## Short Communication

# Mechanism of the Photochemical Activity of Isolated Chloroplasts

## VII. INACTIVATION OF A PHOTOACT BY DILUTE FERRICYANIDE ION[1,2]

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Destructive effects of the most commonly used Hill oxidant, ferricyanide ion, are well documented and include its acceleration of the second order inactivation process in isolated chloroplasts (3). Concentrated ferricyanide (50 mM), especially at pH values above 7, rapidly uncoupled photophosphorylation in spinach chloroplasts (4), thus accounting for its increase in the light-independent rate constant of ferricyanide reduction $k_D$ by chard and beet chloroplasts (1, 9), and it strongly inactivated a light step associated with oxygen evolution (4), which would account for our lowered photochemical efficiency as $k_L$ (1, 9). Ferrocyanide ion was equally effective (4), and the effect on $k_L$ was obtained with all artificial oxidants tried (9). The less concentrated (0.5 mM) ferricyanide ordinarily used to measure the velocity of the Hill reaction also inhibited photophosphorylation below pH 5.5 by potentiating acid lability and by solubilizing the coupling factor $CF_1$ as did ferrocyanide (12). In addition it partially inactivated, independent of pH, a dark step associated with the oxygen-evolving site, but this was prevented by weak illumination and by ferrocyanide, as it was an oxidative effect of ferricyanide (4). We now report, by way of kinetically resolved $k_L$ (9, 13) with chloroplasts from a cultivar of sugar beet under given conditions of measurement, that the limiting photochemical step can also be very labile toward the lower concentration of ferricyanide, and that this characteristic is promoted, as uncoupling was (4), by acidic as well as basic solutions.

Chloroplasts were prepared from leaves of field-grown sugar beets by brief blending and centrifugal washing in 0.5 M sucrose, 10 mM NaCl and 50 mM Tricine buffer at pH 7.8, and by final suspension in the same without buffer for snap-freezing and storage at −55 C. The routine 1-ml reaction mixture contained 100 $\mu$moles of sodium phosphate buffer of various values of pH, 10 $\mu$moles NaCl, 0.375 $\mu$mole of potassium ferricyanide with 0.125 $\mu$mole potassium ferrocyanide to poise the initial potential, or with 10-fold concentration of this couple with or without added DPIP[4], and chloroplast suspension containing 0.10 mg of chlorophyll with 50 $\mu$moles of sucrose. Ferricyanide reduction was recorded potentiometric-
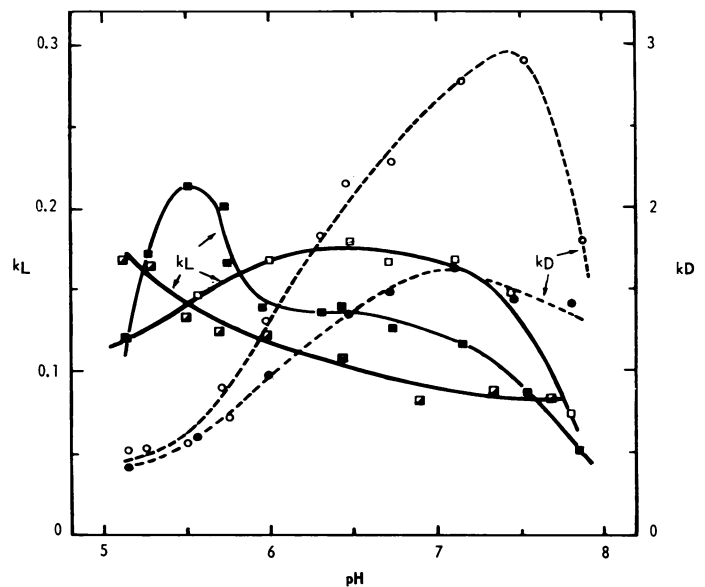


FIG. 1. The pH dependence of $k_L$ (squares) and $k_D$ (circles) as influenced by the method of measurement. Open points computed from measurements made by the older method, solid points from the present procedure, and half-filled points of $k_L$ computed from the light intensity dependence of the latter measurements extrapolated back to zero time, each with a different chloroplast preparation.

ally (13) in a stirred, 1 mm deep suspension thermostatted at 5 C during illumination with 80 to 3000 ergs $cm^{-2}$ $sec^{-1}$ of red light of wavelength 668 nm and 10 nm half bandwidth. The chloroplasts were routinely exposed to the reaction mixtures in the dark for 2 min after or during thawing, while the potential dropped to 5 to 15% reduction of the ferricyanide. This phenomenon corresponded to that which is known to involve the partial, oxidative inactivation of a dark step (4). The ferricyanide-Hill reaction was measured at a number of different light intensities. The phenomenological parameters $k_L$ and $k_D$ were computed as previously described (9, 13) but as macroscopic constants here. The regressions of the linearized hyperbolic plots were highly significant, and the standard errors averaged 12% of $k_L$ and 2% of $k_D$. The second order inactivation rate constants ($k_2'$) were obtained from the linear plots of $k_L^{-1}$ and $k_D^{-1}$ against time over the course of an hour of chloroplastic aging in the reaction mixture at 5 C.

With this method we observed that pH 5.5 was optimal for

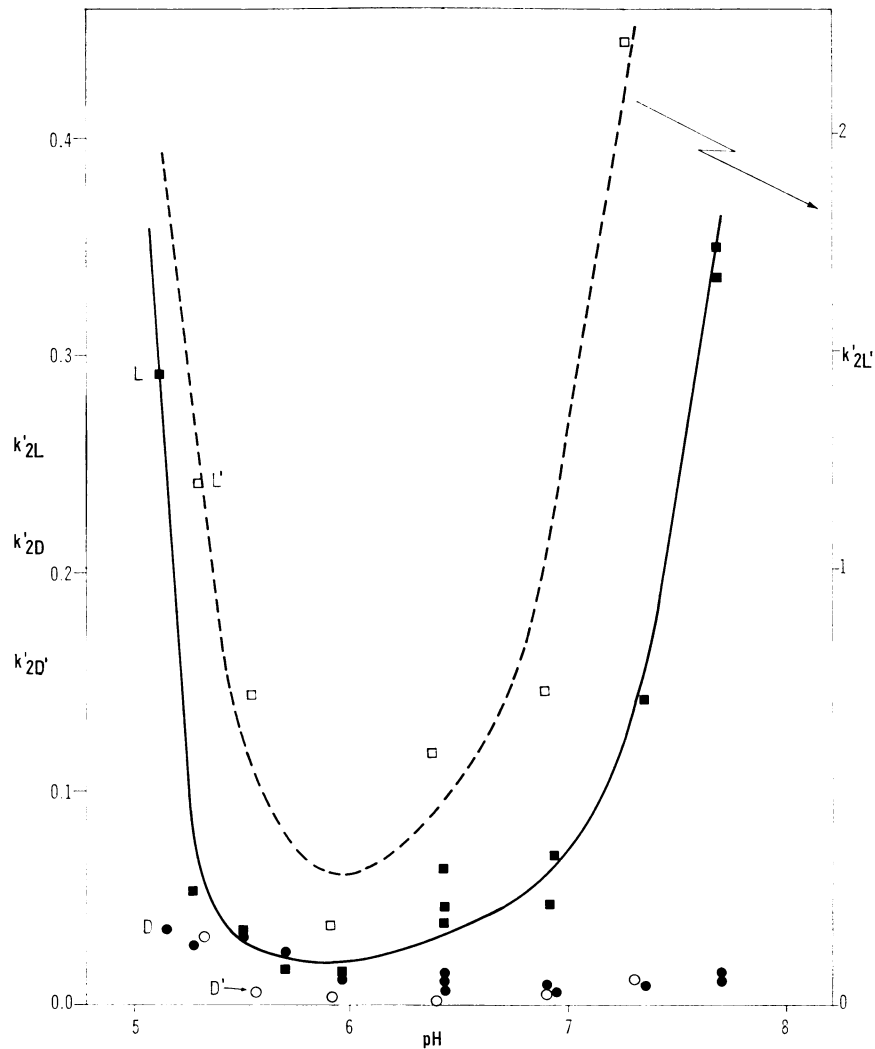[4] Abbreviation: DPIP: 2,6-dichlorophenol indophenol.

FIG. 2. The pH dependence of inactivation, as the second-order rate constants of decrease in $k_L$ (squares) and $k_D$ (circles), in 0.5 mM ferri-ferrocyanide ($k'_{2L}$, $k'_{2D}$, solid points) and in 5 mM ferri-ferrocyanide and 50 $\mu$M DPIP ($k'_{2L}$, $k'_{2D}$, open points).

Table I. *Effects of Different Proportions of Oxidants on the Parameters of the Rate-limiting Reactions*

| DPIP Concentration | Ferricyanide Concentration | $k_L \pm$ SE | $k_D \pm$ SE |
|---|---|---|---|
| $\mu M$ | $mM$ | | |
| 0 | 0.375 | 0.17 ± 0.02 | 0.91 ± 0.01 |
| | 3.75 | 0.13 ± 0.01 | 1.23 ± 0.02 |
| 1 | 0.375 | 0.14 ± 0.06 | 0.94 ± 0.02 |
| | 3.75 | 0.12 ± 0.01 | 1.26 ± 0.02 |
| 50 | 0.375 | 0.10 ± 0.01 | 1.35 ± 0.02 |
| | 3.75 | 0.06 ± 0.01 | 1.64 ± 0.04 |

$k_L$ in contrast to the previously reported (9, 14) optimum value of pH 6.2 which was obtained with a procedure that involved a longer time of incubation of the chloroplasts with the ferricyanide before illumination (Fig. 1). When the time-contingence of the activity was measured with the present procedure and extrapolated back to zero time, the dependence of that activity on light intensity yielded values of $k_L$ that increased with decreasing pH down to pH 5.1 (Fig. 1). Below that pH and above pH 7.7 the inactivation was too rapid to permit valid measurements (Fig. 2). For chloroplast preparations in which

the light-saturated rate, $k_D$, of the ferricyanide-Hill reaction was halved in 1 hr at all values of pH, the value of $k_L$ was also halved at pH 5.2 and 7.4 but decreased by only 10% at pH 6. The light reaction dominating $k_L$ was most stable at pH 5.9 (Fig. 2). The literature does not permit the general conclusion that ferricyanide is a specific oxidant for system II, in the sense of the plastoquinone site, but since the (first) light reaction of the generally more labile system II is the limiting photoact in the intact noncyclic chain of systems so tested (6, 16), it is likely that our parameter $k_L$ predominantly embodied the rate constant of the photochemical reaction (first) of system II. In comparison with our results the pH curve formerly attributed to the photoact of system I was flat over this range of pH (16).

The value of $k_L$, calculated from measurements made after the routine 2 min of mixing, decreased with increase in ferricyanide concentration as previously reported (9) and the effect was enhanced by added DPIP (Table I). DPIP at 50 $\mu$M had more effect with 0.5 mM ferri-ferrocyanide (0.375 mM ferricyanide) than did a 10-fold increase in the latter. The rate of inactivation caused by the concentrated mixed oxidants was more sensitive to pH, as shown in Figure 2, where it is also evident that at pH 6 the inactivation rate constant for $k_L$ was increased in proportion to the ferricyanide concentration, but at pH 5.3 and 7.3 it was two orders of magnitude higher with the mixed

oxidants ($k'_{2L'}$) than with the more dilute ferri-ferrocyanide alone ($k'_{2L}$). In general, the inactivation in the parameter $k_D$ was comparatively little affected by pH and the oxidants (Figs. 1, 2; Table I). The photophosphorylative uncoupling actions of ferri-ferrocyanide (1, 4, 9) and DPIP (5, 7, 8), perhaps by means of enhanced permeation of the thylakoid membranes, account for the stimulations of electron transport (Table I, $k_D$); but the possibility that the mechanism of this action involves a qualitative shift to an earlier electron donor (8, 10) need not be discounted yet.

Like the aging process of isolated chloroplastic material (2, 14) the ferricyanide enhanced inactivation was second order in both $k_L$ and $k_D$. This is a kinetic puzzle for which one possible explanation is that in the reduction of ferricyanide ion by plastoquinone (11, 16) damage to either one of the two electron transport chains that are coupled at the site of plastoquinone reduction (11, 15) may destroy the electron transport ability of both. Investigators who are using artificial oxidants without refined experimental techniques should take warning that the differential responses of the inactivation processes can dominate the results, including the photochemical reaction, even at low concentrations of the oxidants.

### LITERATURE CITED

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