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# CORRELATION OF REDOX LEVELS OF COMPONENT ELECTRON CARRIERS WITH TOTAL ELECTRON FLUX IN AN ELECTRON-TRANSPORT SYSTEM

## *P*-700 AND THE PHOTOREDUCTION OF $NADP^+$ IN CHLOROPLAST FRAGMENTS

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SUMMARY

A mathematical analysis is described which measures the effects of actinic light intensity and concentration of an artificial electron donor on the steady-state light-induced redox level of a reaction-center pigment (e.g. *P*-700) and on the overall light-induced electron flux (e.g. reduction of $NADP^+$). The analysis led to a formulation (somewhat similar to the Michaelis-Menten equation for enzyme kinetics) in which a parameter, $I_{\frac{1}{2}}$, is defined as the actinic light intensity that, at a given concentration of electron donor, renders the reaction-center pigment half oxidized and half reduced. To determine the role of a presumed reaction-center pigment, $I_{\frac{1}{2}}$ is compared with another parameter, equivalent to $I_{\frac{1}{2}}$, that is obtained independently of the reaction-center pigment by measuring the effect of actinic light intensity and concentration of electron donor on the overall electron flow.

The theory was tested and validated in a model system with spinach Photosystem I chloroplast fragments by measurements of photooxidation of *P*-700 and light-induced reduction of $NADP^+$ by reduced 2,6-dichlorophenolindophenol. A possible extension of this mathematical analysis to more general electron-transport systems is discussed.

## INTRODUCTION

In the study of photosynthetic electron transport, absorption spectrophotometry is an important and at times the only non-destructive technique that is suitable for the elucidation of the role of component electron carriers. Ordinary spectrophotometric measurements can provide information about the direction and amplitude of steady-state light-induced redox changes in individual electron carriers but seldom

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

can provide an answer to the question of how the rate of electron flow (electron flux) through a particular electron carrier compares with the total electron flux through the entire electron-transport chain.

Recognizing the importance of this question for the assessment of the role of individual carriers, Hoch, Rurainski, and associates [1–4] developed the steady-state relaxation spectrophotometry technique that permitted measurement and comparison of the photoinduced electron flux through *P*-700 with the rate of $NADP^+$ reduction. Although this technique led to such important findings as that *P*-700 may not be involved in linear electron transport from water to $NADP^+$ [3, 4], it was not adopted by other investigators to whom the required special instrumentation was not available.

We describe here a graphic method that makes possible, without special instrumentation other than a commercially available dual wavelength/split-beam spectrophotometer, quantitative comparisons between the overall light-induced electron flux through an electron-transport chain and redox changes of component electron carriers in the chain. The theoretical formulation of the method was experimentally validated in a model system by measurements of steady-state, light-induced absorbance changes of *P*-700 and of the rate of $NADP^+$ reduction with an artificial electron donor by chloroplast fragments enriched in Photosystem I and lacking Photosystem II activity.

The application of this method to other electron-transport systems and to techniques other than absorption spectrophotometry is also discussed. The companion paper describes the use of this method for an analysis of electron transport in algal membrane fragments [5].

## METHODS

Chloroplast fragments (D-144) were prepared from greenhouse-grown spinach leaves by the digitonin method of Anderson and Boardman [6]. Chlorophyll was measured and spinach ferredoxin and ferredoxin-$NADP^+$ reductase were prepared by procedures previously reported from this laboratory [7–9]. Absorbance changes at 700 nm were measured in an Aminco spectrophotometer (Model DW-2) operated in the split-beam mode. The cell compartment was extensively baffled to minimize interference from fluorescence and scattered light and the photomultiplier tube was shielded from the actinic light by a red cut-off filter (Corning 2-64).

The reaction mixture was placed in a $10 \times 4$ mm quartz cuvette (light path of measuring beam, 10 mm) and illuminated by a light beam from a 150 W tungsten-iodine lamp after it was passed through two blue-green filters (Corning 4-96). Combinations of neutral-density filters (Balzer and Schott) were used to control the actinic light intensity, which was measured at the surface of the sample cuvette with a YSI-Kettering radiometer (Model 65) and an Eppley thermopile. The actinic light beam that entered at a right angle to the axis of the spectrophotometer measuring beam passed through the polished sidewalls of the cuvette.

The rate of $NADP^+$ reduction was measured in the same spectrophotometer under the same conditions except that the wavelength of the measuring beam was 340 nm and the photomultiplier was protected by a Baird-Atomic 340-nm interference filter. All of the experiments were done at room temperature (approx. 20 °C).

THEORY

*Steady-state level of the reaction-center pigment*

The steady-state redox level of an intermediate electron carrier in an electron-transport chain is determined by the rate of its oxidation by an electron acceptor and the rate of its reduction by an electron donor. In this paper, the intermediate electron carrier will be a photochemical reaction-center pigment which will be referred to as $P$ and understood to serve as a photon trap that undergoes photooxidation, i.e. acts as a primary electron donor. The reduction of $P$ by a substrate electron donor (i.e. the formation of $P_{red}$) is a dark reaction that is independent of light. $P_{red}$ is the only fraction of $P$ that is photochemically active. The oxidation of $P_{red}$ (the formation of $P_{ox}$) is a photochemical reaction whose rate is a function only of light intensity ($I$) if the subsequent dark electron-transfer steps are fast enough not to be rate limiting. A model of operation of a photochemical reaction center [10–14] can be formulated in which the rate of the photooxidation of $P_{red}$, that is, the formation of $P_{ox}$, occurs in accordance with Eqn. 1,

$$\frac{dP_{ox}}{dt} = \Phi_r \cdot I \cdot \frac{P_{red}}{P_t} = \Phi_r \cdot I \cdot \frac{P_t - P_{ox}}{P_t} \tag{1}$$

where $P_t = P_{ox} + P_{red}$ and $\Phi_r$ is the relative quantum efficiency.

The relative quantum efficiency, $\Phi_r$, rather than the absolute quantum efficiency, is used here because only the incident light intensity, $I$, needs to be measured (as ergs $\cdot$ cm$^{-2}$ $\cdot$ s$^{-1}$) without the additional determination of absorbed quanta as is necessary for computations of absolute quantum efficiency. The use of the relative quantum efficiency, $\Phi_r$, is deemed to be justified because the same $\Phi_r$ values will be used in the subsequent section that deals with the total electron flux in the same system.

The rate of reduction of $P$ is proportional to $P_{ox}$ and a term, $k$, that may be a function of the concentration of the substrate electron donor.

$$\frac{dP_{red}}{dt} = k \cdot P_{ox} \tag{2}$$

In the steady state, the rate of photooxidation of the reaction-center pigment will equal its rate of reduction in the dark, hence,

$$\frac{dP_{ox}}{dt} = \frac{dP_{red}}{dt} \tag{3}$$

Substituting for the left and right sides of Eqn. 3 the values from Eqns. 1 and 2, we obtain

$$\Phi_r \cdot I \cdot \frac{P_t - P_{ox}}{P_t} = k \cdot P_{ox} \tag{4}$$

from which

$$P_{ox} = \frac{\Phi_r \cdot I \cdot P_t}{k \cdot P_t + \Phi_r \cdot I} \tag{5}$$

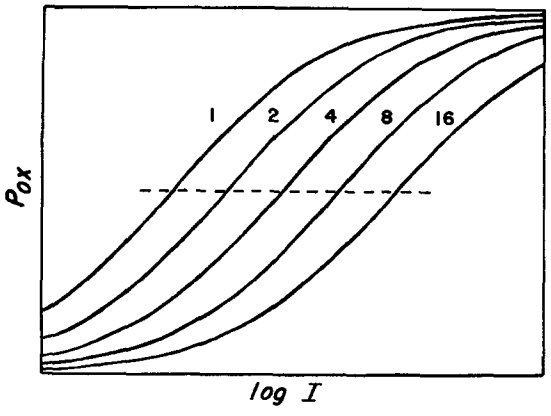


Fig. 1. Sigmoid curves in a plot of $P_{ox}$ versus log $I$, according to Eqn. 6. Numbers indicate arbitrary values of $k$. The dotted line joins the 50 % level of $P$ at which $I = I_{\frac{1}{2}}$ (see discussion in text).

or

$$P_{ox} = \frac{P_t}{\frac{k \cdot P_t}{\Phi_r \cdot I} + 1} \tag{6}$$

Eqn. 6 indicates that when $P_t$, $\Phi_r$, and $k$ are fixed, the values for $P_{ox}$ are determined by light intensity, $I$, and would range from 0 (as $I$ approaches 0) to $P_t$ (as $I$ approaches infinity). As shown in Fig. 1, a plot of $P_{ox}$ against log $I$ would yield a sigmoid curve. Any change in the value of $k$, determined by the concentration of the substrate electron donor, would, according to Eqn. 6, shift the sigmoid curve along the abscissa but would not affect the shape of the curve or its maximum and minimum limits. Thus, different values of $k$ would give a series of sigmoid curves whose horizontal locations depend on $k$.

Eqn. 6, which defines the effect of light intensity on $P_{ox}$, is in a form that is analogous to the Michaelis-Menten equation that defines the effect of substrate concentration on the rate of enzyme reaction. Like the Michaelis-Menten equation, Eqn. 6 can be algebraically changed into other forms that are more useful for plotting experimental data. By taking a reciprocal of both sides of Eqn. 6 it is transformed into Eqn. 7.

$$\frac{1}{P_{ox}} = \frac{k}{\Phi_r} \cdot \frac{1}{I} + \frac{1}{P_t} \tag{7}$$

Eqn. 7 is analogous to the Lineweaver-Burk double-reciprocal modification of the Michaelis-Menten equation, a modification that gives a straight-line plot of the reciprocal of the initial velocity of an enzyme-catalyzed reaction ($1/v$) versus the reciprocal of substrate concentration ($1/[S]$). The Lineweaver-Burk straight-line plot gives a $1/V$ intercept on the $1/v$ axis and a $-1/K_m$ intercept on the $1/[S]$ axis. The negative reciprocal of the $1/[S]$ axis intercept is equal to $K_m$, the Michaelis constant, which is the experimentally determined substrate concentration at which the reaction velocity ($v$) is half-maximal.

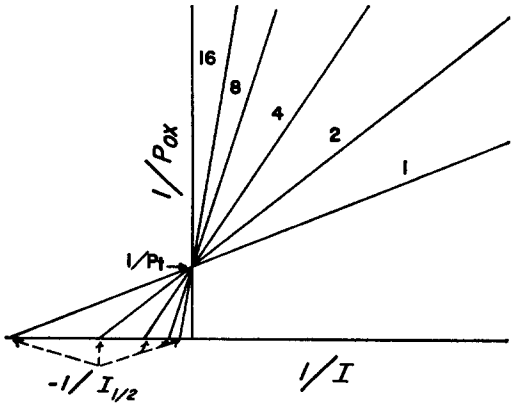


Fig. 2. Straight line double reciprocal plot of $1/P_{ox}$ versus $1/I$ according to Eqn. 7. Numbers indicate arbitrary values of $k$. Corresponding $I_{\frac{1}{2}}$ values are the negative reciprocals of the $X$-axis intercepts (see discussion in text).

Similarly, as shown in Fig. 2, Eqn. 7 gives a straight line plot of $1/P_{ox}$ against $1/I$. This straight line will have $1/P_t$ as intercept on the $1/P_{ox}$ axis and $-\Phi_r/k \cdot P_t$ as the intercept on the $1/I$ axis. The negative reciprocal of the latter intercept, $k \cdot P_t/\Phi_r$, is equal to a new term that is discussed below.

We will now introduce, by analogy to $K_m$, a term, $I_{\frac{1}{2}}$, to denote "half-light intensity" ("half-intensity" for short) which is defined as the light intensity that induces the photooxidation of one-half of $P_t$. By equating $\frac{1}{2}$ of $P_t$ with $P_{ox}$ and $I_{\frac{1}{2}}$ with $I$ and substituting in Eqn. 7 we obtain,

$$I_{\frac{1}{2}} = \frac{k \cdot P_t}{\Phi_r} \tag{8}$$

It will be noted that the value of $I_{\frac{1}{2}}$ in Eqn. 8 is the same as that of the negative reciprocal of the intercept on the $1/I$ axis of the straight line plot of $1/P_{ox}$ against $1/I$, as shown in Fig. 2.

The importance of $I_{\frac{1}{2}}$ will be discussed in the following section.

*Steady-state electron flow*

The preceding section dealt with an analysis of the light-induced steady-state oxidation-reduction level of the photochemical reaction-center pigment, $P$, and introduced the term $I_{\frac{1}{2}}$. Eqn. 8 shows that $I_{\frac{1}{2}}$ is the ratio of $k \cdot P_t$ to $\Phi_r$. In this section it will be shown that $k \cdot P_t$ and $\Phi_r$ can be estimated from an analysis of the overall rate of electron flow alone, without actual measurement of $P$ itself. The $I_{\frac{1}{2}}$ values obtained in this manner can then be compared with the $I_{\frac{1}{2}}$ values calculated from measurements of $P$.

Assume experimental conditions that would keep almost all of $P$ in a reduced state by maintaining its rate of reduction high by a high concentration of the substrate electron donor and its rate of oxidation low by a low incident light intensity. When $P_{red} \simeq P_t$, Eqn. 1 is transformed into Eqn. 9.

$$\frac{dP_{ox}}{dt} \simeq \Phi_r \cdot I \tag{9}$$

When the reaction mixture contains saturating amounts of the electron acceptor to assure that the removal of electrons from $P$ is not rate limiting, the rate of electron flow can be equal to $dP_{ox}/dt$. Under these conditions, $\Phi_r$ may be estimated from the slope of a plot of the rate of electron flow against light intensity, $I$. If we assume other experimental conditions that would keep almost all of $P$ in an oxidized state by maintaining its rate of oxidation high by a high light intensity and its rate of reduction low by a low concentration of the electron donor ($P_{ox} \simeq P_t$), Eqn. 2 could be changed into Eqn. 10.

$$\frac{dP_{red}}{dt} \simeq k \cdot P_t \tag{10}$$

Again, when the electron acceptor is present in excess, the rate of reduction of $P$ ($dP_{red}/dt$) becomes a measure of the rate of overall electron flow, which in turn depends on the concentration of the electron donor. Thus, according to Eqn. 10, $k \cdot P_t$ is equal to the rate of the overall electron flow. By substituting the values so obtained for $k \cdot P_t$ and $\Phi_r$ in Eqn. 8, $I_{\frac{1}{2}}$ may be determined without a separate determination of $P_t$.

In sum, by measuring the steady-state absorbance changes of $P$ as a function of incident light intensity, we obtain a parameter, $I_{\frac{1}{2}}$, which is defined as the light intensity needed to photooxidize one-half of $P$. Furthermore, $I_{\frac{1}{2}}$ can be determined not by measuring changes in the redox state of $P$ but by measuring the rate of the overall electron flow, as reflected in the reduction of the terminal electron acceptor (or the oxidation of the initial electron donor). In this manner, a correlation can be established between the observed redox state changes (usually measured as absorbance changes) of a photochemical reaction-center pigment and the overall rate of electron flow through a given electron-transport chain. This approach may be used to test the role of a photoactive reaction-center pigment in a linear electron-transport chain.

## EXPERIMENTAL RESULTS

### *Effect of light intensity and concentration of electron donor on absorbance changes of P-700*

The aforegoing theory was tested in a model system that included chloroplast fragments (D-144), a substrate electron donor consisting of 2,6-dichlorophenolindophenol (DCIP) maintained in the reduced state by an excess of ascorbate, and $NADP^+$ as the terminal electron acceptor. Work from several laboratories indicates that in this system *P*-700 is the reaction-center pigment that serves as the primary electron donor for $NADP^+$ reduction [15–18].

The light-induced oxidation of *P*-700 was measured spectrophotometrically as described under Methods. The difference (dark minus light) in absorbance ($\Delta A_{700\,nm}$) at the steady state (usually attained in a few seconds) was plotted against the logarithm of light intensity (Fig. 3). As predicted from Eqn. 6 and illustrated by Fig. 1, the plot gave a series of sigmoid curves of identical size and shape whose location along the abscissa was determined by the concentration of the electron donor, DCIP.

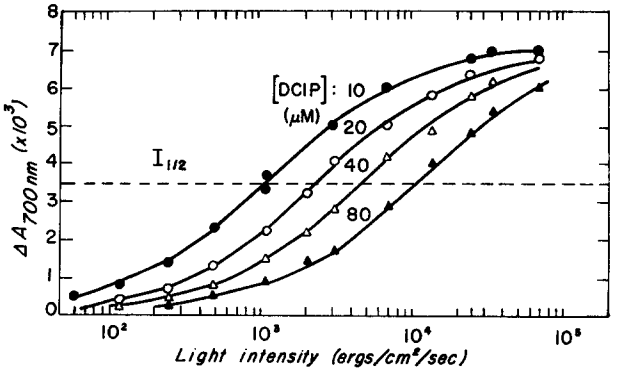


Fig. 3. Effect of light intensity on steady-state absorbance changes at 700 nm in Photosystem I chloroplast fragments at different concentrations of DCIP. The reaction mixture contained chloroplast fragments (chlorophyll *a*, 14 μg/ml), 2.5 mM $MgCl_2$, 20 mM sodium ascorbate, 5 mM NADP, 20 μM ferredoxin, a saturating amount of ferredoxin-NADP$^+$ oxidoreductase, and DCIP as indicated.

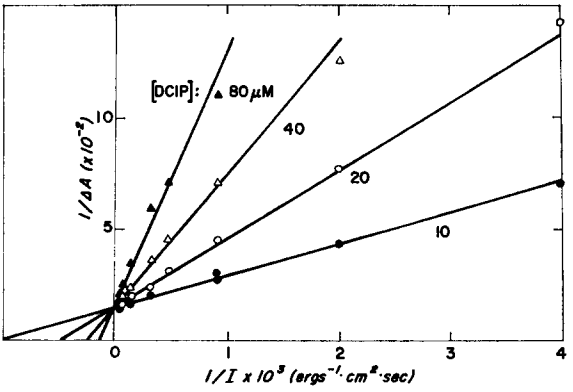


Fig. 4. Plot of reciprocal of absorbance changes at 700 nm against reciprocal of light intensity at different concentrations of DCIP. Experimental conditions as in Fig. 3.

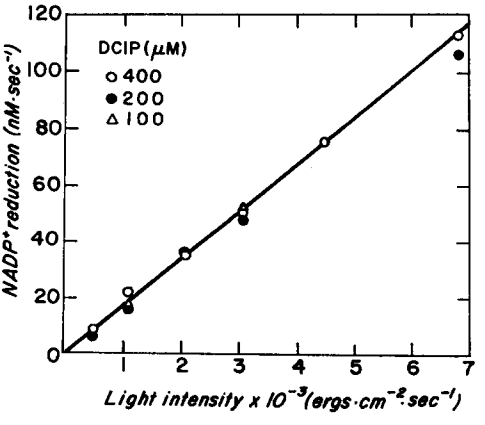


Fig. 5. Effect of light intensity on rate of NADP$^+$ reduction. Reaction mixtures as in Fig. 3 except for the indicated concentrations of DCIP.

The theory was further borne out by a series of double reciprocal plots of $1/\Delta A_{700\ nm}$ against $1/I$ which (as had been predicted from Eqn. 7 and illustrated by Fig. 2) gave a series of straight lines for different concentrations of DCIP (Fig. 4). From the sigmoid curves in Fig. 3 and the double reciprocal plots in Fig. 4, an $I_{\frac{1}{2}}$ value was determined for each concentration of DCIP.

These values of $I_{\frac{1}{2}}$ obtained from measurements of *P*-700 will now be compared with $I_{\frac{1}{2}}$ values obtained from measurements of the overall electron flow, i.e. from measurements of $NADP^+$ reduction.

*Effect of light intensity and concentration of electron donor on $NADP^+$ reduction*

As discussed in the theoretical section, under conditions of low incident light intensity and high concentration of the electron donor, $dP_{ox}/dt$ can be measured as the rate of $NADP^+$ reduction and becomes proportional to light intensity, $I$ (Eqn. 9). Under these conditions, the relative quantum efficiency, $\Phi_r$, will be equal to the slope of the plot of the rate of $NADP^+$ reduction against light intensity.

Fig. 5 shows that a plot of the rate of $NADP^+$ reduction (in lieu of $dP_{ox}/dt$ in Eqn. 9) against light intensity gave a straight line. The straight line relationship was particularly close at low light intensities and at high concentrations of DCIP. The value of $\Phi_r$ (relative, not absolute quantum efficiency; see Theory) determined as the slope of the straight line plot in Fig. 5 was $1.7 \cdot 10^{-11}$ M $\cdot$ erg$^{-1} \cdot$ cm$^{-2}$.

Next, the values of $k \cdot P_t$ were determined from Eqn. 10 at high light intensities when, as discussed in the theoretical section, $dP_{red}/dt$ can also be measured as the rate of $NADP^+$ reduction. As shown in Fig. 6, a log-log plot of the rate of $NADP^+$ reduction (in lieu of $dP_{red}/dt$ in Eqn. 10) against DCIP concentration gave a straight line with a slope of 1, an indication that the rate of $NADP^+$ reduction was proportional to the concentration of the electron donor. Fig. 6 was used, therefore, to determine the values of $k \cdot P_t$ (equal to the rate of $NADP^+$ reduction) at different concentrations of DCIP.

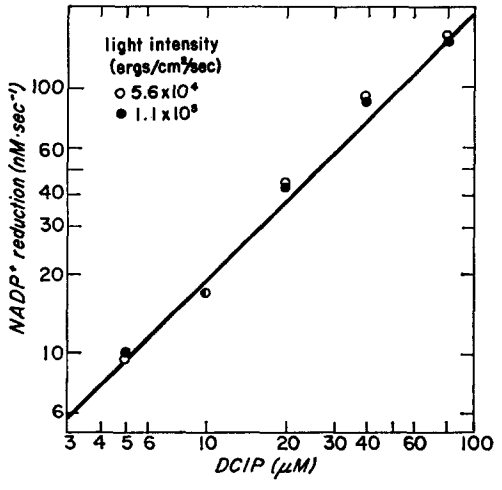


Fig. 6. Effect of DCIP concentration on $NADP^+$ reduction at two actinic light intensities. Reaction mixtures as in Fig. 3.

## VALIDATION OF THEORY

The basic premise of the theoretical section was that, at a given concentration of the electron donor (DCIP), the values of $I_{\frac{1}{2}}$ obtained by two different approaches should be in agreement, i.e. the $I_{\frac{1}{2}}$ values obtained by measurement of the absorbance changes of the photoactive reaction-center pigment (in this instance *P*-700) should agree with the values of $I_{\frac{1}{2}}$ obtained from Eqn. 8. The determinations of $\Phi_r$ and $k \cdot P_t$ needed in Eqn. 8 were made by measurement of $NADP^+$ reduction, as shown in Figs. 5 and 6, respectively.

The validity of this premise is demonstrated in Fig. 7. For the same concentration of the electron donor (DCIP), the values for $I_{\frac{1}{2}}$ obtained from measurements of *P*-700 (full circles) are in good agreement with the values for $I_{\frac{1}{2}}$ obtained, according to Eqn. 8, from measurements of $NADP^+$ reduction (empty circles). Thus, for the D-144 spinach chloroplast fragments, the validity of the equations and assumptions discussed in the theoretical section was experimentally confirmed by measurements of *P*-700 absorbance changes and steady-state rates of $NADP^+$ reduction.

## CONCLUDING REMARKS

The theoretical approach described herein was verified experimentally by a correlation between the total electron flux and the photooxidation of a photochemical reaction-center pigment. The same approach may also be used to determine whether such a correlation exists with other electron carriers. Secondary and tertiary electron donors and acceptors can similarly be examined by this approach provided that the reactions between these carriers and the photochemical reaction-center pigment are sufficiently fast (i.e. not rate limiting). Such intermediate electron carriers might be cytochromes, as Borisov and Ivanovskii [19] showed in a somewhat different treatment of the electron-transport system in photosynthetic bacteria. Furthermore, the method may also be applied to the determination of the steady-state level of an intermediary electron carrier, *C*, in non-photosynthetic electron-transport systems in

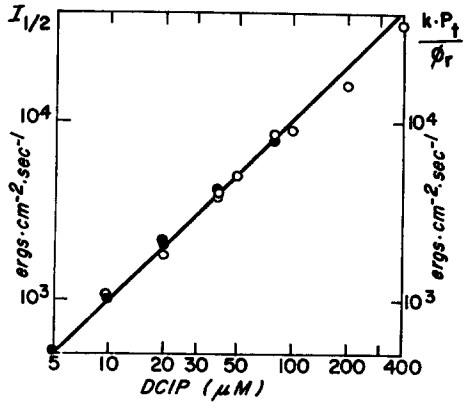


Fig. 7. Comparison of $I_{\frac{1}{2}}$ values from measurements of *P*-700 (full circles) with $I_{\frac{1}{2}}$ values from measurements of $NADP^+$ reduction (empty circles).

which, at the steady state (i) the concentrations of the electron donor ($D$) and the terminal electron acceptor ($A$) remain constant and (ii) $A$ and $D$ do not react with each other because of their steric properties and/or their spatial separation. A theoretical analysis of such a system, similar to that presented here, would yield the relationship:

$$\frac{1}{C_{ox}} = \frac{k_1}{k_2 \cdot C_t} + \frac{1}{C_t} \tag{11}$$

where $C_{ox}$ is the oxidized fraction of $C$, $C_t$ is the total $C$ ($C_{ox}+C_{red}$), $k_1$ is the rate constant for the reduction of $C$, and $k_2$ is the rate constant for the oxidation of $C$.

Eqn. 11 is analogous in form to Eqn. 7. A double reciprocal plot of $1/C_{ox}$ against $1/k_2$ (when $k_1$ is fixed) would give a straight line with a $y$-axis intercept equal to $1/C_t$, i.e. such a plot would provide a graphic method for the determination of $C_t$. Furthermore, by varying the concentration of either the electron acceptor ($A$) or the electron donor ($D$), $k_2$ and $k_1$ could be determined from measurements of the overall rate of electron flow as a function of either $A$ or $D$.

The overall light-induced electron transport was measured here as the rate of $NADP^+$ reduction under two sets of extreme conditions, namely, high light intensity and low concentrations of the electron donor, $D$, and low light intensity and high concentrations of $D$. Similarly, the overall rate of electron transport in a non-photosynthetic system represented by Eqn. 11 could be measured under two sets of extreme conditions, i.e. high concentrations of the electron acceptor, $A$, combined with low concentrations of $D$, and low concentrations of $A$ combined with high concentrations of $D$. The values of $k_1$ and $k_2$ obtained from measurements of the rate of overall electron transport under these sets of experimental conditions could then be compared with direct measurements of $C_{ox}$ under the same conditions.

The techniques applicable to these methods for analyzing diverse electron-transport systems are not limited to spectrophotometry. They include any physical detection method of a non-destructive nature, such as electron paramagnetic resonance (EPR) spectrometry, circular dichroism, optical rotary dispersion, and fluorimetry.

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