KINETICS OF REACTIONS INVOLVING C-550 AND CYTOCHROME b_{559} IN CHLOROPLASTS AT LOW TEMPERATURE. EVIDENCE FOR TWO PHOTOREACTIONS.

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Summary : The kinetics of reduction of C-550 and of oxidation of cytochrome b_{559} are studied with spinach chloroplasts, at -170°, under light-limited conditions, at different light intensities. The rate of reduction of C-550 is proportional to the light intensity I; the rate of oxidation of b_{559} is 2-3 times slower and not proportional to I. We propose that two light reactions occur at the reaction center of Photosystem-II (RC-II) at low temperature.

The photoreduction of C-550 and the photooxidation of cyt b_{559} have been observed in photosynthetic materials at low temperature (1-5). Several arguments allow the identification of C-550 with the primary electron acceptor of Photosystem-II (6,7). The oxidation of cyt b_{559} is assumed to be a secondary dark reaction (5,8). We provided different arguments (9,10) for the location of C-550 and cyt b_{559} at RC-II : several properties of the photoreaction are affected by the state of the reaction center, as defined by Kok et al (11).

In this work, the kinetics of reduction of C-550 and of oxidation of cyt b_{559} are studied at -170°, under light-limited conditions, at different light intensities. In contrast to the results presented by Butler et al (12), we find that the reduction of C-550 is proportional to I. The behaviour of the oxydation of cytochrome b_{559} leads us to propose that at least two photoreactions occur in Photosystem II at low temperature.

MATERIAL AND METHODS.

Chloroplasts, prepared from spinach leaves (9), were resuspended in the grinding buffer (0.4 M sucrose; 0.02 M Tris, pH 7.8; 0.01 M NaCl) plus 5% dimethylsulfoxide. The suspension was distributed in 0.5 ml tubes and kept in liquid N_2.

Differential absorption spectra were recorded with a Perkin-Elmer spectrophotometer (9).

Abbreviation : cyt b 559 : cytochrome b_{559}

For measurements of kinetics of absorbance changes the chloroplasts were thawed and transferred to a cuvette (e = 1 mm) made of lucite windows inserted in a copper frame. The suspension was dark-adapted (2 min at 20°); then the cell was dipped into a liquid N_2 container for 60 s. The cell was then transferred to a partly unsilvered Dewar flask, containing liquid N_2. This procedure was accomplished in the dark. A thermocouple, inserted in the cuvette, indicated $-170^{\circ}(\pm 5^{\circ})$. The suspension was frozen in a microcrystallized state. The actinic and measuring beams, perpendicular to each other, were at 45° of the plane of the cuvette.

The actinic beam, provided by a 625-w tungstene-iodine lamp, was focused on the cuvette. On a parallel path of the beam were inserted : a 2-cm water filter, a Calflex (Balzers) filter, a Compur electronic shutter (opening time 10-90 : 8 ms), a Schott RG 610-3 mm filter, a MTO (630 nm) interference filter. The maximum intensity of the actinic beam at the position of the cuvette was 25 $mW.cm^{-2}$ (measured with a calibrated Eppley thermopile. It will be referred as I = 100; smaller values of I were obtained with Schott neutral filters.

The measuring light was provided by a 108-W tungsten lamp mounted on a Bausch and Lomb 500 mm monochromator (slits 0.5 mm). The beam crossed the sample and was received by a photomultiplier (EMI 9558 B) protected by a Schott BG 18-3 mm and two Wratten 64 filters. The PM output (load resistor : 500 KΩ;a variable capacitor of 10 nF to 1 μF was inserted to have the minimum required electrical bandwith) fed a Tektronix W differential amplifier whose V_c output, fixed at 2 volts, was used as comparison voltage. The amplified signal was measured with a Didac (Intertechnique) multichannel analyser.

The measuring beam was opened 1 min before measurement of the absorbance change; it had no detectable photochemical effect. In most experiments the signals corresponding to several successive cuvettes were stored and added in order to improve the S/N ratio.

RESULTS.

Comparison of the kinetics at different light intensities.

The effect of illumination of chloroplasts at - 170° is reported in Fig. 1a, as a differential absorption spectrum. The negative peak at 556 nm is due to the oxidation of cyt b_{559} and the S-shaped signal (+ at 542 nm, - at 546 nm) to the reduction of C-550.

The kinetics of reduction of C-550 and of oxidation of cytochrome b_{559} were studied at 542 and 556 nm respectively, in the range of excitation inten-

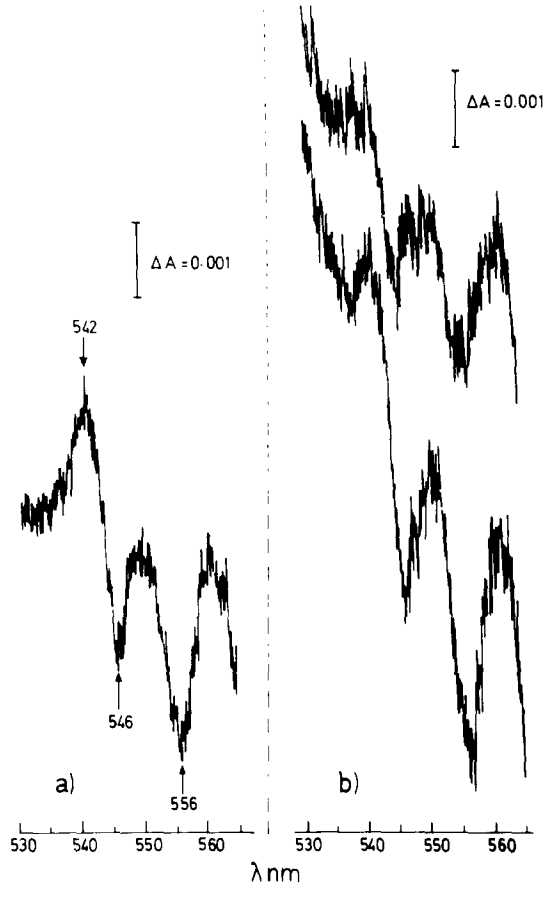

Figure 1. Difference spectra recorded after illumination of the sample cuvette at -196°
a - Chloroplast suspension used for the results of Fig. 2-4. Illumination by white light (I_s). Chlorophyll concentration : 202 $\mu g.ml^{-1}$
b - Illumination during 15 min by monochromatic light (611 nm) of very low intensity (top curve) and then during 1s by white light (bottom). Chlorophyll concentration : 250 $\mu g\ ml^{-1}$.

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sities from 25 $mW.cm^{-2}$ (I = 100) to 0.098 $mW.cm^{-2}$ (I = 0.39). At the saturation, absorbance changes were the same at all intensities. Typical kinetics are presented in Fig. 2; they are practically of first order at both wavelengths.

The reciprocal of the $t_{1/2}$ for reaction is plotted versus I in Fig.3. At 542 nm (reduction of C-550), the rate ($t_{1/2}^{-1}$) is proportional to I, as seen by a straight line with a slope of 1 in the log plot (similar results, not shown, were obtained at 546 nm). This is in agreement with the assumption that the reduction of C-550 is a primary photoreaction. An important departure

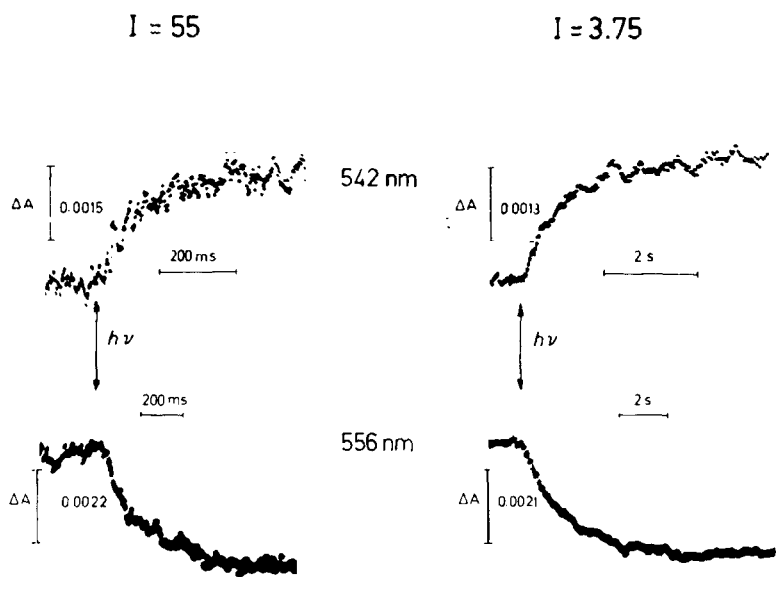

Figure 2. Time course of absorbance changes at 542 and 556 nm, at two light intensities. The arrows indicate the opening of the shutter. Number of averaged experiments : 6 for I = 55, 3 for I = 3.76 .

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from linearity, reported by Butler et al (12) was not observed in our experiments At 556 nm, the rate of absorbance change is 2-3 times slower, with a clear tendency to larger ratio at the highest intensities.

Evolution of absorbance in the dark after a limited reaction.

According to the described kinetics, when the illumination is stopped once the $t_{1/2}$ of reduction of C-550 is achieved, the proportion of reduced C-550 is greater than the proportion of oxidized cyt b_{559}. However such a difference is not observed when difference spectra are recorded with a Perkin-Elmer spectrophotometer, a few minutes after the illumination : at any part of the progressive phototransformation, the difference spectrum has the same shape, as seen in Fig. 1b (see also Fig. 2 in ref. 9). So some dark reaction must occur.

Fig. 4 represents an attempt to follow the evolution of absorbance in the dark after photoreduction of 60% of the C-550 and oxidation of 25-30% of the cyt b_{559}. No evolution is observed, so that we cannot decide whether the dark reaction is a reverse oxidation of C-550 or a dark oxidation of cyt b_{559}. We see however that any evolution has to be very slow ($t_{1/2}$ greater than 5s). A time gap remains between the abilities of the kinetic apparatus (about 10 s, due to slow evolutions of the baseline) and of the Perkin-Elmer spectrophotometer (about 1 minute, due to several manual operations following the illumination).

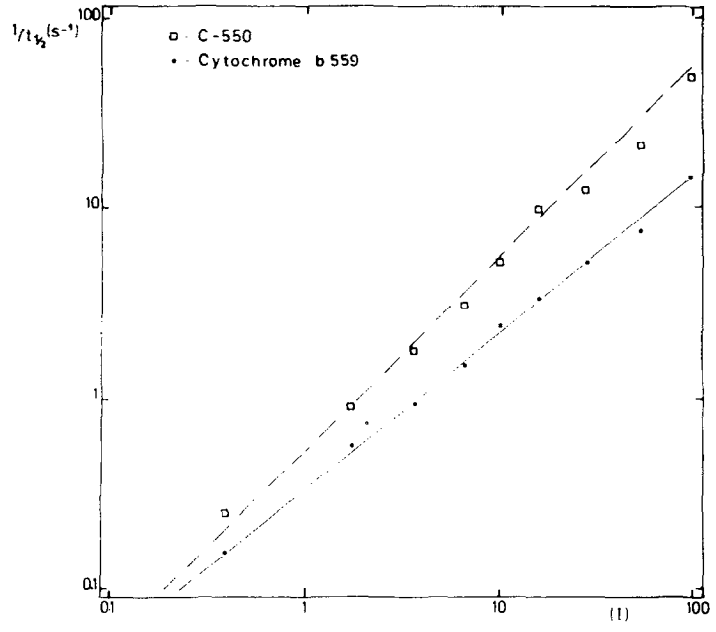

Figure 3. A logarithmic plot the reciprocal of the half-time of absorbance changes at 542 nm (reduction of C-550) and at 556 nm (oxidation of cyt b559) versus light intensity I. The dashed line has a slope of 1. The continuons line is a best fit for cyt b559. In that plot the straight line with a slope different of 1 has no well-defined signification.

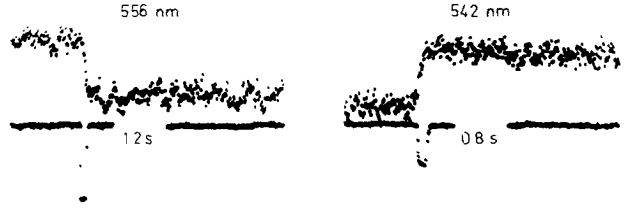

Figure 4. Time course of absorbance changes at 542 and 556 nm, with I = 16.25 and a shutter opening of 1/8 s (negative square signal). At 556 nm, 8 experiments were averaged; the vertical step corresponds to $\Delta A = 1.1 \times 10^{-3}$. At 542 nm, 6 experiments were averaged; the vertical step corresponds to $\Delta A = 1.8 \times 10^{-3}$.

DISCUSSION.

Our results confirm that C-550 behaves as a primary electron acceptor : at -170° its rate of reduction is proportional to I. However they cannot be accounted for by the currently used model for the low-temperature reactions

of Photosystem-II (8,9) :

$$C_{550} - Chl - b_{559} \underset{k_r}{\overset{h\nu}{\rightleftharpoons}} C_{550}^{-} - Chl^{+} - b_{559} \xrightarrow{k_b} C_{550}^{-} - Chl - b_{559}^{+}$$

where k_b is a dark reaction. Indeed for no value of k_b this model can predict a ratio of 2-3 between the rates of reduction of C-550 and of oxidation of cyt b_{559} in our range of intensities. We are led to the proposal of a second light reaction, e.g. according to one of the two following models :

$$a : C_{550} - Chl - b_{559} \underset{k_r}{\overset{\alpha I}{\rightleftharpoons}} C_{550}^{-} - Chl^{+} - b_{559} \underset{k_b}{\overset{\beta I}{\rightleftharpoons}} C_{550}^{-} - Chl - b_{559}^{+}$$

$$b : \begin{cases} C_{550} - (Chl - D) \underset{k_r}{\overset{\alpha I}{\rightleftharpoons}} C_{550}^{-} - (Chl - D)^{+} & b_1 \\ (A - Chl) - b_{559} \xrightarrow{\beta I} (A - Chl)^{-} - b_{559}^{+} & b_2 \end{cases}$$

(D : electron donor - A : electron acceptor)

Model a/ can readily explain the observed variation of the reaction kinetics with I and the identical amount of reduced C-550 and of oxidized cyt b_{559} some time after a partial phototransformation. However it predicts, for the photooxidation of cyt b_{559}, a definite lag that we were not able to detect. The second model correctly predict the time course of the absorbance changes; however additional assumptions are needed to account for the non-linearity in the rate of oxidation of cyt b_{559} (versus I) and some interaction has to be postulated between the products of photoreactions b_1 and b_2.

Model b/ presents similarities with the proposal by Joliot and Joliot of two donor-acceptor pairs at RC-II (13).

We have no information on the possible occurence of these two reactions in normal photosynthesis, at room temperature. The second reaction could explain the parallelism between photooxidation of cyt b_{559} and fluorescence induction in cases where cyt b_{559} is the electron donor : very low temperatures (12,14), -55° in the S_2 and S_3 states (10,15).

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