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NEW RESULTS ON THE MODE OF ACTION OF 3,-(3,4-DICHLOROPHENYL)-1,1-DIMETHYLUREA IN SPINACH CHLOROPLASTS

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SUMMARY

Simultaneous measurements of hydroxylamine photo-oxidation and fluorescence induction were performed in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The results provide a justification for the common use of fluorescence data to estimate the concentration of active System II centers in the presence of inhibitors.

The addition of DCMU to dark-adapted chloroplasts under special conditions induces a large increase of the initial yield of fluorescence. A reversible inactivation of part of the System II centers is responsible for this effect. Similar data were obtained with other classical inhibitors of oxygen evolution.

INTRODUCTION

According to Duysens and Sweers[1], the electron acceptor Q of Photoreaction II acts as a quencher of chlorophyll fluorescence in the oxidized state. Q is reduced in the light by System II and reoxidized indirectly by System I. This reoxidation is inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The fluorescence yield is not a linear measurement of the ratio $[Q^-]/[Q]_{total}$ (refs 2 and 3). However, a linear relationship does exist, in the absence of inhibitors, between the photochemical rate measured by oxygen evolution and the variable part of fluorescence[4]. Therefore, as noticed by Forbush and Kok[5], the area over the rise curve of fluorescence in isolated chloroplasts, which is a product of photochemical rate and time, represents the pool size of electron acceptors of System II (non-photochemical quenching is assumed to be constant).

The fluorescence rise curve in the presence of DCMU follows the progressive reduction of Q in the light and is not modified by hydroxylamine[6]. Oxygen evolution is inhibited by DCMU, but a transitory photooxidation of hydroxylamine by System II centers can be observed in the presence of this inhibitor. This result confirms that DCMU is acting on the reducing side of System II[6,7].

Whenever the area over the fluorescence rise curve in the presence of DCMU has been taken as a measurement of the concentration of active System II centers,

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea.

linearity between photochemical rate and variable fluorescence has been assumed to exist. However, this assumption is not supported by any experimental data. Simultaneous measurements of hydroxylamine photooxidation and fluorescence induction were performed in the presence of DCMU in order to clarify this problem.

During this study, it appeared that, under special conditions, addition of DCMU to dark adapted chloroplasts led to a large increase of the initial fluorescence yield and a parallel decrease of the initial rate of hydroxylamine photooxidation. Previously, Joliot[8] noticed that 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU), added to dark adapted algae, slightly increased the initial fluorescente yield (f_0). Delosme assumed that DCMU prevents the action of the non-photochemical quencher *R* (ref. 3) In the present case, the DCMU-induced increase of f_0 corresponds to the inactivation of part of the System II centers into a non-quenching form.

MATERIALS AND METHODS

Spinach chloroplasts were prepared according to the procedure of Avron[9]. Isolated chloroplasts were resuspended in 0.05 M Tris–HCl buffer, pH 7.5, with 0.4 M sucrose and 0.5 g/l serum albumin.

Fluorescence measurements in continuous light were performed with the apparatus described previously[7].

Simultaneous measurements of polarographic current and fluorescence emission were performed under modulated light with the electrode described by Joliot *et al.*[10,11]. A Radiotechnique XP 1002 photomultiplier was set up 8 cm above the platinum electrode and fitted with red filters (Rubilyth Amberlith Ulano+

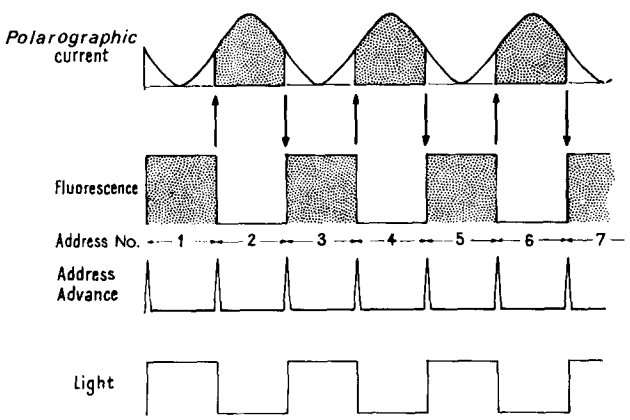

Fig. 1. Input of polarographic and fluorescence signals to signal digitizer. The fluorescence signal is in phase with the light because the frequency of modulation is low (25 cycles/s). The phase of the amperometric current is dependent on the frequency of the light and other parameters (see Joliot *et al.*[10]). In our experimental conditions, the phase shift between light and polarographic current due to the photooxidized hydroxylamine derivative is approximately 180°. The frequency of the pulses triggering the address advantage is fixed at twice the frequency of the light. The phase of this signal is adjusted so that each pulse corresponds to a dark to light or a light to dark transition. At the onset of the illumination, the first light pulse accounts for the multiplier signal, the second one for the polarographic signal and so on. Such a process achieves the demodulation of both signals.

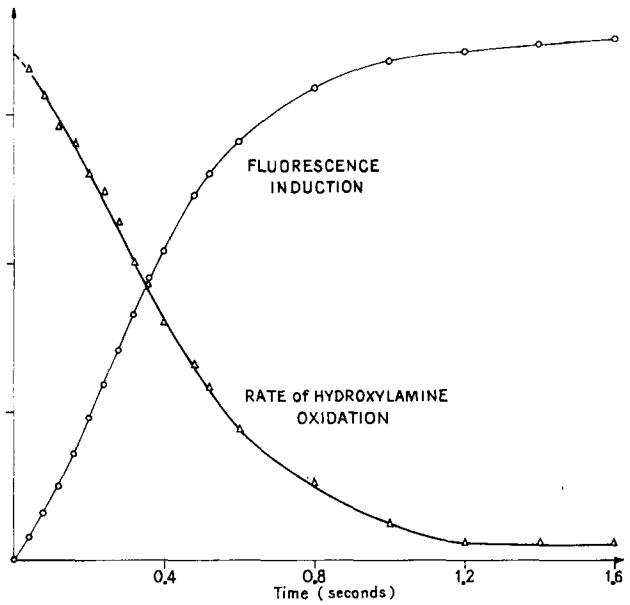

Fig. 2. Simultaneous measurements of the rate of hydroxylamine oxidation (△—△) and fluorescence rise (○ — ○) with spinach chloroplasts (relative units). Chloroplasts incubated in the dark with 10 mM hydroxylamine for 10 min. 10 μM DCMU is added 2 min before the experiment. Frequency of modulation of the light, 25 cycles/s. Temperature, 20 °C.

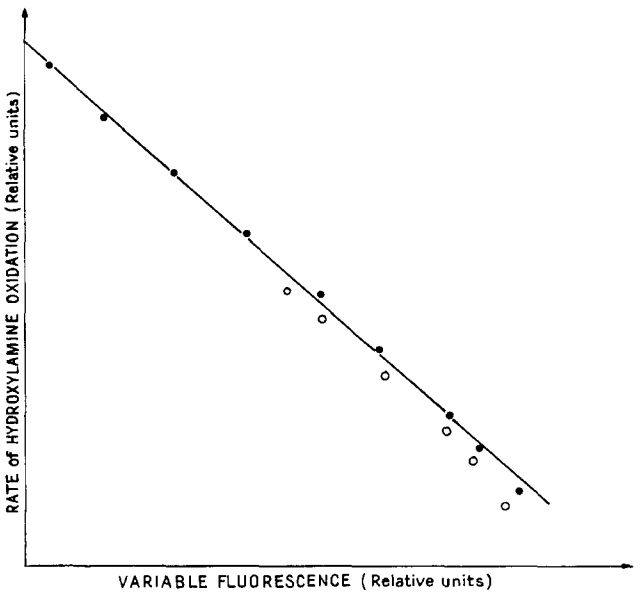

Fig. 3. Rate of hydroxylamine oxidation *versus* variable fluorescence. Black circles represent data from Fig. 2. Open circles are values from Fig. 4 (solid lines).

Kodak Wratten 70) transmitting light beyond 650 nm. The modulated beam was filtered through two blue filters (Schott BG 38 10 mm) and one yellow filter (Schott GG 14 3 mm) selecting wavelengths from 500 to 600 nm. Because there is a minimum absorption of light in this wavelength range, uniform illumination of the sample on the electrode was assured. Both photomultiplier and polarographic signals were applied after appropriate adaptation to a signal digitizer CAT 400 C set in multiplexing mode. The principle of the measurement is described in Fig. 1.

RESULTS

Fig. 2 shows typical results of simultaneous measurements of fluorescence and hydroxylamine oxidation in the presence of DCMU. A linear relationship between variable fluorescence and photochemical rate is observed (Fig. 3). There-

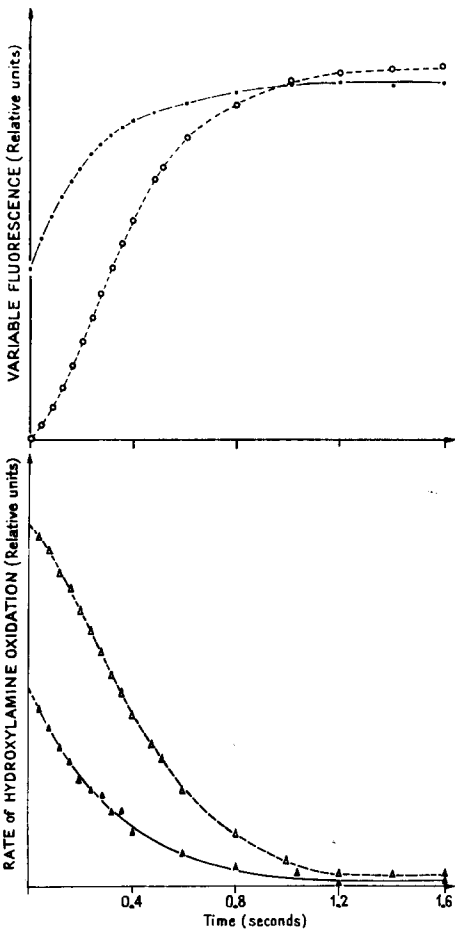

Fig. 4. Simultaneous measurements of the rate of hydroxylamine oxidation and fluorescence rise with spinach chloroplasts. Frequency of modulation of the light, 25 cycles/s. Temperature, 20 °C. △, ○, same conditions as Fig. 2; ▲, ●, chloroplasts incubated in the dark with 10 mM hydroxylamine for 5 min, then illuminated for 30 s with white light (3000 lux) and incubated for another 5 min in the dark. 10 μM DCMU is then added 2 min before the experiment starts.

fore, the measurement of the concentration of active System II centers by the area over the fluorescence rise curve is justified.

In the first experiment, chloroplasts were first incubated in the dark with hydroxylamine and then DCMU was added in the dark prior to the measurement. It appears that, when hydroxylamine-treated chloroplasts are exposed to strong white light and further incubated in the dark, the addition of DCMU induces a large increase of the initial fluorescence yield together with a decrease of the initial rate of hydroxylamine oxidation. From the experiment depicted in Fig. 4, it can be computed both by the area over the fluorescence curve or below the hydroxylamine oxidation curve that 60% of the centers have been inactivated by this treatment. An important increase of f_0 was still observed when DCMU was added as long as 40 min after the preillumination.

However, if no DCMU is added, the initial fluorescence yield goes back to the normal f_0 value within 5 min following the preillumination and the fluorescence rise is not modified. No increase of f_0 could be observed when the preillumination was given in the absence of hydroxylamine. The phenomenon described here is not DCMU specific: similar data were obtained with phenylurethane, dinitrophenol and orthophenanthroline. However, the largest increase of f_0 was induced by DCMU.

In order to test the reversibility of this effect, *o*-phenanthroline was used since the inhibition due to this compound can be reversed by bivalent cations such as Zn^{2+}(ref. 12). The effects of *o*-phenanthroline are depicted in Fig. 5. For non-preilluminated chloroplasts incubated with hydroxylamine, a fast fluorescence rise

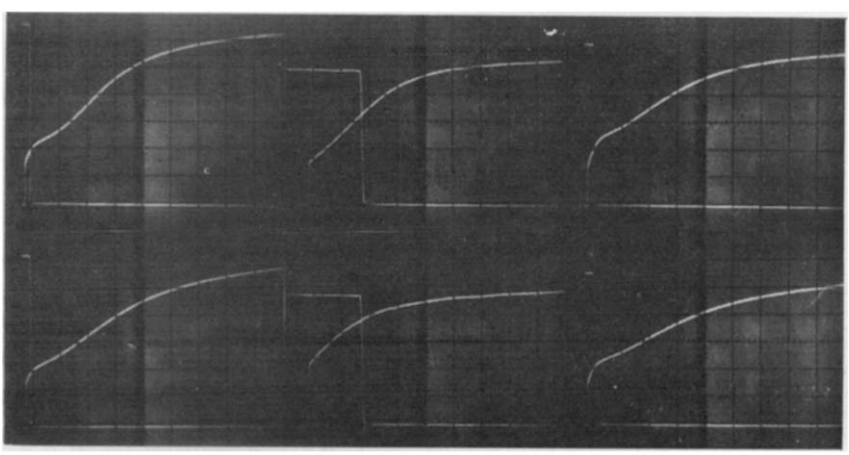

Fig. 5. Fluorescence induction under continuous light. Spinach chloroplasts, 20 °C. Top left: chloroplasts incubated with 10 mM hydroxylamine for 10 min in the dark; 100 ms/division. Top middle: same conditions as top left + 0.2 mM *o*-phenanthroline added 1 min before the experiment; 10 ms/division. Top right: same conditions as top middle *plus* 0.2 mM $ZnSO_4$ added 1 min before the experiment; 100 ms/division. Bottom left: chloroplasts incubated with 10 mM hydroxylamine for 5 min in the dark, then illuminated for 30 s in white light (5000 lux) and incubated in the dark for another 5 min; 100 ms/division. Bottom middle: same conditions as bottom left *plus* 0.2 mM *o*-phenanthroline added 1 min before the experiment; 10 ms/division. Bottom right: same conditions as bottom middle *plus* 0.2 mM $ZnSO_4$ added 1 min before the experiment; 100 ms/division.

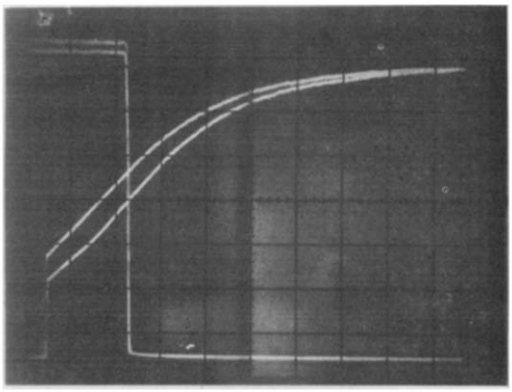

Fig. 6. Fluorescence induction under continuous light with spinach chloroplasts at 20 °C. DCMU is added to dark-adapted chloroplasts 3 min before the experiment. Lower curve, 4 μM DCMU; Upper curve, 250 μM DCMU. The same ethanol concentration (5%) was used for both experiments. Stationary levels of fluorescence are figured in the upper left corner of the picture. The higher level is obtained for the lower DCMU concentration (10 ms/division).

is observed in the presence of *o*-phenanthroline corresponding to the inhibition of Q^- reoxidation through the pool of electron acceptors. The addition of Zn^{2+} reverses this inhibition. For preilluminated chloroplasts, a fast fluorescence rise is also observed in the presence of *o*-phenanthroline, but starting from a high initial value compared to that obtained without preillumination. This high value is no longer observed after the addition of Zn^{2+}. The above effects of *o*-phenanthroline are then reversible and similar to those induced by DCMU.

Fig. 6 shows the effect of very high DCMU concentrations (250 μM). It appears that in the absence of any pretreatment a high concentration of DCMU induces a large increase of f_0 compared to the level observed for the usual concentration (4 μM). This increase of f_0 is associated with a decrease of the area over the fluorescence rise curve. Therefore DCMU alone is inactivating part of the System II centers into a non-quenching form.

DISCUSSION

It can be seen in Fig. 4 that the preillumination does not increase the half time of either the fluorescence rise or the hydroxylamine oxidation curve. The same observation holds for the fluorescence rise curves in the presence of *o*-phenanthroline (Fig. 5). Also, the addition of a very high concentration of DCMU (Fig. 6) does not increase the half time of the fluorescence rise. As shown in Fig. 3, the relationship between rate and fluorescence remains the same after the preillumination treatment: the same slope is observed.

Therefore, the observed increase of f_0 does not correspond to a decrease of the quenching efficiency of every center. Rather, this new mode of action of the tested inhibitors results in the transformation of a part of the active centers into a non-quenching form. This modification is reversible. The quenching efficiency of the remaining centers is unchanged.

The effect of high concentrations of DCMU described in Fig. 6, together

with the known fact that even moderate concentrations of DCMU increase f_0, leads us to believe that preillumination of hydroxylamine-treated chloroplasts simply magnifies an effect which appears to a lesser extent under normal conditions. The reversibility of the phenomenon also favors this view.

It is quite remarkable that various chemicals can simultaneously induce an inhibition of electron transfer between Q and the pool of oxidants and the inactivation of a part of System II centers into a non-quenching form. These two effects may corresponds to two different sites of action of the inhibitors. The loss of quenching capacity of the centers could either be due to a chemical modification induced by the inhibitor or a reversible disconnection of the quenchers from the centers. In the latter case, one could speculate that a reversible binding of the quenchers to the centers may be part of the current process of electron transfer.

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