we wanted to specifically inhibit them. The charge transfer at photosystem II can be written schematically as follows:

\[
\begin{align*}
    M Z P_{680} Q_a & \rightarrow M Z P_{680}^+ Q_a \\
    M Z^+ P_{680} Q_a & \rightarrow M^+ Z P_{680} Q_a,
\end{align*}
\]

where \(M\) is the oxygen-evolving system, \(Z\) is a charge carrier, and \(P_{680}\) is the reaction-center chlorophyll. Thylakoids washed with Tris at alkaline pH have reaction 2 blocked (40-42). In this way any contribution of \(M\) to the absorption change at 325 nm could be eliminated. The oxidation of \(Z\), which is not inhibited by Tris washing, does not have an absorption change at 325 nm (39, 43). Figure 2 shows that the contribution due to S-state transitions on the first flash is about 15% of the control absorption change and in the same direction.

Based on these measurements the absorption change observed at 325 nm on the first flash in a control sample can be converted to reflect \(Q_a\) with the contribution of all other reactions being eliminated. The control signal is increased by 18% to compensate for photosystem I and its associated charge carriers and decreased by 15% to compensate for S-state transitions. The 325-nm absorption change is corrected in this manner for the determination of \(Q_a\) concentrations. The amount of RCII was equated to \(Q_a\), assuming that only one molecule of \(Q_a\) is reduced for every reaction center II that undergoes charge separation.

A typical signal for the absorption change at 705 nm is shown in Fig. 3. When measuring this absorption change the sample was dark-adapted for 1 min or longer. The shutter that blocked the measuring beam was opened 2 s prior to a series of excitation flashes given at 4 Hz. The reaction medium included the following: 4 \(\mu\)M DCMU to block photosystem II reaction after the first flash, 0.4 \(\mu\)M gramicidin to eliminate absorption changes due to membrane potential, 600 \(\mu\)M ascorbate to completely reduce P700 in the dark, and 100 \(\mu\)M methyl viologen to serve as a photosystem I acceptor. Multiple flashes are given to assure complete oxidation of the quinone pool and P700. This is observed as a jump in absorption after a flash, Fig. 3. The jumps eventually stop when all charges between the DCMU block and P700 have been removed. This final level gives the absorption when P700 is completely oxidized. The amount of RCII was equated to the amount of P700. The spikes are Chl \(a\) fluorescence generated during the flash. The amount of P700 was found to be dependent on the growth-light intensity. In this sample grown at 45 W/m\(^2\) the Chl/P700 was 825. Other samples grown
FLASH-YIELD DETERMINATION OF PHOTOSYSTEM II CENTERS

Cl

FIG. 3. Absorption change at 705 nm in pea thylakoids. The sample was dark-adapted at least 1 min prior to measurement. A downward deflection is a decrease in absorption, which corresponds to P700 oxidation. The samples were measured at a chlorophyll concentration of 25 μg/ml with 4 μM DCMU, 0.4 μM gramicidin D, 600 μM ascorbate, and 100 μM methyl viologen. Flash groups were given at 4 Hz, and this data trace is an average of nine flash groups. The electronic rise time was 2 ms.

at 75 W/m² had Chl/P700 of 600 (data not shown).

The identities of the light-induced absorption changes at 325 and 705 nm, under the experimental conditions used here, were verified by the light-minus-dark difference spectra of these changes. Figure 4A shows the absorption difference spectrum around 325 nm. This spectrum is in good agreement with those that have been published (3, 18) and has been attributed to the reduction of the primary photosystem II acceptor to its semiquinone form, QA. Figure 4B shows the absorption difference spectrum around 705 nm. Published spectra (17) associated with the oxidation of the photosystem I reaction center, P700, are similar to Fig. 4B.

Figure 5 is a double-reciprocal atrazine binding plot (1/[bound] vs 1/[free atrazine]) for thylakoids dark-adapted for 10 min or longer. Typical binding data are shown for pea thylakoids. The atrazine binding study was carried out at 20°C. The linear portion of these data is due to specific binding at photosystem II and corresponds to a dissociation constant of 0.057 μM. When extrapolated to the y axis, the intercept is equal to milligrams Chl per nanomole bound atrazine. For these data there are 322 Chl per bound atrazine.

Table I shows a compilation of data from the various sample types used. All the methods for estimating RCII concentration except atrazine binding gave values that are consistent within experimental error. The internal consistency of these values allows confidence in the assumptions and methods used in making these measurements. This is of particular interest for the 325-nm absorption change, which has come under attack because of uncertainty in the value of the extinction coefficient used and the validity of the flattening-factor correction (8). The stoichiometry of RCII/RCI shown in Table I, based on all the methods except atrazine binding, is greater than unity. This ratio varies with sample growth conditions (11, 44); however, these phenomena will not be treated here.

In thylakoids, the RCII concentration based on atrazine binding is found to be twice that found by all the other methods (see Table I). One possible explanation for this discrepancy in RCII concentration is that preparation of thylakoids caused inhibition of the oxygen evolution system and hence electron flow; this would result in a low value for the RCII concentration in the measurement of O₂, H⁺, QA, and DCIP. The binding of atrazine it is presumed would not be affected by thylakoid preparation. To determine if this possibility was reasonable, oxygen evolution per flash and atrazine binding measurements were carried out on whole cells of Chlamydomonas and Chlorella where thylakoid isolation procedures are not a factor. The data are shown in Table I and, as in thylakoid preparations, the RCII concentration based on oxygen evolution is about half that found with atrazine binding. The Chl/RCII value of 827 in Chlamydomonas seems rather high but it was quite reproducible under our growth conditions. In Chlorella we found a Chl/RCII value of 381, which is similar to values previously reported (45). The 325-nm absorption change could not be measured in whole-cell preparations due to...
large scattering signals. We conclude that the low values for the RCII concentration calculated from the flash measurements of $O_2$, $H^+$, and DCIP in thylakoids are not a result of damage incurred in the thylakoid isolation procedure since the same result is found in whole cells of *Chlamydomonas* and *Chlorella*.

It is possible that the atrazine binding in whole cells is unrelated to RCII concentration because of nonspecific binding to other membrane sites (46). In the concentration range of atrazine used in this work (Figs. 5 and 6) the inverse-inverse plots were linear with a single slope, which is indicative of specific binding (22, 46).

**DISCUSSION**

This study has shown an agreement in values for RCII concentration obtained by

<table>
<thead>
<tr>
<th>Sample material</th>
<th>Pea thylakoids</th>
<th><em>Chlamydomonas</em></th>
<th><em>Chlorella</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta$Abs 325 nm</td>
<td>574 ± 60</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$O_2$ Yield</td>
<td>616 ± 30</td>
<td>827 ± 40</td>
<td>381 ± 20</td>
</tr>
<tr>
<td>DCIP reduction</td>
<td>586 ± 70</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$H^+$ evolution</td>
<td>592 ± 60</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Atrazine binding</td>
<td>322 ± 50</td>
<td>444 ± 60</td>
<td>222 ± 30</td>
</tr>
<tr>
<td>$\Delta$Abs 705 nm</td>
<td>825 ± 60</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RCII/RCI</td>
<td>1.39 ± .14</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*All entries are reported as Chl/RC. The absorption change at 705 nm is equivalent to RC1 concentration, and all other measurements are RCII concentration. For calculating the RCII/RCI ratio all RCII measurements, except atrazine binding, were averaged. The flattening correction for the absorption change at 325 nm was 1.85. The deviations shown are standard deviations for five repeat measurements.
FLASH-YIELD DETERMINATION OF PHOTOSYSTEM II CENTERS 547

Fig. 6. Double-reciprocal plot of [14C]atrazine binding to whole cells of the alga *Chlamydomonas.*

325-nm absorption change and flash yields of oxygen evolution, proton evolution, and DCIP reduction. With these methods, the RCII/RCI stoichiometry is $1.39 \pm 10\%$ for pea thylakoids, and is believed to be correct and not attributable to errors in a particular experimental method.

Values of RCII/RCI stoichiometry of 2 or more have been reported (3, 10, 11, 44). In these investigations the 325-nm absorption change was used to determine RCII concentration and the 705-nm absorption change to determine RCI concentration. The 325-nm absorption change measurements in these earlier investigations were performed differently than those done here. Ferricyanide was included in the reaction medium of these earlier measurements. It was observed that with ferricyanide present the 325-nm absorption change signal increased by 70 to 100%, which gives rise to RCII concentrations proportionally larger than what is reported here. We have shown (32) that ferricyanide oxidizes additional photosystem II acceptors besides Qa. These acceptors have an absorption change at 325 nm and produce a high value for RCII concentration when they are not differentiated from absorption changes due to Qa. In this work ferricyanide was not used; thus, these secondary acceptors remained reduced and artifacts due to them in this 325-nm absorption change signal were avoided. Correction of the 325-nm absorption change signal for contributions of S-state transitions, cytochromes, and photosystem I were then made. The resultant 325-nm absorption change is then solely from Qa, and calculations of Chl/RCII based on these signals are in very close agreement with other methods (Table I).

In this work the RCII concentration based on atrazine binding is larger by about a factor of two in thylakoids and whole cells of *Chlamydomonas* and *Chlorella.* An earlier report (44) incorrectly presented RCII concentrations based on oxygen evolution that were in agreement with determinations by atrazine binding and 325-nm absorption change. In this earlier work the oxygen flash yields were calculated incorrectly.

To explain the differences found here in RCII concentration based on 325-nm absorption change and flash-yield measurements vs atrazine binding, it is proposed that there are two types of photosystem II centers; functional and nonfunctional for oxygen evolution and electron flow. Both kinds of centers have binding sites for atrazine. From the linear plots of the binding data (Figs. 5 and 6) these two different binding sites must have binding affinities for atrazine that are identical within experimental error. However, based on the data of Table I, about 50% of all the centers are functional for oxygen evolution and electron flow under steady-state flash conditions. This cannot be attributed to inhibition of oxygen evolution during thylakoid preparation, because essentially the same result is found in whole cells of *Chlamydomonas* and *Chlorella.* One difficulty with this hypothesis is that the existence of a large portion of nonfunctional photosystem II centers is inconsistent with reports of quantum requirements of 2.0 for the $\text{H}_2\text{O} \rightarrow \text{NADP}$ reaction (47). However, this deficiency is of no consequence if quanta that arrive at an inactive center can be transferred with high probability to an active center in close proximity. An interesting possibility is that those centers that are nonfunctional for oxygen evolution are identical to the non-B-type centers of Lavergne (48). The non-B-type centers
are hypothesized to be unconnected to the oxygen evolution system.

Another possibility is that each photosystem II reaction center is associated with a single oxygen-evolving complex but each reaction center has multiple quinone electron acceptors, each with an apoprotein with a Qb (herbicide) binding site. The existence of multiple acceptors has been postulated previously (31, 49-53). This work can be interpreted to mean that for every oxygen-evolving complex, there are approximately two quinone electron acceptors capable of being reduced and the same number of herbicide binding sites as acceptors. One of these quinone acceptors, Q400, is only oxidized when ferricyanide is present. Again, these two binding sites associated with the Qa and Q400 apoproteins must have binding affinities for atrazine that are the same within experimental error.

The results reported here emphasize the importance of defining how the reaction center concentrations are determined when one discusses stoichiometry of reaction centers. Based on atrazine binding or 325-nm absorption change with ferricyanide present, RCI/RCl ratios much greater than two are found. However, based on photosystem II measurements made without ferricyanide present, RCI/RCl ratios closer to unity are observed. Since electron flow in photosynthesis originates from the dehydrogenation of water, the RCI/RCl stoichiometries based on oxygen evolution are the most meaningful for understanding photosynthetic efficiency. The RCI/RCl stoichiometries based on atrazine binding or 325-nm absorption changes in the presence of ferricyanide give additional information about photosystem II structure, not about the relationship between photosystem I and II.

The important question now is what significance these stoichiometries have for plant function. One would expect with RCI/RCl near 2 that maximum oxygen yield per flash would only occur with supplemental photosystem I light, which would keep the plastoquinone pool oxidized. This was found not to be the case (4). It may be that RCI/RCl stoichiometry is not important for overall photosynthetic capacity, but instead other rate limitations are paramount. For instance, RCI/RCl stoichiometry has been reported to be altered by light intensity during growth (11). We find the P700 concentration to be sensitive to light intensity during growth. However, the limiting factors for photosynthetic capacity in cells grown under low light intensities are found to be the redox carriers of the electron transport chain (54). Clearly, the number of reaction centers and their stoichiometries vary with growth conditions. The importance this may have in photosynthetic capacity of the plant will be appreciated only with further research.

ACKNOWLEDGMENTS

The authors thank Dr. Alan Stemler for his reading of this manuscript and helpful discussions of this research. We also thank Dr. Homer LeBaron, CIBA-Geigy Corporation (Greensboro, N. C.), for the radioactively labeled atrazine used in this study; and the Ferry-Morse seed company (Orchard Park, N. Y.), for the Progress No. 9 peas.

REFERENCES