The effects of changes in light intensity on the rate of electron transport from photosystem II to artificial electron acceptor 2,6-Dichlorophenolindophenol in the light reactions of photosynthesis in *Spinacia oleracea L.*

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BIOL 240

Dr. Loni Walker

December 15, 2016

Introduction

Plants are able to reduce CO_2 into organic compounds for use in metabolism. Because plants use energy from sunlight to carry out this process, the process is called photosynthesis. [1] Photosynthesis can be divided into two major parts: the light-dependent reactions and the light-independent reactions. In the light-dependent reactions, plants use electrons from water to generate chemical energy in the form of ATP and NADPH. The energy stored in these molecules is then used to reduce CO_2 into glucose precursors in the light-independent reactions. [1]

The chloroplast is the organelle where photosynthesis occurs inside of a plant cell. The chloroplast has three membranes: an inner and outer membrane separated by an intermembrane space, and the thylakoid membrane, located interior to the inner membrane in a space called the stroma. [1] The thylakoid membrane, which surrounds a space called the thylakoid lumen, contains different pigments (compounds that absorb specific wavelengths of light), including chlorophyll a, chlorophyll b, and carotenoids. Chlorophyll a and b absorb light in the blue and red regions of the visible light spectrum and are responsible for most of the light-absorption for photosynthesis. [2] Carotenoids absorb blue-green light, though their primary function is to dissipate excess energy as heat. [2] The light-dependent reactions of photosynthesis begin upon the absorption of light by these pigments.

The light-dependent reactions occur in the thylakoid membrane and thylakoid lumen of the chloroplast. [1] Within the thylakoid membrane are light-harvesting complexes containing 200-300 chlorophyll molecules. Each light harvesting complex surrounds a large embedded protein called a photosystem, that holds a dimer of chlorophyll. [2] When radiant energy is absorbed by the pigments of the light-harvesting complex, their electrons become excited, and the energy is then transferred to the chlorophyll dimer special pair located in the adjacent photosystem, photosystem II. The transferred energy causes the electron held by the special pair to become excited. The excited electron is then transferred to a primary electron acceptor, thus oxidizing the special chlorophyll pair of photosystem II, the first of many oxidation-reduction reactions in the light reactions. [2] The

electron is passed through a series of electron carriers including plastoquinone, cytochrome b_6f, and plastocyanin via oxidation-reduction reactions, with each electron acceptor having a higher electron affinity than the previous acceptor. During electron transfer between photosystem II and cytochrome b_6f, protons are transferred across the thylakoid membrane from the stroma to the thylakoid lumen using energy released from oxidation. This generates a concentration gradient that is used to form ATP. [2] The mobile carrier plastocyanin passes its electron to the special pair of another photosystem, photosystem I. The electron is excited by energy from light absorbed by the associated light harvesting complex and reduces a number of carriers before arriving to the final electron acceptor, NADP+. Upon receiving two electrons and a proton, NADP+ is reduced to NADPH in the stroma. [2]

The light reactions are a continuous process, provided light, electrons, and sufficient substrates are present. The electrons in the light reactions come from the splitting of water (photolysis), which occurs in the oxygen-evolving complex associated with photosystem II. [1] The complex consists of several proteins surrounding a cluster of manganese (Mn) and calcium (Ca) and is capable of holding 4 electrons. [2] When the chlorophyll special pair of photosystem II is oxidized, one electron is passed from a nearby tyrosine residue to the special pair, and an electron from the Mn-Ca cluster reduces tyrosine. As photons are continually absorbed and photosystem II is oxidized, electrons travel from the Mn-Ca cluster to tyrosine to the special pair. When the Mn-Ca complex is completely empty of electrons, it acts as an oxidizing agent, pulling four electrons from two water molecules. The protons are released into the thylakoid lumen, contributing to the proton gradient for ATP synthesis, the four electrons reduce the Mn-Ca cluster, and O_2 is released to the environment as waste. [2]

In the light-independent reactions, the ATP and NADPH generated in the light reactions are used to fix CO_2 into glyceraldehyde 3-phosphate, a three-carbon precursor to starch, glucose, sucrose, or other carbohydrates needed by the plant. [2] Without the chemical energy from the light

dependent reactions, plants would not have a sufficient supply of these important compounds necessary for metabolism.

This experiment focuses on the light reactions of photosynthesis, specifically on the Hill reaction, where, in response to light, an electron acceptor is reduced by electrons from water, and oxygen is evolved. The Hill reaction is named after Robert Hill, who in 1937 discovered that this reaction can occur in the absence of CO_2, which was previously hypothesized to be the source of electrons and O_2. [3] Provided that an electron acceptor was available, O_2 generation still occurred in the absence of CO_2, giving evidence that the ultimate source of O_2 release and therefore electrons for photosynthesis is from water and not CO_2. [3]

To analyze the Hill reaction, we used an artificial electron acceptor, 2,6-Dichlorophenolindophenol (DCIP), to determine the rate of photosynthetic electron transfer under three different light intensities. Qualitatively, the reduction of DCIP can be easily seen, as the normally blue oxidized dye turns colorless when reduced. In addition, DCIP provides an ideal measurement standard, as it is a simple absorber (a substance which adheres to the Beer-Lambert law) . [4] The Beer-Lambert law, quantified by the equation $A = \epsilon L c$, states that the absorbance (A) of a sample changes proportionally as either the path length through the sample (L), the molar absorption coefficient (ϵ), or the concentration(c) of the simple absorber changes. [5] In this case, the proportionality between change in absorbance and concentration of reduced DCIP is examined. By spectrophotometrically measuring the absorbances of a solution containing thylakoids and DCIP over time, the rate of electron transfer to DCIP can be determined using stoichiometry and the molar absorption coefficient derived from previous experiments. It is expected that the lowest light intensity has the lowest rate of electron transfer to DCIP, because less intense light means fewer photons absorbed and therefore a slower electron transport chain. With increasing LUX, more photons will be absorbed, and the rate of DCIP reduction is therefore expected to increase.

Materials and Methods [4]

*All chemicals, unless otherwise specified, were purchased from Fisher Scientific.

*Samples were kept cold when possible to avoid protein denaturation.

Thylakoid membrane preparation

Approximately 25 grams of *Spinacia oleracea L* (purchased at Kroger) were washed, deveined, and blended with 100 mL of cold thylakoid buffer (250mM sucrose, 50 mM Tris*HCl pH 8.0, 10 mM NaCl, 2 mg/mL fatty acid-free BSA, pH 8.0) in a pre-chilled blender. The blended mixture was then passed through cheese cloth into a pre-chilled beaker and kept on ice until centrifugation.

The sample was transferred into a chilled centrifuge tube and centrifuged for 10 minutes at 4°C and 6750 RPM in a Beckman J2-MC centrifuge to isolate the thylakoids from the chloroplasts in the leaves. The pellet that formed was the thylakoids; the contents of the stroma remained in solution, removing stromal electron acceptors. After decanting the supernatant, the thylakoid pellet was re-suspended in 5 mL thylakoid buffer. The sample was kept on ice until centrifuging for five minutes at 4°C and 3550 RPM. The supernatant was decanted, and the pellet was re-suspended in 5 mL thylakoid buffer.

To determine the amount of chlorophyll present in the thylakoids, 0.1 mL of the thylakoid suspension, 1.9 mL water, and 8 mL acetone were added to a small flask and kept in the dark for 10 minutes following filtration through a Whatman filter. The absorption of this mixture was then read at 652 nm (average of the maximum absorptions of chlorophyll a and b) [4] on a ThermoScientific Spectronic 20D+ spectrophotometer. Using the equation: mg chlorophyll/mL = (Absorbance/34.5)*Dilution, [4] the mass of chlorophyll present per milliliter was determined and then diluted to a concentration of 0.5 mg/mL. Thylakoids were kept in the dark at room temperature for the rest of the experiment.

The effect of light and chloroplasts on DCIP reduction

LUX, or the intensity of light at various distances, was measured using a Pasco model 8020 photometer. A 0.05 mM DCIP solution prepared in thylakoid buffer was added to a cuvette. After

blanking the spectrophotometer at 600 nm, 200 µg of thylakoids were added to the cuvette. The cuvette was quickly inverted to evenly distribute the thylakoids, and the absorbance was immediately read at 600 nm (Time 0). [4] The cuvette was then moved up and down in front of an AFT fiber optic light source, Model LS87/110, at a LUX of 140, for 30 seconds before inverting and quickly re-measuring the absorbance at 600 nm. This process of light exposure, inversion, and measurement was repeated every 30 seconds for 3.5 minutes. Then, the experiment was repeated 3 more times, beginning with a new sample of DCIP, buffer, and thylakoids each time.

This portion of the procedure was repeated using two additional LUX values to determine the effect on DCIP reduction. Four trials each at LUX 120 and 90 were tested for 3.5 minutes. For controls, the absorbances of a solution with no thylakoids and a solution with no DCIP were read at each LUX at 600 nm for 3.5 minutes.

Transformation of Data: Finding Rate of Electron Transport from Absorbance

The total change of absorbance for each 30-second interval of each trial was determined by subtracting the absorbance observed at each time from the initial absorbance at time 0. Using change of absorbance, concentration (moles per liter) of reduced DCIP (C) was calculated using the Beer-Lambert equation $A=\epsilon CL$, with A= absorbance, L=length of light path through sample (1 cm), and $\epsilon= 8000$, the molar absorption coefficient determined previously. [4] Moles of DCIP reduced was determined by dividing the concentration of reduced DCIP by 0.0005 liters, the volume of DCIP used in each solution. This number was then converted to nanomoles by multiplying by 10^9. For each trial, nanomoles of DCIP reduced was plotted against time and analyzed using linear regression with an intercept of 0. The slope of each trial's trendline was the rate of DCIP reduction in nanomoles per minute. DCIP requires 2 electrons for reduction, so multiplying the rate of DCIP reduction by 2 yielded the rate of electron transport.

The average rate of DCIP reduction for each LUX was determined by averaging the slopes of the trendlines of all four trials for that LUX. Percent deviation and standard deviation were calculated to examine precision between trials of DCIP reduction at a given LUX. Three separate two-tailed unpaired t tests between LUX values were used to determine the significant difference, if any, between the average rates of DCIP reduction at LUX 140, 120 and 90.

Results

Figure 1 depicts the nanomoles of DCIP reduced versus time at LUX 140. Trials 1-4 at LUX 140 demonstrate high precision, as the nanomoles of DCIP reduced at two minutes ranges from 23.875 to 25 nanomoles (Fig. 1). The slope of the trendline gives the rate of DCIP reduction. The rate of DCIP reduction of Trial 3 is the highest, at 11.792 nanomoles per minute, while Trial 1 is the lowest, at 11.458 nanomoles per minute (Fig. 1). The average percent deviation between the rates of trials was 1.6%, and the standard deviation was 0.133, again confirming the high precision between trials. Though samples were tested for 3.5 minutes, most trials demonstrated a decrease in the rate of DCIP reduction after 2 minutes, likely because only a small amount of DCIP remained available to be reduced (data not shown). Due to this, the time used for data presentation and analysis is 2 minutes.

Figure 2 shows the nanomoles of DCIP reduced versus time at LUX 120. Trials at LUX 120 were considerably more variable than the other two LUX values. Nanomoles of DCIP reduced at 2 minutes ranged from 10.625 to 13.75 nanomoles, and rates of DCIP reduction ranged from 5.375 nanomoles per minute in Trial 4 to 6.6667 in Trial 1 (Fig. 2). The average percent deviation was 12.1%, and the standard deviation was 0.490, confirming the relatively low precision in comparison with the other two LUX.

Figure 3 depicts the nanomoles of DCIP reduced versus time at LUX 90. Contrary to what was expected, nanomoles of DCIP reduced at LUX 90 is quite similar to LUX 120, ranging from 11.25 to

11.875 nanomoles at two minutes (Fig. 3). The lowest rate of DCIP reduction was 5.7083 nanomoles per minute in Trial 2 and the highest was 5.875 in Trials 1 and 4 (Fig. 3). LUX 90 demonstrates the highest precision, with an average percent deviation of 1.5%, and standard deviation of 0.068.

Figure 4 shows the average rate of DCIP reduction for each LUX. Though LUX 90 and 120 differ by 30 units of light intensity, no statistical difference was observed between their average rates. The average rates of DCIP reduction were 11.602, 6.010425, and 5.8229 nanomoles per minute, for LUX 140, 120, and 90, respectively (Fig. 4). The results of the t tests between average rates of DCIP reduction are as follows, with degrees of freedom equal to 6 in all three t tests. Between LUX 140 and 120, t value=31, and $p < 0.001$. Between LUX 140 and 90, t value=110, and $p < 0.001$. Between LUX 120 and 90, t value=1.1, and $p > 0.20$. As before, the time used for calculations is 2 minutes. The lack of statistical difference between LUX 90 and 120 was not expected; rather, the rate of LUX 120 was expected to be a statistically different rate between those of LUX 90 and 120.

Discussion

The rate of electron transport was expected to increase with increasing LUX, because increased LUX means an increased amount of light for absorption by the light-harvesting complex. Increased photon absorbance was expected to cause increased oxidation of photosystem II, which produces a higher rate of DCIP reduction. [4] The highest LUX has the highest rate of DCIP reduction, as expected; however, the statistically insignificant rates ($p > 0.20$) for LUX 120 and 90 were not anticipated (Fig. 4). Rather, the rate of electron transport at LUX 120 was expected to be a statistically different rate between the rates at LUX 140 and 90.

Sources of Error

Although the lack of statistical difference ($p > .20$) between the rate of DCIP reduction at LUX 120 and 90 was surprising, in hindsight a combination of investigative errors (including imprecise technique or holding the thylakoids at the wrong LUX) could be responsible for the observed results.

Using the standard deviations of the trials presented in Figures 1-3, one can easily see that, of the 3 LUX values tested, the trials at LUX 120 exhibited the least precision, with a standard deviation of 0.490 (Fig. 2). One possible source of this imprecision is the pipetting of thylakoids. Pipetting more thylakoids would have caused a higher initial absorbance and faster rate of DCIP reduction, perhaps explaining why the rate of Trial 1 at LUX 120 was 6.6667 nanomoles per minute as compared to the other trials' rates of 6.25, 5.75, and 5.375 nanomoles per minute (Fig. 2). Conversely, pipetting a smaller volume of thylakoids would lead to a slower rate, perhaps explaining why Trial 4, at 5.375 nanomoles per minute, was below the other rates (Fig. 2). Similarly, inconsistent inversion of the cuvette containing the thylakoids could have generated some imprecision. If the thylakoids weren't mixed thoroughly, the absorbance would be lower and the rate would be slower. Perhaps if these errors were resolved and LUX 120 was more precise, we would have observed a statistical difference between LUX 120 and 90, though this would need to be verified by repeating the experiment.

Though LUX 90 was expected to have the lowest rate of DCIP reduction, it also exhibits the greatest precision between trials (Fig. 3), suggesting that conditions between trials for this LUX were kept relatively uniform. Therefore, the underlying error is more likely something held consistent, rather than micropipetting error or other errors in technique observed for LUX 120.

Due to the high precision of measurement for all trials and the fact that LUX 90 and 120 yielded nearly identical rates of electron transport (Fig. 4), the most apparent possible source of error is that both were in fact measured at the same actual LUX. Distances from the light source corresponding to specific LUX values were marked with tape. The cuvette could have mistakenly been held at the distance corresponding to LUX 120 or 90 for both trials. Or, alternatively, the light source could have been bumped towards the mark for LUX 90, thus decreasing the distance and increasing the intensity of light, explaining the highly precise yet inaccurate measurements.

__Further Investigation: Determination of DCIP reduction site using three photosynthesis inhibitors__

To further investigate the properties of DCIP as an electron acceptor, three photosynthesis inhibitors (DCMU, DBMIB, and paraquat) can be used to determine the location of DCIP reduction in the electron transport chain. DCMU is known to inhibit the reduction of the mobile electron carrier plastoquinone, DBMIB inhibits electron flow to cytochrome b_6f, and paraquat blocks electron flow to ferrodoxin. [6] By observing the change in absorbance of samples containing both DCIP and one inhibitor, the approximate location of DCIP reduction can be determined, because the absorbance will stop decreasing when reduction is not occurring. To resolve the possible sources of error of the DCIP experiment, extra care will be taken to hold the cuvette at the correct distance and to not bump or disturb any equipment.

__Conclusion__

Though the rate of electron transport to DCIP was expected to decrease as LUX decreased, this trend was only partially observed in the results of this experiment, even though our analysis suggests the measurements we made were relatively precise. This suggests the underlying error was something held consistent, such as holding the cuvette at the same location for LUX 120 and 90. Due to the unexpected results of this experiment, the procedure should be performed again in order to verify whether the rate of DCIP reduction at LUX 90 and 120 is truly nearly identical and not statistically different.

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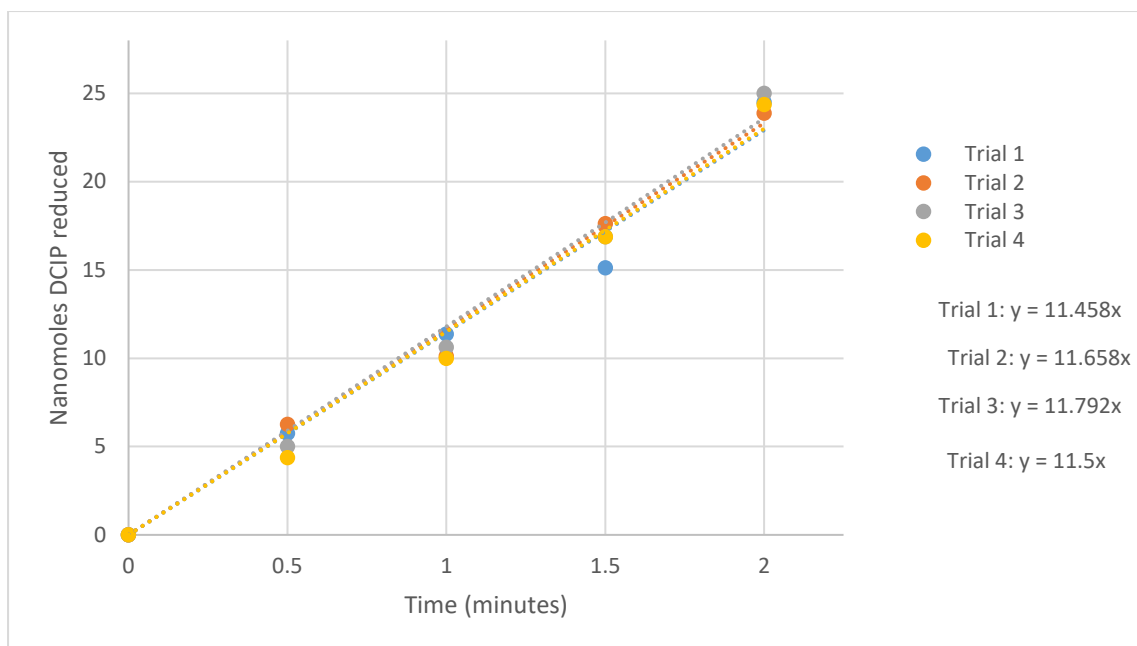
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Figure 1: Nanomoles of DCIP reduced at LUX 140. The number of nanomoles of DCIP reduced at two minutes was highly precise between trials, with values ranging from 23.875 to 25 nanomoles. The rate of DCIP reduction is given by the slope of the trendline; thus, rates of DCIP reduction ranged from 11.458 to 11.792 nanomoles per minute. The average percent deviation between trials was 1.6%, and the standard deviation was 0.133. Each sample was prepared with thylakoid buffer to a concentration of 0.05 mM DCIP. N=1.
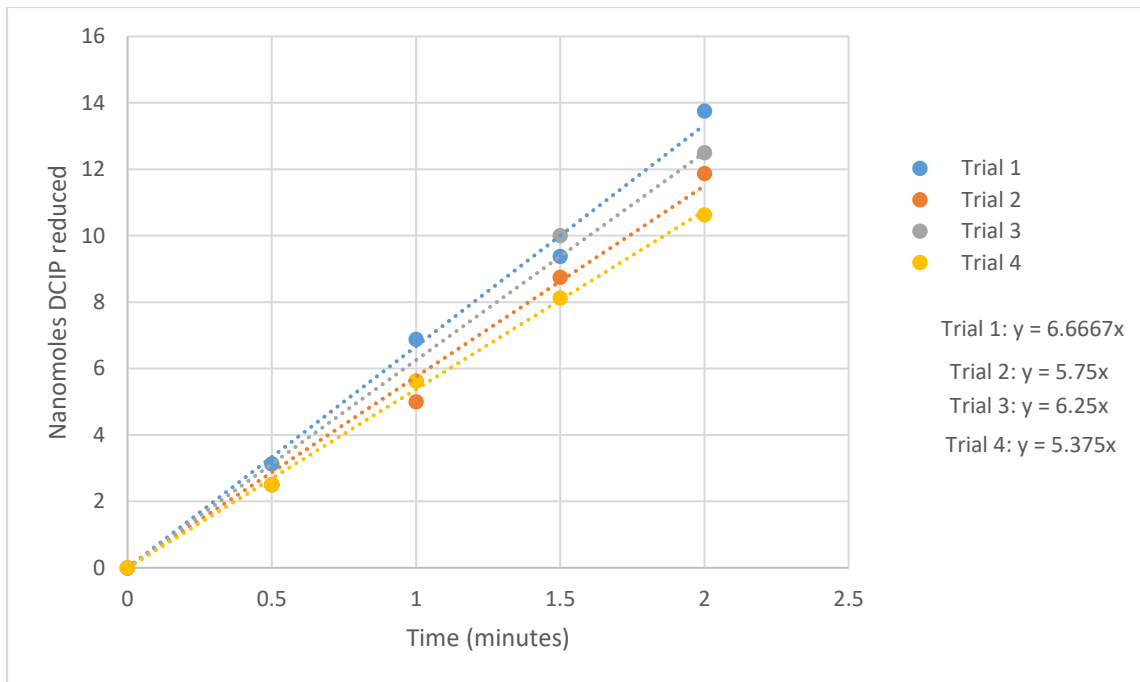
Figure 2: Nanomoles of DCIP reduced at LUX 120. Nanomoles of DCIP reduced at two minutes showed more variation than LUX 140 and 90, ranging from 10.625 to 13.75 nanomoles. The rates of DCIP reduction, equivalent to the slope of the trendline, range from 5.375 to 6.6667 nanomoles per minute. The average percent deviation between rates of DCIP reduction was 12.1%, and the standard deviation was 0.490, the highest of all 3 LUX. All samples were prepared in thylakoid buffer to a concentration of 0.05 mM DCIP. N=1.
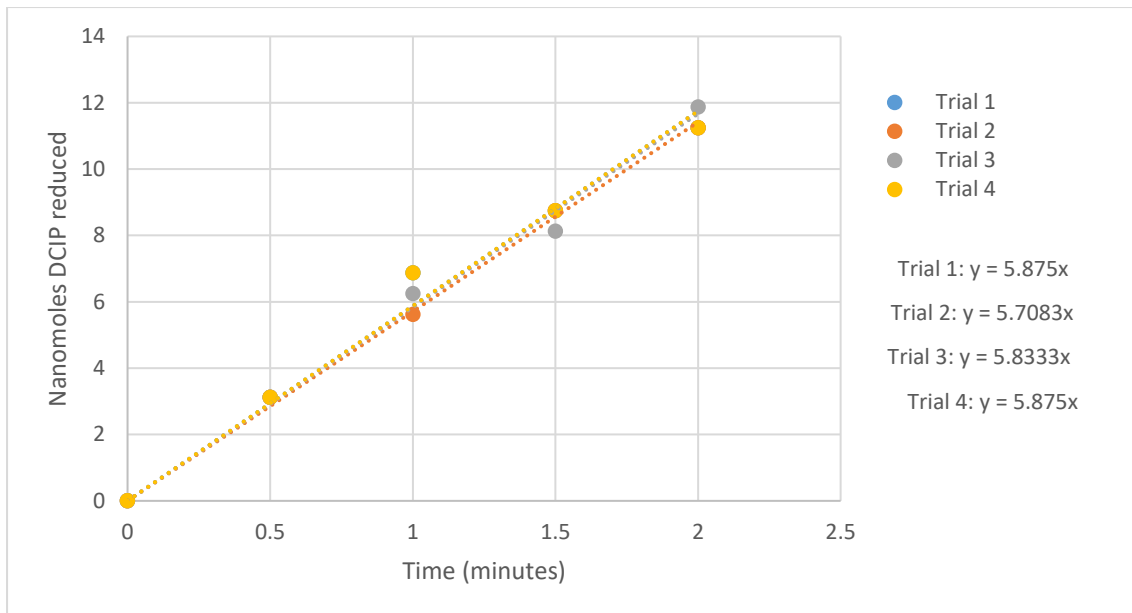
Figure 3: Nanomoles of DCIP reduced at LUX 90. Precision between trials at LUX 90 is much greater than LUX 140 and 120, and the number of nanomoles of DCIP reduced is quite similar to that at LUX 120. The number of nanomoles reduced at 2 minutes had a smaller range than LUX 140 and 120, ranging from 11.25 to 11.875 nanomoles. The rate of DCIP reduction, given by the slope of the trendline, ranged from 5.7083 nanomoles per minute in Trial 2 to 5.875 nanomoles per minute in Trials 1 and 4. The average percent deviation between rates was 1.5%, and the standard deviation was 0.068, significantly smaller than LUX 140 and 120. All samples were prepared in thylakoid buffer to a concentration of 0.05 mM DCIP. N=1.
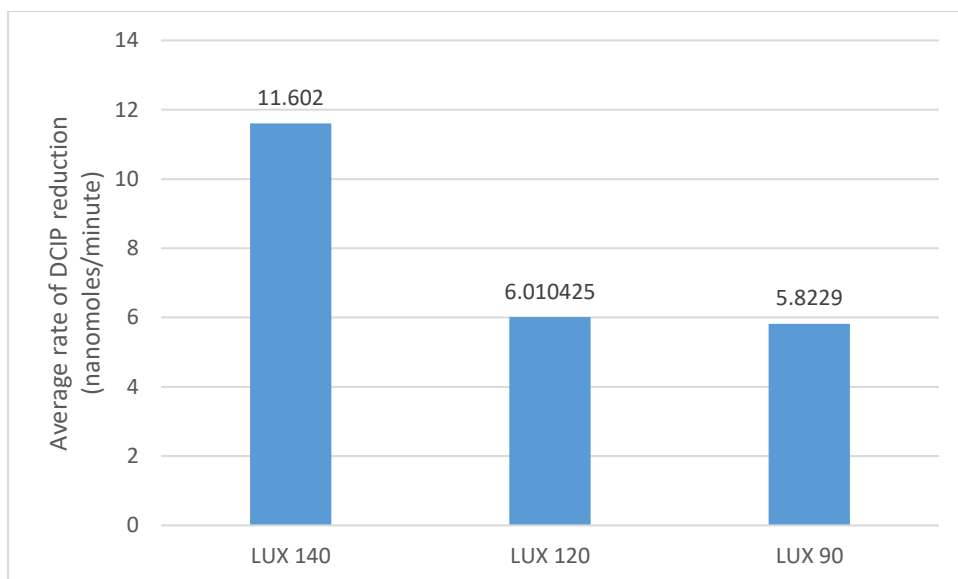
Figure 4: Average rate of DCIP reduction at LUX 140, 120, and 90. The average rates of DCIP

reduction at LUX 120 and 90 are quite similar, differing by only 3.1% despite the 30-unit difference in

light intensity. The average rates of DCIP reduction were 11.602, 6.010425, and 5.8229 nanomoles

per minute for LUX 140, 120, and 90, respectively. The t value between LUX 140 and 120 is 31, and p

< 0.001. The t value between LUX 140 and 90 is 110, with p < 0.001. The t value between LUX 120

and 90 is 1.1, and p > 0.2. Thus, there is a sharp decrease and statistical difference between rate of

DCIP reduction at LUX 140 and 120, but no statistical difference between LUX 120 and 90. Degrees of

freedom= 6 for all three t-tests. All samples were prepared in thylakoid buffer to a concentration of

0.05 mM DCIP. N=4 for each LUX.