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## *Laboratory Exercises*

# Diphenyl Carbazide Restores Electron Transport in Isolated, Illuminated Chloroplasts After Electron Transport from Water Has Been Eliminated by Mild Heat Treatment

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**Freshly isolated, illuminated chloroplasts oxidize water and transfer the resulting electrons through the photosynthetic electron transport chains in their thylakoid membranes to the artificial electron acceptor, dichlorophenol indophenol (DCPIP). As a consequence, DCPIP is reduced and the decline in absorbance over time can be used to measure the rate of electron transfer. When gently heated, chloroplasts lose the capacity to oxidize water and the transfer of electrons to DCPIP is eliminated. Electron transport through chloroplasts to DCPIP is restored in the presence of the artificial electron donor diphenylcarbazide (DPC). If students gain experience with the DCPIP photoreduction assay and are given information on normal chloroplast function, they should be able to predict the behavior of heat-treated chloroplasts in a variety of experimental conditions. A number of such predictions are outlined and tested. The experiments can all be conducted with a limited repertoire of equipment and easily prepared solutions. Consequently, this work is well suited to an investigative study in which each student group, in consultation with instructors, can make and test its own prediction. The ways in which changing different variables can affect the quality of the experimental results is emphasized. Additional studies, on measurements of rates of oxygen evolution and emitted chlorophyll fluorescence, are briefly described to support the inferences that heat-treated chloroplasts do not oxidize water and that the vectorial transfer of electrons through them to DCPIP is identical to that in untreated chloroplasts.**

*Keywords*: Isolated chloroplasts, electron transport rate, heat treatment, artificial electron donor, dichlorophenol indophenol, diphenyl carbazide, uncoupling, ammonium chloride, gramicidin D.

We teach part of a mandatory laboratory course for second-year undergraduates entitled "Scientific Methods in Biology." The course deals with experimental design, instrumentation, evaluation of data, and how results are communicated to other scientists. Because our students write about some of their work in the form of a scientific paper, we try to introduce new experimental systems that exemplify similar principles so that the writing assignments can be varied. One experimental system has been particularly fruitful in providing a variety of experimental questions, namely, the measurement of rates of photoreduction of dichlorophenol indophenol (DCPIP)[1] by isolated, illuminated chloroplasts.

Chloroplasts, the organelles responsible for photosynthesis in green plants, have an outer limiting envelope and an internal system of thylakoid membranes that separate two compartments; an outer stromal compartment and an inner lumen. The thylakoid membrane includes, or is associated with, a number of molecular complexes involved in converting light energy to chemical energy. Those relevant to this article are illustrated in Fig. 1. Photons in the 380- to 700-nm range are absorbed by pigment molecules in the light-harvesting complexes (LHC II) associated with photosystem II (PS II). Some of the absorbed energy is funneled toward the reaction center (P 680) of PS II where it causes an electron ($e^-$) to be lost to pheophytin (pheo). This oxidation of the reaction center is the primary photochemical event in photosynthesis. Pheophytin then transfers an electron to a quinone ($Q_A$) that is tightly bound to PS II. $Q_A$ subsequently transfers electrons to $Q_B$. Plastoquinones (PQ), which diffuse within the thlakoid membrane, bind to $Q_B$. They become fully reduced ($PQH_2$) by accepting a pair of electrons from $Q_B$ and two protons ($H^+$) from the aqueous environment of the stroma. The pool of reduced plastoquinones within the membrane becomes oxidized as each molecule deposits two protons into the lumen and transfers two electrons to the cytochrome $b_6f$ complex. These electrons then pass through a further series of redox reactions to the final electron acceptor, $NADP^+$.

Electrons lost from the reaction center of PS II are replaced by electrons derived from the oxidation of water

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[1] The abbreviations used are: DCPIP, dichlorophenol indophenol; LHC, light-harvesting complex; PS, photosystem; pheo, pheophytin; $Q_A$, quinone; PQ, plastoquinone; PQH, reduced plastoquinone; OEC, oxygen-evolving complex; DPC, diphenylcarbazide; DCMU, dichlorophenyl dimethyl urea.

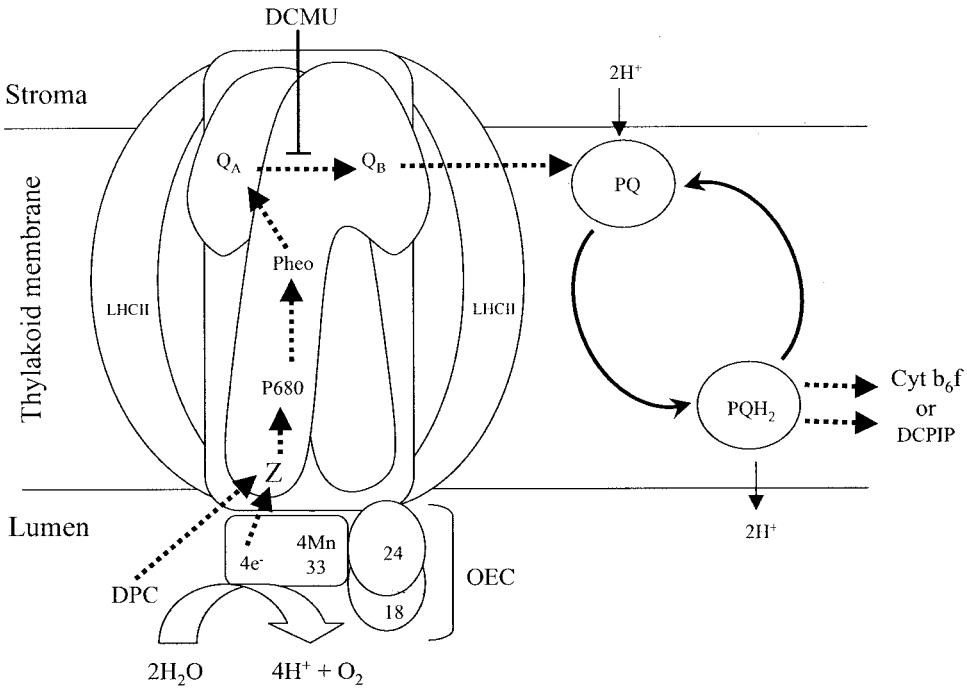


FIG. 1. **Schematic of electron flow through PS II in the thylakoid membranes of chloroplasts.** The OEC catalyzes the oxidation of water. The resulting electrons are transferred to a tyrosine residue (Z) and on to the reaction center (P 680) if it has been oxidized as a result of excitation by energy transferred from the light-harvesting complexes (LHC II). Electrons then flow through pheophytin (Pheo) to $Q_A$ and then to $Q_B$. $Q_B$ is a binding site for the plastoquinones (PQ) that diffuse within the membrane. Electrons accepted by PQ are typically donated to the cytochrome $b_6f$ complex or to DCPIP in the conditions of our assay. Heat-treatment damages the OEC so that water is not oxidized and electron transport is abolished. Electron transport can be restored by DPC that donates electrons directly to Z. DCMU binds to $Q_B$ and prevents it from accepting electrons from $Q_A$.

($H_2O$). Water oxidation is catalyzed by the oxygen-evolving complex (OEC) that consists of three polypeptides with relative molecular masses of ~18, 24, and 33 kDa and four atoms of manganese. The electrons pass from the OEC to a tyrosine residue (Z) on a constituent protein of PS II and then to the reaction center. Consequently, light energy is used to generate a flow of electrons from the oxidation of water to the reduction of $NADP^+$ in a stepwise manner. In our DCPIP reduction assay, electrons pass from the intramembrane plastoquinone pool to DCPIP instead of cytochrome $b_6f$. As the oxidized, blue form of DCPIP accepts the electrons, it is converted to the colorless, reduced form. The consequent decline in absorbance at 600 nm ($A_{600}$) over time is proportional to the rate at which DCPIP accepts electrons. To date, we have exploited the fact that rates of photoreduction of DCPIP change with irradiance level [1], in response to pH and an inhibitor [2] and in the presence of uncoupling reagents that abolish the proton gradient across the thylakoid membrane [3].

A useful approach to finding new experimental systems is to comb the research literature for results that, with modification for the skill level of students and the equipment available to them, can be adapted for class use. One such finding, that heat-treated chloroplasts will not photoreduce DCPIP but their capacity to photoreduce DCPIP is restored in the presence of 1,5- diphenylcarbazide (DPC) [4], forms the basis of our article. The heat-treatment damages the OEC so that water is not oxidized, oxygen is not produced, and no electrons are available to reduce DCPIP. Heat-treated, illuminated chloroplasts will, however, accept electrons from DPC, an artificial electron donor, and subsequently reduce DCPIP. DPC reportedly donates electrons to Z [5]. If the pathway of electrons from DPC to DCPIP in heat-treated chloroplasts is identical to that from water to DCPIP in untreated chloroplasts, we can predict the response of heat-treated chloroplasts to a number of experimental conditions. For example, if the electrons pass through the normal pathway in the chloroplast before they reduce DCPIP, the reaction should be sensitive to dichlorophenyl dimethyl urea (DCMU), a reagent that blocks the transfer of electrons from PS II to the plastoquinones. Other predictions are included in "Experimental Procedures."

Because many of the predictions can be tested using a limited number of stock reagents, this experimental system is highly suited to an investigative approach. If students are given experience with the basic assay and are introduced to the information on chloroplast function outlined in Fig. 1, they can be asked to come up with a single prediction and design an experimental protocol to test it.

One further point of pedagogical interest becomes apparent from this work. Protocols designed to measure the effects of changing four possible variables, namely the quantity of light or the concentrations of the chloroplast preparation, DPC, or DCPIP, generate experiments of widely divergent quality. This provides the basis for discussions on introducing unintended variables in experimental design.

Finally, the DCPIP reduction assay cannot directly show that heat-treated chloroplasts do not oxidize water or that electrons actually pass through PS II prior to reducing DCPIP. Consequently, evidence supporting these points is

TABLE I

*Basic composition of reaction mixtures (volumes in milliliters)*

The reference blank for reaction mixtures A–E contains no DCPIP and an additional 1.5 ml of distilled water.

| Code | Stock DCPIP | Stock buffer | Distilled water | DMSO | Stock DPC[a] | Methanol[b] | Stock DCMU[b] |
|---|---|---|---|---|---|---|---|
| A | 1.5 | 1.0 | 2.50 | – | – | – | – |
| B | 1.5 | 1.0 | 2.45 | 0.05 | – | – | – |
| C | 1.5 | 1.0 | 2.45 | – | 0.05 | – | – |
| D | 1.5 | 1.0 | 2.40 | 0.05 | – | 0.05 | – |
| E | 1.5 | 1.0 | 2.40 | – | 0.05 | * | * |

[a] Aliquots of stock DPC should not be added until the start of the reaction.

[b] Asterisks indicate that volumes of methanol and DCMU in methanol were adjusted to give the DCMU concentrations described in the results with a total of 50 $\mu$l of methanol in each reaction mixture.

presented from measurements of oxygen evolution rates and chlorophyll fluorescence.

## EXPERIMENTAL PROCEDURES

### *Equipment*

- Visible range spectrophotometers (*e.g.* Spectronic 20) and suitable cuvettes or tubes
- 5- and 10-ml capacity glass pipettes
- Variable volume mechanical pipettes (10- to 100-$\mu$l capacity) with tips
- Domestic blender
- Clinical centrifuge with graduated tubes
- Lamps with 100-Watt frosted bulbs
- Cheesecloth, Parafilm
- Constant-temperature circulating water bath

(A Li-Cor LI-189 quantum/radiometer/photometer with an attached LI-190 SA quantum sensor is very useful but not essential.)

### *Stock Solutions*

- Chloroplast isolation buffer: 50 mM tricine, 400 mM sorbitol, 10 mM NaCl, 2.5 mM $MgCl_2 \cdot 6H_2O$, 1.25 mM $MnCl_2$, 0.3 mM $Na_2EDTA$ adjusted to pH 7.8 with saturated NaOH
- Chloroplast reaction buffer: prepared at 5× the concentration used
- $NaH_2PO_4$: 29.4 g/liter
- $Na_2HPO_4$: 11.995 g/liter
- Sorbitol: 91.085 g/liter

When diluted 5-fold in the reaction mixtures, this produces a 66 mM phosphate buffer containing 100 mM sorbitol (the osmoticum) at pH 6.3 with an ionic strength of 0.1 mM [6].

- 90% (v/v) aqueous acetone
- 0.1 mM 2,6-dichlorophenol indophenol (DCPIP) (BDH Chemicals, Poole, United Kingdom)
- Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO)
- 50 mM 1,5-diphenylcarbazide (DPC) (Sigma) in DMSO
- Methanol (BDH Chemicals)
- 1.0 mM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) in methanol (and diluted further as necessary)

### *Reaction Mixtures*

All reaction mixtures, to which the chloroplast preparation was added, contained 30 $\mu$M DCPIP and 66 mM phosphate buffer (pH 6.3) containing 100 mM sorbitol in a total volume of 5.0 ml. Reaction mixtures containing DMSO, DPC in DMSO, methanol, or DCMU in methanol are summarized in Table I. These reaction mixtures, coded A to E, will subsequently be referred to by the code letter.

### *Chloroplast Isolation*

Weigh 25 g of spinach leaves after removing the petioles and major veins. Cut the leaves into smaller pieces and place them in a blender cup that has been stored in the freezer. Add 100 ml of cold chloroplast isolation buffer and blend the leaves with three 5-s bursts at full speed. Filter the blended mixture through four layers of cheesecloth into a beaker on ice. Transfer six 10-ml aliquots to 15-ml capacity centrifuge tubes. Centrifuge for 5 min at 1,300 × *g*. Discard the supernatant and add 2.0–2.5 ml of cold isolation buffer to each pellet. Resuspend the pellets with a paintbrush and pool them in a single tube. The resulting 12–15 ml of chloroplast preparation is enough for a minimum of 300 assays and can be stored on ice for hours with minimal loss of activity.

### *Estimation of the Chlorophyll Concentration in the Chloroplast Preparation*

Add 50 $\mu$l of chloroplast suspension to 5.0 ml of 90% acetone. Cover the tube with Parafilm and shake to dissolve the chlorophyll. Centrifuge at 1,300 × *g* for 3 min. Read the absorbance of the supernatant at 652 nm ($A_{652}$). The chlorophyll concentration, in micrograms of chlorophyll per microliters of chloroplast preparation, is calculated by multiplying the measured $A_{652}$ by 100 and dividing by 34.5 [3]. A volume of chloroplast preparation containing 20 $\mu$g of chlorophyll was used in all experiments unless otherwise stated in the figure legends.

### *Assay Protocol*

Add a volume of chloroplast preparation containing the chosen quantity of chlorophyll to the reference blank, cap the tube with Parafilm, and invert three times to mix. Use the blank to set the spectrophotometer to zero absorbance.

The remainder of the work is carried out in a darkened room [3]. For each reaction, in turn, add the chosen volume of chloroplast preparation, cap the tube, and invert to mix. Take the initial $A_{600}$ reading, place the tube in a beaker at the selected distance from the lamp, and start timing when the lamp is turned on. Take $A_{600}$ readings at 1, 2, and 3 min from the time the lamp was turned on. Return the tube to the beaker between readings. Dark controls are treated identically except that they are wrapped in aluminum foil between readings.

Reaction mixtures containing DCMU can be made in bulk before running the reactions. DPC should not be added to the reaction mixtures until after the chloroplast preparation is added because, at a slow rate, it directly reduces DCPIP.

In the work reported here, all reactions were run at 20 cm from the lamp at a photon fluence rate of ~140 $\mu$mol of photons/$m^2$/s unless stated otherwise in the figure legends.

### *Rate Calculations*

As previously explained [3], the average rate during the first 2 min of the reactions produces satisfactory data. To calculate these rates:

a) Calculate the cumulative change in absorbance during the first 2 min ($\Delta A_{600}$/2 min) for each reaction by subtracting the $A_{600}$ at 2 min from that at zero time.

b) Subtract the $\Delta A_{600}$/2 min for the dark control from the $\Delta A_{600}$/2 min for each of the reactions. This gives the corrected $\Delta A_{600}$/2 min that reflects only the decline in absorbance due to the presence of illuminated chloroplasts.

c) The molar absorption coefficient ($\epsilon$) for DCPIP is derived from the slope of a standard curve of $A_{600}$ against DCPIP concentration (in moles/liter) in the same buffer as is used for the reactions.

d) The corrected $\Delta A_{600}$/2 min for each reaction is divided by the molar absorption coefficient to derive the change in concentration of DCPIP in the first 2 min of the reaction ($\Delta c$/2 min).

e) Divide the $\Delta c/2$ min by 2 to derive the $\Delta c$/min.
f) Correct for the total volume of the reaction mixture by multiplying the $\Delta c$/min by the total volume of the reaction mixture (*i.e.* 5.0 ml plus the volume of the chloroplast preparation added) and dividing by 1,000 ml/liter. This gives the rate in moles of DCPIP photoreduced/minute.
g) Divide the rate from step f by the number of micrograms of chlorophyll added to the reaction mixture to give the final rate in moles of DCPIP photoreduced per minute per microgram of chlorophyll.

### *Heat Treatment of the Chloroplast Preparation*

One-milliliter aliquots of fresh chloroplast preparation in 1.7-ml capacity disposable micro-centrifuge tubes were floated in a plastic microfuge tube rack in a constant-temperature circulating water bath at 55 °C for 0 (untreated), 0.5, 1.0, 1.5, 2.0, or 2.5 min. After heating, the tubes were cooled in ice-water. (If a water bath is not available, a large bucket containing water mixed to 55 °C with hot and cold tap water is equally effective).

### *Predictions and Experimental Design*

The predictions, numbered i-vii, are shown in italics. Any further experimental details are given after each prediction.

i) *Heat-treated chloroplasts will not reduce DCPIP when illuminated unless DPC is present and will not reduce DCPIP in the dark even in the presence of DPC.*

The $A_{600}$ of reaction mixtures (mixture A; Table I) containing chloroplast preparation heated at 55 °C for 2.5 min was read at 1-min intervals in the light or in the dark. At the end of the second minute, 50 $\mu$l of 50 mM DPC in DMSO was added to the tubes and $A_{600}$ values were read for a further 3 or 4 min.

ii) *If the function of the oxygen-evolving complex is impaired by heat treatment, a progressive loss of function should be seen with increasing time of exposure of the chloroplasts to 55 °C.*

The rate of photoreduction of DCPIP by illuminated, untreated chloroplasts and of chloroplasts exposed to 55 °C for 0.5, 1.0, 1.5, 2.0, or 2.5 min was determined in the absence of DPC and DMSO, the presence of DMSO, and the presence of 0.5 mM DPC (reaction mixtures A, B, and C, respectively; Table I).

The following four predictions (iii-vi) can be made by thinking of the transfer of electrons from DPC to DCPIP via the heat-treated chloroplasts as a single chemical reaction in which a light-activated catalyst (the illuminated chloroplasts) mediates the reduction of DCPIP by DPC. In this case, according to the Rate Law, increasing the quantity or concentration of the light, the chloroplasts, the DPC, or the DCPIP should increase the rate of photoreduction of DCPIP.

iii) *At light levels lower than light-saturation, the degree of oxidation of the reaction center in PS II depends on light quantity. The rate of photoreduction of DCPIP by heat-treated chloroplasts in the presence of DPC should therefore decline with declining irradiance levels.*

The rate of photoreduction of DCPIP by chloroplasts exposed to 55 °C for 2.5 min was measured in quadruplicate in the presence of 0.5 mM DPC (reaction mixture C; Table I) at 10, 20, 30, 40, 50, or 60 cm from a 100-Watt lamp and in the dark. The rate for untreated chloroplasts was measured at 10 cm from the lamp in the presence and absence of DPC (reaction mixtures C and B, respectively; Table I).

iv) *If DPC donates electrons to PS II in heat-treated chloroplasts, there should be no photoreduction of DCPIP in its absence and increasing concentrations of DPC should increase the rate of photoreduction of DCPIP.*

Variants of reaction mixture C (Table I) were used with volumes of the stock DPC solution adjusted to produce DPC concentrations from 0 to 0.5 mM. Compensating volumes of DMSO were added. Rates of photoreduction of DCPIP by chloroplasts exposed to 55 °C for 2.5 min were measured in the absence and the presence of 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 mM DPC.

v) *The reaction center of PS II must be oxidized before PS II can accept electrons from DPC. Increasing the quantity of oxidized PS II (i.e. the quantity of illuminated chloroplast preparation) in the reaction mixture should increase the rate of transfer of electrons from DPC to DCPIP.*

Reactions were run in quadruplicate in reaction mixture C (Table I) using volumes of chloroplast preparation, treated for 2.5 min at 55 °C, containing 10, 20, or 30 $\mu$g of chlorophyll.

vi) *Since DCPIP is the final electron acceptor in the DCPIP reduction assay, increasing the concentration of DCPIP should increase the rate of transfer of electrons from DPC to DCPIP.*

Variants of reaction mixture C (Table I) were used with volumes of stock DCPIP solution adjusted to produce DCPIP concentrations of 20, 30, or 40 $\mu$M. Compensating volumes of distilled water were added. Reactions were run in quadruplicate with dark controls.

vii) *If electrons pass through PS II of heat-treated chloroplasts before reducing DCPIP, a) no DCPIP reduction should occur in the presence of high concentrations of DCMU and b) the rate of DCPIP reduction will be affected in a dose-dependent manner at lower DCMU concentrations.*

Rates of photoreduction of DCPIP by untreated and heat-treated (2.5 min at 55 °C) chloroplasts were determined in variants of reaction mixtures D and E (Table I) containing no DCMU or various DCMU concentrations.

From results obtained using the DCPIP photoreduction assay we can only infer that, in heat-treated chloroplasts, a) the function of the oxygen-evolving complex is impaired and b) the remaining components of PS II retain their normal activities. To provide instructors with evidence that these inferences appear to be true, we will present data from studies using an oxygen electrode and a device that measures chlorophyll fluorescence emitted by chloroplast preparations. The method and rationale for measuring oxygen produced by isolated chloroplasts has been previously presented [3]. However, because we regard this work as unsuitable for a majority of undergraduates, and because the equipment to measure chlorophyll fluorescence is available in only a few research laboratories, the methods will not be described. Data from oxygen-evolution and fluorescence measurements are presented and interpreted in the next section.

## RESULTS AND DISCUSSION

The results are presented in the same numerical order (i to vii) as the predictions in the preceding section.

i) Heat-treated chloroplasts do not photoreduce DCPIP in the absence of DPC but some DCPIP reduction occurs in the presence of DPC in the dark (Fig. 2). These data suggest that chloroplasts exposed to 55 °C for 2.5 min a) do not oxidize water because no DCPIP reduction occurs when they are illuminated in the absence of DPC and b) can accept electrons from DPC only if the reaction center of PS II has been excited by energy derived from light. Moreover, because DPC lowers the $A_{600}$ value in the dark, DPC may be directly reducing the DCPIP to a measurable degree. This last point contradicts the suggestion that the rate of direct reduction of DCPIP by DPC is "too slow to interfere" [7] with the DCPIP photoreduction assay and that appropriate dark controls are mandatory.

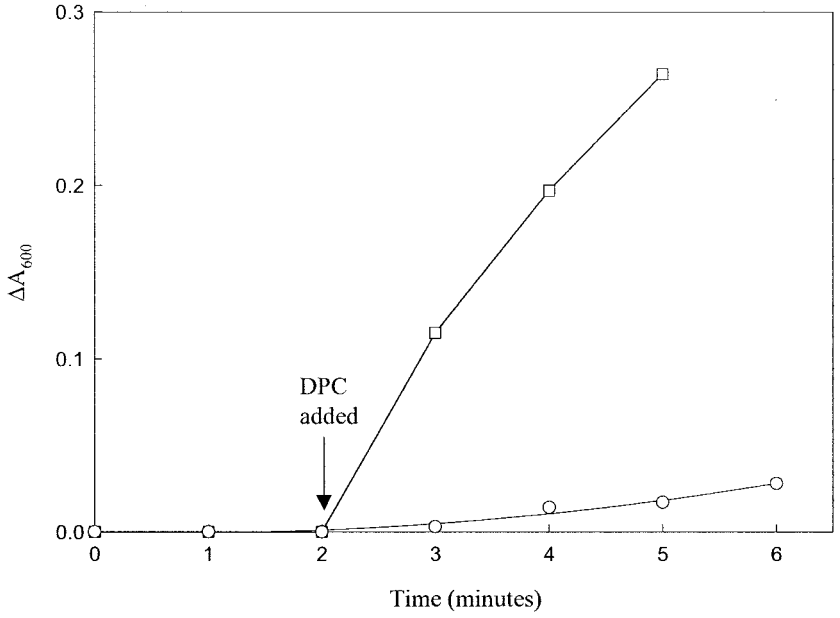


FIG. 2. **The change in absorbance at 600 nm ($\Delta A_{600}$) over time in reaction mixtures containing heat-treated chloroplasts in the light (20 cm from the lamp at 120 $\mu$mol of photons/m$^2$/s, *open squares*) and in darkness (*open circles*).** DPC was added at 2 min to a concentration of 0.5 mM (*arrow*). No DCPIP reduction is seen in the absence of DPC. When DPC is added, a high rate of photoreduction is evident in reactions exposed to light and some direct reduction of DCPIP by DPC is seen in reactions run in darkness.

ii) Chloroplasts exposed to 55 °C for 2 or more minutes completely lose the capacity to transfer electrons from water to DCPIP (Table II). Chloroplasts heat-treated for 1 or 1.5 min show a progressive loss of this function. DPC, at a concentration of 0.5 mM, restores the ability of chloroplasts treated for 2 min to photoreduce DCPIP to ~76.5% of the rate for untreated chloroplasts (Table II). These data are consistent with the hypothesis that the effect of heat-treatment is localized to the OEC and that the electron transport chain downstream is functioning in a relatively normal way.

iii) The rate of transfer of electrons from DPC to DCPIP by heat-treated (2.5 min at 55 °C) chloroplasts declines with declining photon fluence rate (Fig. 3). Rates decline from 82% of the control at 10 cm to 23% of the control at 60 cm from the lamp. All differences are significant ($p < 0.05$). This is the expected result because the reaction centers of PS II will only accept electrons from DPC (or water) if they have been oxidized as a result of energy input from the light-harvesting complexes, and the degree of oxidation of the reaction centers at irradiance levels below light saturation will decline with declining irradiance levels.

These data include an unexpected outcome; the rate of DCPIP photoreduction by untreated chloroplasts in the presence of DPC is significantly higher than in its absence even though the rates have been corrected by subtracting the value for the dark control. Again, this observation contradicts an earlier study that presented evidence that "Only as the oxygen evolution system is inactivated does the ability of the DPC to interact with photosystem 2 become apparent" [8]. Our data suggest that the DPC may be donating electrons to PS II while the latter is simultaneously accepting electrons from the oxidation of water.

iv) Chloroplasts heated at 55 °C for 2.5 min do not photoreduce DCPIP in the absence of DPC. When DPC is present, the rate of DCPIP photoreduction increases with increasing DPC concentration (Fig. 4). The rates of DCPIP reduction in Fig. 4 have not been corrected using dark controls because the extremely small amount of direct reduction by DPC at low concentration cannot be reliably measured using this type of assay. If it is assumed that the rate of direct reduction of DCPIP is proportional to DPC concentration, then the conclusion is valid even though the rate values may be inflated at the higher DPC concentrations.

TABLE II

*The progressive loss of the capacity of isolated, illuminated chloroplasts to reduce DCPIP as a function of time at 55 °C and the restoration of electron transport to DCPIP in the presence of 0.5 mM DPC*

| Exposure to 55 °C (min) | DCPIP reduction rate (mol/min/$\mu$g chlorophyll $\times 10^{-9}$) | | |
|---|---|---|---|
| | − DMSO, −DPC | 50 $\mu$l of DMSO | 50 $\mu$l of 50 mM DPC in DMSO |
| 0 | 1.93 | 1.70 | 1.70 |
| 0.5 | 1.86 | 1.86 | 1.74 |
| 1.0 | 1.45 | 1.53 | 1.80 |
| 1.5 | 0.08 | 0.14 | 1.32 |
| 2.0 | 0.00 | 0.00 | 1.30 |
| 2.5 | 0.00 | 0.00 | 1.20 |

v) Increasing the quantity of the heat-treated chloroplast preparation in the reaction mixture increases the rate of transfer of electrons from DPC to DCPIP (Fig. 5*a*). This is the expected result because when more oxidized PS II reaction centers are present, more electrons can be accepted from the DPC and be subsequently transferred to the DCPIP. However, this protocol has more than one experimental variable. Increasing the quantity of chloroplast preparation in the reaction mixture also makes the latter less transparent. Consequently, at higher chloroplast concentrations, each light-harvesting complex has a lower probability of absorbing a photon. Two pieces of evidence support this contention: a) the absolute rate of DCPIP reduction (in moles/minute) is close to doubled as the chlorophyll

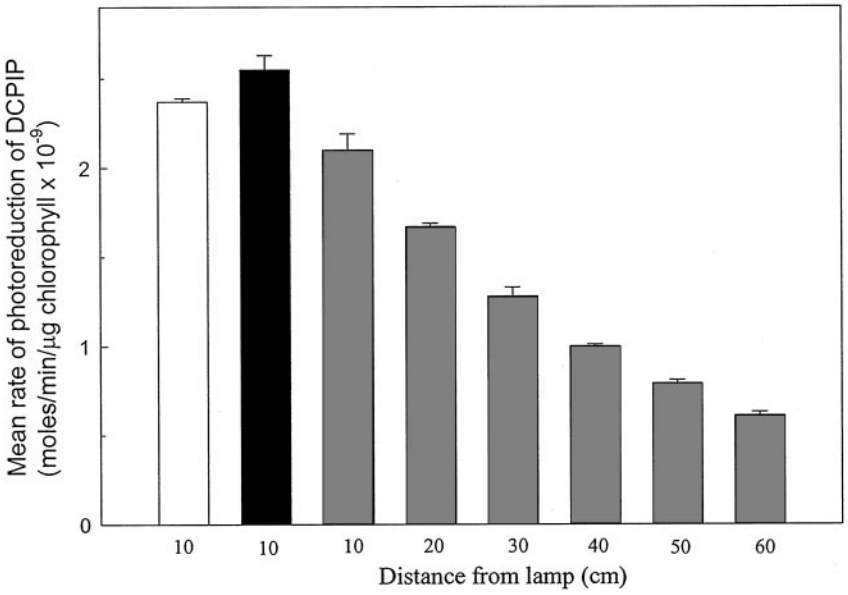


FIG. 3. **Mean rates of photoreduction of DCPIP by untreated (*white* and *black bars*) and heat-treated chloroplasts (*gray bars*) at 10, 20, 30, 40, 50, or 60 cm from a lamp (photon fluence rates of 274, 113, 61, 39, 27, or 20 μmol of photons/m$^2$/s, respectively).** Reaction mixtures contained DMSO only (*white bar*) or DPC in DMSO at a concentration of 0.5 mM (*black* and *gray bars*). The rate of transfer of electrons from DPC to DCPIP by illuminated chloroplasts declines with declining irradiance level. *Error bars* are ± 2 × S.E., $n = 4$.

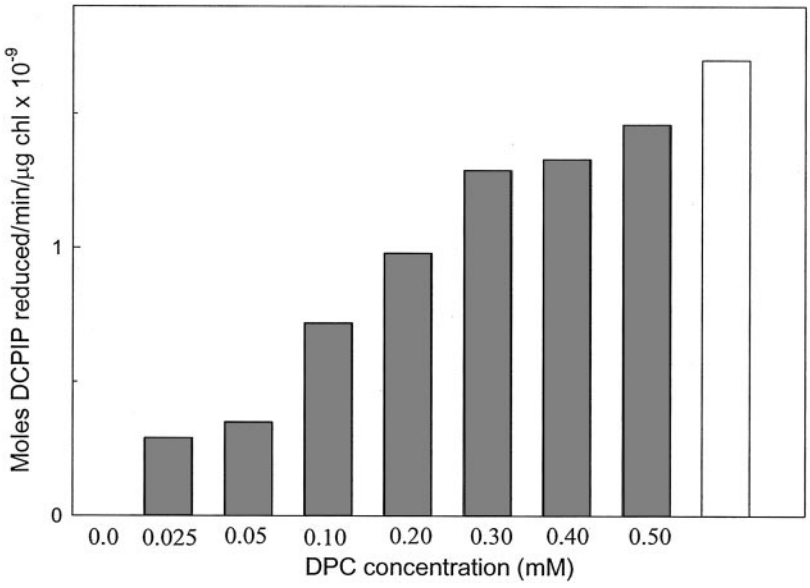


FIG. 4. **Rates of photoreduction of DCPIP, by heat-treated (*gray bars*) chloroplasts in the presence of different concentrations of DPC.** The rate of DCPIP reduction by untreated chloroplasts in the absence of DPC is included (*white bar*). All reactions were run at 20 cm from the lamp at 116 μmol of photons/m$^2$/s. The rate of DCPIP photoreduction increases with increasing DPC concentration. No DCPIP reduction is detectable in the absence of DPC.

concentration in the chloroplast preparation is increased from 10 to 20 μg but not trebled when the chlorophyll concentration is increased from 10 to 30 μg (Fig. 5*a*), and b) the rate of DCPIP photoreduction per microgram of chlorophyll is significantly lower ($p < 0.05$) in reactions involving 30 μg of chlorophyll than in those containing 10 μg (Fig. 5*b*).

vi) Mean DCPIP photoreduction rates by heat-treated chloroplasts in reactions containing 20, 30, and 40 mM DCPIP were 1.17 ± 0.07, 1.24 ± 0.03, and 1.26 ± 0.10 (±2 × S.E., $n = 4$) mol of DCPIP reduced/min/μg of chlorophyll × $10^{-9}$, respectively. The differences are not significant ($p < 0.05$).

Clearly the effect of changing the DCPIP concentration in the reaction mixture cannot be investigated using the DCPIP photoreduction assay. Because increasing the DCPIP concentration greatly increases the absorbance of the reaction mixture, the reactions are, in fact, being run at different irradiance levels.

Taken together, the last four results (iii–vi) form an instructive basis for discussions on experimental design. Simply changing one of four possible variables produces experiments of widely varying quality.

The experiment with different light levels (iii) is very clean with only one variable. Varying the DPC concentration (iv) introduces a second variable because DPC directly reduces DCPIP at a rate that at low DPC concentrations is impossible to measure. Similarly, a second variable is introduced when chloroplast quantity is varied (v) because higher chloroplast concentrations make the reaction mixtures more opaque. In the last two cases, the introduced errors are not fatal and interpretable results can be obtained. However, when the concentration of DCPIP is changed the effect of the unintended variable, that chloroplasts are exposed to different amounts of light, is insurmountable. It is not possible to quantify what is causing the measured changes.

vii) DCMU, the active ingredient of the herbicide Diuron™, binds to $Q_B$ of PS II (Fig. 1) and prevents it from becoming reduced by accepting electrons

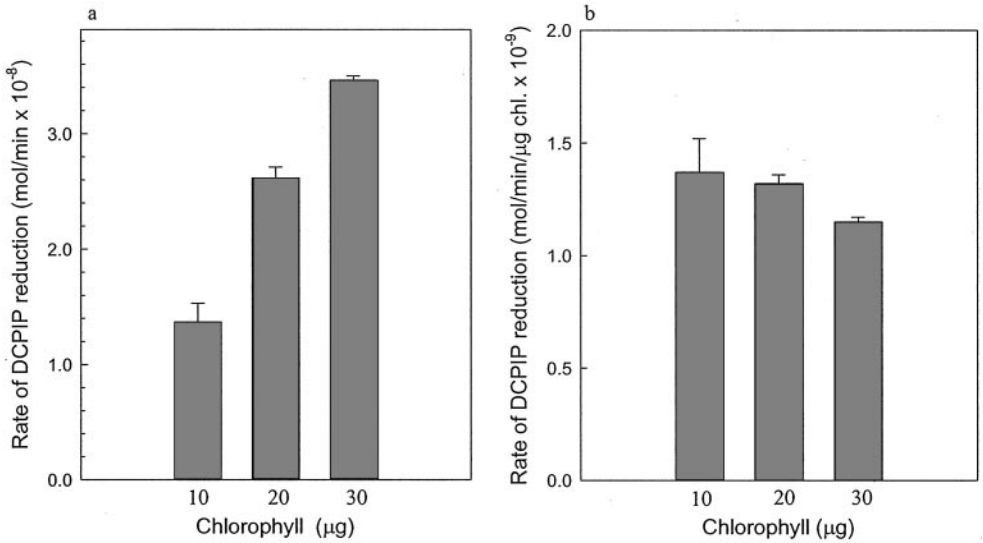


FIG. 5. **The absolute rate of DCPIP reduction (mol/min, *a*) and the rate of DCPIP reduction per microgram of chlorophyll (*b*) in the presence of volumes of chloroplast preparations containing 10, 20, or 30 $\mu$g of chlorophyll.** The reaction rate increases with increasing quantities of chlorophyll but not in direct proportion to the chlorophyll concentration. Reactions were run at 20 cm from the lamp at a photon fluence rate of 118 $\mu$mol of photons/m$^2$/s. *Error bars* are $\pm$ 2 $\times$ S.E., $n$ = 4.

from $Q_A$. As a consequence, $Q_B$ is unable to reduce the plastoquinones and DCPIP is not photoreduced. Because one molecule of DCMU binds to each $Q_B$ site, and because there are many $Q_B$ sites in each chloroplast, we expect that DCMU would inhibit the rate of photoreduction of DCPIP in a dose-dependent manner. At a concentration of 20 $\mu$M, DCMU completely prevents the photoreduction of DCPIP by untreated chloroplasts in the conditions of our assay (not shown). This concentration does not completely inhibit the reaction by heat-treated chloroplasts in the presence of DPC. The latter photoreduce DCPIP at ~9% of the rate seen in the absence of DCMU. This raises the possibility that heat-treated chloroplasts are less sensitive to DCMU than untreated chloroplasts; an idea that is supported by two trials using overlapping DCMU concentrations (Table III). At all DCMU concentrations between 0.05 and 0.5 $\mu$M, there is reduced inhibition of the photoreduction of DCPIP by heat-treated chloroplasts compared with that by untreated chloroplasts (Table III). Although the sensitivity to DCMU is apparently lower in heat-treated chloroplasts, it seems likely that DCMU is blocking the efflux of electrons from PS II because the degree of inhibition is dose-dependent.

So far, the data presented are consistent with the hypotheses that heat-treated chloroplasts do not oxidize water and that electrons derived from DPC are transferred through PS II to DCPIP in heat-treated chloroplasts via the same pathway as those derived from water in untreated chloroplasts.

Aliquots from a single chloroplast preparation were used to compare the rates of photoreduction of DCPIP, the rate of oxygen evolution and the emitted chlorophyll fluorescence by untreated samples and those exposed to 55 °C for 2.5 min. Heat treatment reduces the mean rate of oxygen evolution from 65.9 to 4.0 $\mu$mol/mg of chlorophyll/h and the mean rate of DCPIP reduction from 1.87 $\times$ $10^{-9}$ mol/min/$\mu$g of chlorophyll to zero. Heating the chloroplast preparation therefore results in a 94% reduction in the mean rate of oxygen evolution indicating that this treatment destroys the function of the OEC almost completely. Calculations based on the stoichiometry of equations for water oxidation and DCPIP reduction show that transfer of electrons from water to DCPIP through chloroplasts is ~85–90% efficient. This may explain why no photoreduction of DCPIP by heat-treated chloroplasts was observed because the rate of electron production when oxygen is being produced at 4.0 $\mu$mol/mg of chlorophyll/h falls below the threshold for detection when using the DCPIP photoreduction assay.

When isolated chloroplasts are dark adapted, $Q_A$ and other molecules in the photosynthetic electron transport chain become oxidized. Subsequent excitation by a pulse of light at a very low irradiance level (~0.07 $\mu$mol of photons/m$^2$/s) causes a transitory rise in emitted chlorophyll fluorescence to a level called the minimal fluorescence yield or $F_o$. $F_o$ represents the fluorescence emitted by excited chlorophyll *a* molecules in the light-harvesting complexes before the excitation energy is transferred to the PS II reaction centers [9]. Mean $F_o$ values for untreated, heat-treated, and heat-treated chloroplasts in the presence of DPC and DCPIP were identical, indicating that the functional integrity of the PS II light-harvesting complexes is maintained in heat-treated chloroplasts.

Maximum fluorescence yield ($F_m$) is attained when dark-adapted chloroplasts are exposed to a pulse of saturating light (~5,000 $\mu$mol of photons/m$^2$/s). The rise in fluorescence from $F_o$ to $F_m$ is termed variable fluorescence ($F_v$), which is thought to emanate from the chlorophyll *a* molecules in the PS II reaction centers. The saturating light pulse causes $Q_A$ to become fully reduced so it cannot accept electrons from the reaction center. Consequently,

TABLE III

*Summary of two trials to determine the rates of photoreduction of DCPIP by untreated and heat-treated chloroplasts in the presence of different concentrations of DCMU (the percentage of the non-DCMU treated control rates in parentheses)*

| DCMU ($\mu$M) | Rate of photoreduction of DCPIP (mol/min/$\mu$g chl $\times 10^{-9}$) | | | |
|---|---|---|---|---|
| | Untreated chloroplasts | | Chloroplasts 2 min at 55 °C[a] | |
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| 0.00 | 2.06 (100) | 1.59 (100) | 1.36 (100) | 1.21 (100) |
| 0.05 | – | 1.05 (66.0) | – | 0.95 (78.5) |
| 0.10 | 0.76 (36.9) | 0.72 (45.3) | 0.75 (55.1) | 0.73 (60.3) |
| 0.15 | – | 0.53 (33.3) | – | 0.55 (45.5) |
| 0.20 | 0.53 (25.7) | 0.46 (28.9) | 0.57 (41.4) | 0.50 (41.3) |
| 0.25 | – | 0.44 (27.7) | – | 0.48 (39.7) |
| 0.30 | 0.38 (18.4) | – | 0.49 (36.0) | – |
| 0.40 | 0.26 (12.6) | – | 0.45 (33.1) | – |
| 0.50 | 0.26 (12.6) | – | 0.41 (30.1) | – |

[a] In the presence of 0.5 mM DPC.

when excited electrons in the reaction centers fall back to their ground state, the energy is emitted as fluorescence and heat. Differences between $F_v$ values in chloroplasts treated in various ways are a measure of the functional integrity of the reaction centers and subsequently their ability to accept electrons from water or DPC. Derived $F_v$ values are high in untreated chloroplasts, much reduced in heat-treated chloroplasts, and elevated in heat-treated chloroplasts exposed to DPC. These findings indicate that the reaction centers of untreated chloroplasts are accepting electrons from water, that heat-treated chloroplasts are not accepting electrons from water (or are accepting electrons at a much reduced rate), and that heat-treated chloroplasts are accepting electrons from DPC when it is present. In summary, fluorescence studies provide evidence that, in heat-treated chloroplasts, the light-harvesting complexes are functional and that electrons are transferred from DPC to the reaction centers.

## CONCLUSION

In addition to extending the repertoire of laboratory exercises using isolated chloroplasts that are available for laboratory education, the work described in our article is highly suited to investigative studies. Clearly, students will need to prepare for such studies. Precisely how this is done will depend on whether all classes are held in the laboratory or if tutorial sessions are also timetabled. Our students spend several laboratory periods gaining necessary experience and knowledge before the investigative work is attempted. The first class is an introduction to spectrophotometry that includes how the instrument works, the relationship between transmittance and absorbance, and the use of the machine to generate absorption spectra and a standard curve. In the second class, students gain experience in estimating reaction rates by measuring the decline in $A_{600}$ over time as DCPIP solutions are reduced by different initial concentrations of sodium hydrosulfite. These data are used to calculate the rate of DCPIP reduction in moles/minute. In the third class, the instructor gives a detailed account of light-dependent electron flow in the thylakoid membrane, as outlined in our introduction, using a projected image like that in Fig. 1. They are also informed of the effect of heat treatment and DCMU on electron transport and the use of DPC as an artificial electron donor. (Students have a summary of this information in the course Resource Manual and are also referred to their current cell and molecular biology textbook). During the remainder of this class, students measure the rate of photoreduction of DCPIP by isolated, illuminated, untreated chloroplasts as a function of light quantity as previously described [1]. The fourth class is used for planning. The instructor reviews light-dependent electron transport in the thylakoid membrane and student groups are asked to think of an experimental variable to test using heat-treated chloroplasts and to predict the outcome. They discuss this in their working groups while the instructors circulate to prompt and answer questions. Students write their protocol in class. Because range-finding is time consuming, the instructors suggest irradiance levels and the concentrations of solutions to be used. The protocols, including a table to show the composition of the reaction mixtures, are approved by, or modified in consultation with, the instructors. In the next class, students perform their experiment and analyze the data. We ask our students to write up their work in the style of a scientific paper but, if time permits, it would be valuable for students to communicate their findings to the rest of the class as a poster presentation or a short, illustrated talk. An advantage of using this system for investigative studies is purely pragmatic; the experimental work we have presented can all be undertaken using limited amounts of easily prepared material. All that is required is chloroplast preparation and stock solutions of buffers, DCPIP, DPC, DMSO, DCMU, and methanol.

We also suggested that changing some of the possible experimental parameters can introduce unintended variables that make the data more difficult or, in one case, impossible to analyze. These problems are a useful basis for a discussion on experimental design.

Finally, we have only cursorily referred to the measurement of fluorescence and rates of oxygen evolution because we regard this work as unsuitable for a majority of undergraduates. This information has been included to provide course instructors with other lines of evidence that heat-treatment impairs the function of the OEC and that electrons from DPC are transferred to DCPIP through the normal pathway in the thylakoid membranes. Instructors who would like more information on the rationale, methods, or data interpretation when using these methods are encouraged to contact us by E-mail.

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