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Fluorescence Properties of Wild-Type Chlamydomonas reinhardi and Three Mutant Strains Having Impaired Photosynthesis

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Abstract. **The wild-type strain of *Chlamydomonas reinhardi* and 3 mutant strains *ac-21*, *ac-141*, and *ac-115* have been compared for their fluorescence (and luminescence) properties. The different fluorescence levels, the rapid and slow photochemical responses affecting fluorescence, and the intensity of luminescence have been studied under various conditions: air, nitrogen, 3(*p*-chlorophenyl)-1,1-dimethylurea. The strain *ac-21* exhibits fluorescence properties only quantitatively different from those of the wild-type strain, and it is believed to be affected in some component of the electron transport chain between the 2 light reactions. Both *ac-141* and *ac-115* have an abnormally high initial fluorescence level; *ac-115* does not show the normal photochemical response associated with System II and has a very low luminescence. Mutant strains *ac-141* and *ac-115* both seem to be modified in the System II photochemical center. These conclusions are compared with a previous analysis based on absorbance changes of cytochrome 559.**

The fluorescence properties of chlorophyll *a in vivo* and its variations during the induction period are believed to be closely associated with the operation of System II, one of the 2 photochemical systems in green plant photosynthesis (7). However, the relationship of these properties to the photosynthetic electron transport chain is not fully understood.

One method to study this relationship is to utilize certain mutant strains of the unicellular green alga *Chlamydomonas reinhardi* that are known to be unable to carry out normal photosynthesis as a consequence of mutations that affect the photosynthetic electron transport system (15, 16, 19). In the present report, we describe the fluorescence of the wild-type and of 3 different mutant strains having impaired photosynthesis. The results obtained with the 3 mutant strains are interpreted in terms of what is known regarding the manner in which their photosynthetic electron transport systems have been altered.

The fluorescence properties of *Chlorella* have been studied in some detail (12), and several general statements can be made. A low or minimum (m) initial level of fluorescence yield is characteristic of dark adapted cells. This state of minimum fluorescence yield is seen at the onset of illumination and will be called the O state. A light-induced maximum (M) of fluorescence yield can be obtained within about 0.1 second of actinic illumination (about 10^5 ergs sec^{-1} cm^{-2} at 480 nm). The fluorescence yield obtained at this level characterizes what will be called the P state. The fluorescence level of the O relative to that of the P state can be measured by the ratio m/M; a value of the order of 0.3 is typical for *Chlorella*. The burst of fluorescence (transition from the O to the P state) at the onset of illumination, the so-called Kautsky effect, is a well known general feature of the induction period, and there is a striking correlation between this burst of fluorescence and the transients detected in the rate of oxygen evolution (5) and carbon dioxide uptake (17).

The principal properties of the transition from the O to the P state are the following: 1) Under moderate light intensities (*e.g.*, 10^3 ergs sec^{-1} cm^{-2} red light), there is a strict complementarity between the fluorescence yield and the rate of oxygen evolution except for the initial "activation" phase (5).

2) Under high light intensities (*e.g.*, 10^5 ergs sec^{-1} cm^{-2} red light), the transition from the O to the P state appears to be a purely photochemical process, for the extent of the transition is temperature insensitive and depends only on the amount of absorbed light (6). The action spectrum for this transition is characteristic of System II (12).

3) Concomitant with the photochemical process described above, there is a rapid thermal process that tends to decrease the fluorescence yield. This is the P to O transition (13). The acceleration of the O to P transition that has been detected in the presence of inhibitors of oxygen evolution such as CMU [3 (*p*-chlorophenyl)-1,1-dimethylurea] is most simply explained as an inhibition of the P to O transition (14).

[1] A portion of this work was done in Gif-sur-Yvette in 1964 under the tenure of a National Science Foundation Senior Postdoctoral Fellowship.

A simple kinetic model embodying assumptions made by several authors can be proposed to explain these properties. The basic assumption of this model is that put forward by Duysens and Sweers (7). The fluorescence yield is controlled by the redox state of the system Q^-/Q, the primary electron acceptor of System II, Q being associated with a low yield and Q^- with a high yield. In the O state, the quencher Q is fully oxidized, whereas it is fully reduced in the P state. The various properties described above are qualitatively accounted for by the following set of equations:

$$Q + e \rightarrow Q^- \quad (I)$$

$$Q^- + A \rightleftharpoons Q + A^- \quad (II)$$

$$A^- \rightarrow A + e \quad (III)$$

Equation I describes the System II photoreaction; e is the electron ultimately coming from water, and it is assumed that the supply of e is never limiting. The symbol A (or A^-) in equation II and equation III stands for a kinetic entity that may embody a number of known (plastoquinones, cytochromes, plastocyanin) or unknown electron transport components. From Joliot's work on transients of oxygen evolution (11), there appear to be about 10 equivalents of A per equivalent of Q. In equation III it is understood that e is conveyed to the terminal acceptor of the photosynthetic electron transport chain through photoreaction I.

Under moderate light intensity, the equilibrium in equation II, which in the dark lies on the oxidation side, is progressively shifted towards reduction because of the production of Q^- by photoreaction II (equation I). Although the final steady state depends on the relative rates of the "reducing" photoreaction II and the "oxidizing" photoreaction I, there seem to be some limiting steps in equation III so that the light induced maximum M lies slightly under the level corresponding to the P state. Only under high light intensity, or equivalent conditions such as low temperature or presence of inhibitors, may one see a rapid, accumulation of Q^- almost unperturbed by the reoxidation by A (equation II); then, the level M truly corresponds to the P state. In all cases, however, the initial positive transient is accompanied with a more or less complete shift of the system Q^-/Q and possibly A^-/A towards reduction and it will be called the O to P transition. Obviously, the reoxidation of Q^- in equation II, which can be monitored under special conditions (see Methods), must be identified with the opposite transition, that is the P to O transition, and the effect of CMU must be explained as a specific inhibition of this reaction.

It has been proposed (14) that the changes in the system Q^-/Q might be related to the so-called "delayed" fluorescence or luminescence. The redox state of the quencher Q should determine the luminescence intensity in 2 ways: 1) as a yield factor, since the redox state of Q controls the fluorescence expression of any quantum of electronic excitation circulating within the photosynthetic unit regardless of whether it has been produced by an electronic transition or by any other process; 2) as a reaction rate factor, for the luminescence quantum could be generated during the reoxidation of Q^-/Q merely by reversal of photoreaction II (equation I) or by any other sufficiently energetic side reaction.

Hence, it is expected that some connection exists between the P to O transition and luminescence.

It is well known that under constant light intensity the fluorescence burst is followed by a complex decay to a steady state S, the level of which is usually closer to that of the O than to that of the P state. The P to S transition is not well understood, and it will not be considered in the work reported here except under the special circumstances of preillumination to be described below.

Materials and Methods

The wild-type strain, 137c, of *Chlamydomonas reinhardi*, and 3 mutant strains, *ac-21*, *ac-115*, and *ac-141*, were used in the experiments described here. The wild-type and mutant strains were grown in 300 ml cultures of high salt medium (20) supplemented with 0.2 % sodium acetate. The light intensity from fluorescent lamps was 4000 lux, and the temperature was 25°. Cells were harvested from cultures in the logarithmic phase of growth. They were washed once and resuspended in minimal medium. Chlorophyll concentration, determined by a modification (1) of the method of Mackinney (18), was about 20 μg/ml in all the experiments.

Fluorescence was studied with a flow method described in detail elsewhere (13). About 300 ml of cell suspension was maintained in a thermostated reservoir at 20°. The cell suspension was circulated intermittently (*e.g.*, 1 sec flow, 1 sec rest), or continuously from the reservoir to a capillary where fluorescence was analyzed, and then back to the reservoir. Cells in the capillary passed through an analytic beam of 480 nm exciting the fluorescence which routinely was observed at 690 ± 15 nm with a Dumont 6911 photomultiplier. This method is tantamount to withdrawing (with replacement) small samples from the reservoir and analyzing them for the different fluorescence levels:

1) For locating the fluorescence levels in the O and P states, one simply uses the intermittent circulation and records the minimum m level during the flow phase and the maximum M level induced by the analytic beam during the rest phase. It was stated above that M characterizes the P state only under high enough intensity. Similarly, a simple calculation (13) shows that m is always in excess of the O level, unless the amount of analytic light seen by the cells is very low. Extrapolation procedures allow a precise measurement of the fluorescence levels in the O and P states. However, a compromise can be practically found whereby m and M give reasonably good approximation of these levels.

2) It is also possible to adapt the cells to light by means of an incandescent bulb immersed in the reservoir, either operated at full power (30 w) giving "high white light" or at half power or less giving "low red light". Such preillumination induces changes in the m and M levels that are analyzed as in 1) after a delay of 3 seconds. The envelopes of the successive values of M and m are the time curves of the long induction changes produced by preillumination in the reservoir. The M curve is very similar to that which one would obtain in an ordinary induction experiment, but the present procedure gives additional information: the changes in M are easily followed after the end of preillumination and the m curve shows how preillumination affects the end of the P to O transition (after about 3 sec darkness). Platinum and reference *Ag/Ag* Cl electrodes inserted in the flow unit monitor the changes in oxygen concentration.

3) For analyzing the P to O transition properly, the observation of fluorescence must involve much shorter time intervals than with the preillumination experiment. The latter gave induction curves, whereas the former will give decay curves when continuous circulation is used, for the cells see a strong white actinic beam of about 10^6 ergs sec^{-1} during 30 msec, which produces a light stimulated level, $m + \Delta m$. This is analyzed along the capillary from 8 to 250 msec after the end of the flash and compared with the level in the absence of actinic beam (m). The time curve of the ratio $\Delta m/m$ serves as a measure of the spontaneous decay from the P to the O state.

4) Comparison of the luminescence, *Lu*, with the light stimulated fluorescence, $m + \Delta m$, is obtained with an operation similar to 3), the analytic beam being turned off when luminescence is recorded. Both effects are monitored in succession under exactly the same conditions, that is 30 msec of actinic illumination, 8 msec of darkness and 30 msec of observation.

For the effect of anaerobiosis on fluorescence, nitrogen was bubbled into the reservoir. The effect of CMU was studied after introducing the inhibitor into the reservoir.

Results

The O *to* P *Transition.* The fluorescence responses of the wild-type and the mutant strains are given in table I. It can be seen (line no. 1) that the O state is lowest in case of the wild-type strain. The response of *ac-21* is similar, but the relative position of the O state can be somewhat higher. However, both *ac-115* and *ac-141* have a strikingly different behavior. Both mutant strains have an exceptionally high value for the O state; in the case of *ac-115*, the O to P transition is almost absent, and in some instances it has even been seen to reverse (M slightly lower than m). The value of m exceeds

Table I. *Fluorescence and Luminescence Characteristics of* Chlamydomonas *Strains*

Line No 1. m is the fluorescence level in the initial O state, M that in the maximum P state; measurements are made from separate cultures. Line No 2. Calculated from data of Smillie and Levine (19); notice that the variation of the figures is opposite to that of m/M (line No 1). Lines No 3 to 6. $R\phi_1$ and $R\phi_2$ are measures (see text) respectively of the fast component and the slow component of the decay of light stimulated fluorescence (P to O transition). The 2 components may be seen in figure 1. Notice the absence of effect of CMU on the slow component (lines No 5 and 6). Lines No 7 to 10. $\Delta m/m$ is a measure of the light-stimulated fluorescence (see text); the luminescence intensity (Lu) is given in arbitrary units, however, figures in different columns can be directly compared. The 2 quantities are measured simultaneously on the same samples. Notice the absence of light stimulated fluorescence and of luminescence in *ac-115*. Line No 11. Fluorescence intensities measured with continuous flow at indicated wavelengths ± 15 nm, under excitation at 480 nm.

	Line No	Wild-type	*ac-21*	*ac-141*	*ac-115*
Position of O state					
m/M	1	0.37; 0.35; 0.31	0.39; 0.48; 0.28	0.82; 0.85; 0.87	0.97; 0.96; 1.04
Plastoquinone to chlorophyll ratio (mole per mole)	2	0.067	0.033	0.0135	0.010
P to O transition					
$R\phi_1$ control	3	2.03	1.62	...	0
$R\phi_1$ 10^{-5} M CMP	4	1.41	1.41	...	...
$R\phi_2$ control	5	1.22	1.20	...	0
$R\phi_2$ 10^{-5} M CMU	6	1.23	1.21	...	...
Fluorescence and luminescence					
$\Delta m/m$ control	7	0.22	0.14	...	0.007
$\Delta m/m$ 10^{-5} M CMU	8	0.34	0.24	...	...
Lu control	9	51	28	...	9
Lu 10^{-5} M CMU	10	83	39	...	...
Amplitude of the 720 nm component relative to the 685 nm component in the O state.					
m (720)/m (685)	11	0.31	0.28	0.27	0.26

95 % of the total response with *ac-115*. One of us (J. L.) has suggested the use of such fluorescence anomalies as a test for screening mutants deficient in the System II apparatus (8, see also ref 2).

The P to O Transition. When seen under our experimental conditions, that is after a rather long actinic flash (30 msec) and an observation time of up to 250 msec, the decay of the photo-induced fluorescence level is biphasic. A convenient, although admittedly arbitrary, way to quantitate this decay is to use the ratios:

$$\text{fast component, } R\,\phi_1 = \frac{\triangle m \text{ at } 8.4 \text{ msec}}{\triangle m \text{ at } 88.5 \text{ msec}}$$

$$\text{slow component, } R\,\phi_2 = \frac{\triangle m \text{ at } 88.5 \text{ msec}}{\triangle m \text{ at } 168 \text{ msec}}$$

where the times indicated are after the end of the flash.

CMU and other System II inhibitors have been shown to affect only the fast component (13).

Figure 1 and table I show that the fast component is somewhat slower in the case of *ac-21* than in the case of the wild-type strain and that the effect of CMU is correspondingly smaller. On comparing the $R\phi_1$'s an inhibition of 13 % is computed for *ac-21* and 30.4 % for the wild-type strain.

Luminescence. Under our experimental conditions, there appears to be some definite connection between luminescence and the light-stimulated fluorescence as well as its rate of decay. In table I, from the vertical and horizontal comparisons of lines no. 7 and 9 for the control and lines no. 8 and 10 with CMU, it is seen that the intensity of luminescence *Lu* parallels the light-stimulated fluorescence as measured by $\triangle m/m$. Both effects decrease in the order wild type-*ac-21*-*ac-115*. From the horizontal comparison of lines no. 3 and 9, we also note that the intensity of luminescence is directly related to the rate of decay of the fast component of light-stimulated fluorescence as measured by $R\phi_1$; but the vertical correlation is opposite since CMU decreases $R\phi_1$ (lines no. 3 and 4) whereas it increases *Lu* (lines no. 9 and 10). The general relationship between luminescence and light-stimulated fluorescence is best demonstrated in the case of strain *ac-115* where both quantities are nil or almost so. Strain *ac-141* has not been analyzed.

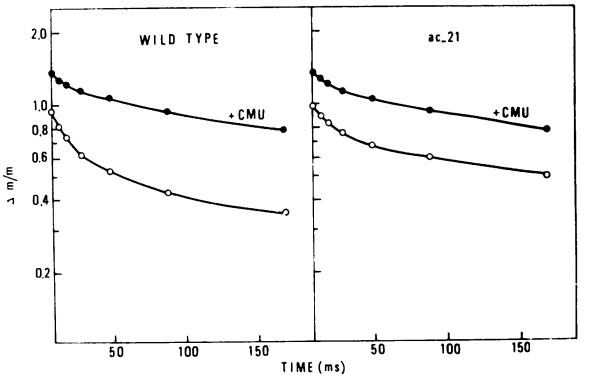

FIG. 1. The *P* to *O* transition with wild type and *ac-21*. The cell suspension is continuously flowing; it sees a white light flash of 30 msec duration and the fluorescence level $m + \Delta m$ is measured at various distances down stream along the capillary, that is at various times after the flash as indicated on the abscissa; or, as a control, the level *m* is measured in absence of a flash. The end of the flash is 8 msec before the zero of the time scale. The biphasic decay is clearly seen on the semi-log plot. Open circles: normal cells; dark circles: 10^{-5} M CMU.

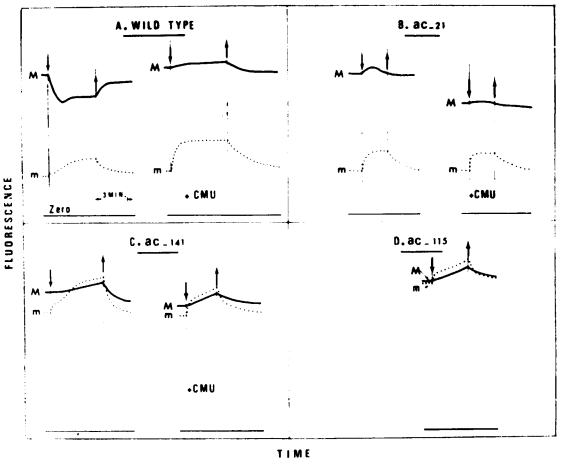

FIG. 2. Long induction effects on *m* and *M* induced by preillumination in wild-type and mutant strains. The *M* and *m* lines are envelopes of recorded oscillations of fluorescence during intermittent flow with a 2 sec recurrence period (see text). For each strain (except *ac-115*), the left drawing is the induction of control, the right is that following administration of 10^{-5} M CMU. Downward arrow: high white light preillumination; upward arrow: preillumination off.

The Effect of Preillumination. When a suspension of wild-type cells is preilluminated with white light, characteristic changes are produced in the *O* and *P* states which seen on the recording as displacements of the *m* and *M* lines (fig 2). Addition of CMU to the cell suspension results in a substantial increase in the level of the *O* state.

In the case of *ac-21*, preillumination in the absence or in the presence of CMU (fig 2B) gives results similar to those obtained with the wild type in the presence of CMU (fig 2A).

Both *ac-115* and *ac-141* exhibit differences compared to both wild type and *ac-21* with respect to the effect of preillumination (fig 2, C and D). The *m* line rises much more than does the *M* line, and the *m* line soon crosses over that of *M*. Thus, there is again an effect of inverted induction. In the presence of CMU, the extent of the inversion is slightly more pronounced, and the decay of *m* at the end of preillumination is slower. These results may be compared to those reported by Butler and Bishop (4).

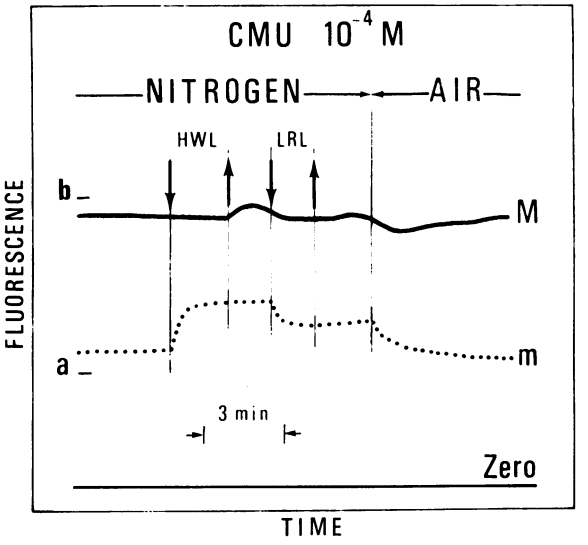

FIG. 3. Long induction effects on *m* and *M* during preillumination in the wild-type strain under nitrogen and 10^{-4} M CMU. See figure 2 for details. a and b stand for *m* and *M* respectively prior to bubbling nitrogen. HWL: high white light; LRL; low red light (see text).

The Effect of Nitrogen. When nitrogen was bubbled into a suspension of wild-type cells or cells of *ac-21*, changes resulted in the relative positions of the *m* and *M* levels. The former decreased whereas the latter increased in magnitude. The effects of nitrogen, CMU, and preillumination on wild-type cells are shown in figure 3. Preillumination with strong white light produces a large increase in *m* level which does not disappear at the end of preillumination. Then, with low intensity red light preillumination, exciting principally System I, the value of *m* decreases somewhat, but once again, after preillumination, the value of *m* does not decline. Only upon the admission of air does the *m* level decrease to its original value. When this experiment is performed with cells of *ac-21*, essentially identical results are obtained. When cells of *ac-141* are placed under nitrogen, both the *M* and *m* levels slowly slope downward. The decay of the *m* level at the end of preillumination is also slowed down under nitrogen.

Discussion

The simplest relationship that can be postulated from our results concerns the relative position of the *O* state as compared to the plastoquinone to chlorophyll ratio that has been obtained in the wild-type and mutant strains (19). From table I, lines no. 1 and 2, the relative position of the *O* state can be compared to the plastoquinone to chlorophyll ratio. A distinct correlation appears: the *O* state is higher when the plastoquinone to chlorophyll ratio is lower. This correlation finds a possible explanation if we assume that, within the plastoquinone fraction, there is 1 special component that can be identified with the quencher *Q* and that the change of the plastoquinone to chlorophyll ratio more or less reflects the change of the *Q* to chlorophyll ratio. As the latter ratio presumably determines strictly the position of the *O* state, the observed relationship is easily understandable. This would assign all of the defects of a given mutant to the System II chlorophyll complex. However, there are alternative explanations. First, we note that throughout the series of strains the position of the *P* state on a chlorophyll basis does not exhibit any large systematic variation and that similarly the chlorophyll content per cell does not vary significantly. Second, it has been shown that the fluorescence emission spectrum in the *O* state has a small component with an emission maximum at 720 nm and that this constant component is somehow associated with the System I sensitizer, whereas the main component with a maximum of 685 nm is emitted by the System II sensitizer and is also responsible for the variable part of the fluorescence emission (12). On comparing line no. 11 with line no. 1 in table I it is seen that the ratio of fluorescence levels at 720 nm and 685 nm in the *O* state decreases significantly throughout the series wild type, *ac-21*, *ac-141*, *ac-115* as if the fluorescence coming from a fixed 720 component was more and more "diluted" by the 685 main component. All this speaks in favor of some constancy of the chlorophyll apparatus throughout the series we have investigated. Thus, the possibility must be kept in mind that the chlorophyll complex, its quantity and composition, is not changed but that, for some structural reason, only the dark redox level of *Q* is different from one strain to the other. However, this is very unlikely because *Q* in normal dark adapted cells in aerated suspensions has always been found to accumulate in the fully oxidized form. Also it must be stressed that a mutation can suppress some specific response not because the component responsible for it is missing but because the component is rendered inactive following some structural changes (21) in the chloroplast. Summarizing the argument, we may trace back the effects of mutation to a change in the quantity or redox state of *Q* or to a change in its physio-chemical environment that affects its activity.

Of the 3 mutant strains, the case of *ac-21* seems the simplest. It only differs quantitatively from the wild-type strain; the fast component of the *P* to *O* transition is slower and less inhibited by CMU, and during preillumination, the *m* line rises more, indicating a larger accumulation of Q^-. All this can be explained by a slowing down of the electron transport chain, perhaps at the site of the reaction between *Q* and *A* (equation II), provided some leakage of electrons towards System I is permitted (15). The evidence from luminescence is less conclusive since one would expect the accumu-

lation of Q^- to favor the reversal of the photoreaction II and hence luminescence, which is the opposite of what is observed. One may think that some secondary effect of mutation tends to decrease the reactivity of Q^-.

From our fluorescence results, it is tempting to propose that both *ac-141* and *ac-115* are defective in the System II chlorophyll complex and more specifically, at least for *ac-115*, that Q is either missing or inactive. With this assumption, the high O level and the absence of P to O transition are self explanatory. If we follow the rule postulated earlier for luminescence which states that the quencher must be in reduced form Q^- and that it must be engaged in an energetic oxidizing reaction, the absence of luminescence can also be easily understood. In any case, it may be significant that the anomaly near or within the System II chlorophyll complex that we assume for *ac-141* and *ac-115* results in the absence of luminescence. In this respect, it is interesting to note that a mutant strain of *Scenedesmus* reported by Bertsch *et al.* (3) shows no photoreaction II and only a very weak luminescence.

It is very difficult to draw any conclusion from the preillumination experiments in which both *ac-115* and *ac-141* show a fluorescence yield that rises slowly above the P state and a limited extent of inverted induction. Too little is understood of the effect of preillumination even in the normal case. We wish to point out, however, that preillumination conditions are such as to allow for the appearance of slow interactions between System I and System II. This is exemplified in figure 3 where the redox state of Q^-, as indicated by the m level, is clearly subjected to the antagonistic action of the 2 photoreactions (and also controlled by external conditions, such as air or nitrogen). It has been demonstrated in 1 case (hydrogen adapted *Scenedesmus*) that the inverted induction was due to the photoreaction I (see ref 20). It may be that, in the present instance also, the observed effects arise from the fact that System I is still operative.

We have attempted to interpret the fluorescence properties of *ac-115* and *ac-141* on the basis of an absent or inactive Q. This interpretation, however, must be considered in terms of the evidence (15) showing that in these mutant strains cytochrome 559 is either missing or inactive. Clearly, Q cannot be identified with cytochrome 559, for CMU inhibits the photoreduction of the cytochrome but not of Q. Both *ac-115* and *ac-141* are single gene mutations, and they are unlinked (10). It is possible that each locus plays a role in the formation or activity of both cytochrome 559 and Q. It is also possible that these loci have only 1 function, the formation of cytochrome 559 or Q, and that when the capacity to form 1 component is lost as a consequence of mutation some feed-back mechanism prevents the formation of the other component.

The genetic control of the formation of components lying in the portion of the photosynthetic electron transport chain close to System II may be complex, for there are at least 2 additional mutant strains in which cytochrome 559 is affected (E. Cosbey and R. P. Levine, unpublished observations). There is no genetic linkage between any of the mutant strains in which cytochrome 559 is affected, and each gene locus should be considered as being functionally distinct. Until these distinctions become understood little can be said regarding the genetic relationship between cytochrome 559 and Q, but at least for the 2 mutant strains described here the loss or inactivity of 1 component seems to be correlated with loss or inactivity of the other.

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