Northern Illinois University

Regulation of Rubisco in Purple Nonsulfur Bacteria

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Honors Thesis Abstract
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ABSTRACT:

Rubisco is an enzyme that catalyzes the first reaction in the Calvin Cycle in photosynthesis. Since it is a poor catalyst, research has attempted to improve the rate at which it fixes carbon because this could in turn increase crop yields. In plants, Rubisco is regulated by light but the evolution of regulatory proteins and mechanisms is unknown. By studying Rubisco activity in photosynthetic bacteria, the origins of its regulation can be explored. We grew purple nonsulfur bacteria on two main substrates and used a culture grown in constant light conditions as a control. The experimental culture was grown in cyclic light and dark conditions to imitate the natural environment. Enzyme assays were performed on samples taken from both the experimental culture during both the light and dark parts of the cycle. We then analyzed the assay in terms of how much carbon was fixed per milligram of protein per hour. We found no evidence of inhibition of Rubisco in the dark. However, we conducted experiments in which we found a set of variables that gave optimal Rubisco activity.

Introduction

In a world where populations are growing at rates that will double the number of people well within our lifetimes, the need to find ways to support the burden is becoming more urgent. One approach is to try to prevent births with increased birth control awareness, but another approach is to try to increase food supply without increasing agricultural land use. Many scientists all over the world are researching ways to manipulate the way plants convert sunlight and water into carbohydrates to boost crop yields. Photosynthesis is the complex process by which plants essentially turn solar energy into molecules fit for consumption and metabolism by animals. There are two major sets of reactions that take place, one is light dependent and one is light independent. My research has focused on the so-called dark reactions, specifically within the carbon reduction cycle, or Calvin cycle. In particular, we are looking at an enzyme that catalyzes the first reaction in the Calvin cycle. This enzyme is not only the most abundant protein on earth but also a very poor catalyst, thus leading scientists to believe they can increase its activity (Mann, 1999). We are interested in finding out the evolution of certain molecules that regulate the activity of this enzyme.

Ribulose-1,5-bisphosphate carboxylase/oxygenase, more frequently referred to as RuBisCo (or Rubisco), is the enzyme of interest, and it is found in most photosynthetic organisms. The Calvin cycle is not only operates in plants but

also in more primitive organisms such as cyanobacteria and several photosynthetic bacteria. Molecules that regulate Rubisco are known in plants, and if the origin of those regulators can be found in organisms ancestral to plants, then the operation of Rubisco in relation to all the other compounds with which it interacts may be better understood. Hence, an approach to increasing the rate of carbon fixation in plants, and thus increasing crop yield may be elucidated and utilized.

Rubisco Catalysis and Regulation

Rubisco is involved in making the first product of the Calvin Cycle and its activity is rate limiting for the dark reactions. Rubisco works by adding CO2 to Ribulose-1,5-bisphosphate (RuBP), a 5-keto sugar. An unstable 6-carbon intermediate forms, then hydrolyzes to two molecules of 3-phosphoglycerate (3-PGA). However, Rubisco is a bifunctional enzyme, meaning it catalyzes two main reactions. Besides carboxylating RuBP, it also oxidizes RuBP via RuBP oxygenase.

RuBP + CO2→2 3-PGA RuBP + O2→ 3-PGA + phosphoglycolate

Our experiments have been designed to eliminate competition from the oxygenase reaction and focuses on carboxylation of RuBP catalyzed by Rubisco.

In plants Rubisco and other key enzymes involved in carbon fixation are active in the light and inactive in the dark. Rubisco first must be converted to an

active form through the process of carbamylation. **Figure 1** shows how Rubisco interacts with various molecules to form its activated or deactivated state. Carbamylation involves the addition of Mg2+ and non-substrate CO2 to the enzyme. In the active form, the CA1P inhibitor can bind to and inhibit Rubisco. RuBP can also inhibit Rubisco by binding to Rubisco that has not undergone carbamylation. Yet another enzyme, Rubisco activase, can reactivate Rubisco in the light by facilitating dissociation of CA1P and RuBP from the enzyme (Portis 1992). Regulation of Rubisco is not well characterized in organisms ancestral to plants, such as photosynthetic bacteria.

Purple Non-Sulfur Bacteria

Our research uses two species of photosynthetic purple nonsulfur bacteria, *Rhodobacter sphaeroides* and *Rhodospirillum rubrum*. They are gram negative and contain bacteriochlorophyll pigments, which color them brown and pink, respectively. They are found in the anoxic zone of stratified lakes where light still penetrates or in shallow ponds associated with algae. They are some of the most metabolically diverse organisms on earth since they can grow aerobically or anaerobically, with or without light, and with various carbon sources.

Materials and Methods

Growth of *R. rubrum* and *R. sphaeroides*

We chose the set of conditions that would allow the bacteria to grow anaerobically and photoheterotrophically. Oxygen was excluded from the system, and illumination as well as a carbon-containing substrate were provided. We used two kinds of substrates: malate and butyrate (with and without bicarbonate). The carbon in the substrate served as the electron donor instead of atmospheric CO_2 (Tabita 1988, 1995). The medium contained 10%$(NH_4)_2SO_4$, 0.5%$FeSO_4*7H_2O$, 1%EDTA, 20%$MgSO_4*2H_2O$, Trace elements, 7.5%$CaCl_2*H_2O$, 1/20 diluted Biotin, and 10% Malic acid or Butyric acid. 0.1%Thiamine*HCl and Niacin were also added for *R. sphaeroides* only. The solution then was brought up to pH 6.8 before adding 0.64M phosphate buffer.

Using pure cultures, we grew the bacteria in smaller containers, transferring them to larger containers as they became dense, finally ending up with one liter cultures. Each time we transferred the culture we used a 10% innoculation. One was set in front of spotlights for a constant light-grown control. The second bottle was set in a dark photoshed with lights connected to a timer, set to switch on and off every 12 hours. Growth rates of the cultures were monitored starting with 300 ml side arm flasks. For this we used the Spectronic 20 set at 600nm because it reads absorbency directly. Once the cultures were innoculated into one-liter bottles, samples were removed with a syringe using sterile technique in a laminar flow hood. These samples were read from a 3-ml cuvette in a spectrophotometer on 600nm light.

Preparation of Cells

We did not harvest the culture grown in constant light. However, the culture grown in the photoshed was harvested twice, once after it had been in the light for at least 5 hours, and once after it had been in the dark for at least 5 hours. The entire harvesting procedure was executed in a darkened lab for the half of the culture taken during the dark part of the cycle. For the remaining culture taken in the light, normal laboratory illumination was used.

First, the bottle was wrapped in foil before being taken from the shed. In a dark room, some of the liquid was quickly poured into a GSA 250 bottle while the airspace of the 1-liter bottle was gassed with nitrogen then capped to avoid contact of the culture with air. The GSA bottle was centrifuged with another bottle to balance for ten minutes at 7000 rpm in the GSA rotor in the refrigerated centrifuge. After pouring off the liquid supernatant 5ml nitrogenated distilled water was added, the airspace was gassed, then it was vortexed to resuspend. The liquid was then poured into a 40ml tube and centrifuged again in the smaller rotor for ten minutes, followed by another removal of the supernatant, resuspension, and centrifugation for 5 minutes. Finally, it was resuspended a final time so the ratio of cells to water was one to one. All the while, the containers were gassed with nitrogen. The suspension was poured into labeled cryotubes and frozen immediately in liquid nitrogen.

Preparation of Cell Extracts

First the frozen samples of bacteria were thawed, then about 0.25ml of the suspension was transferred to a microfuge tube which was microfuged for

one minute. We discarded the supernatant and weighed the pellet. To the pellet, 0.75ml TEMB assay buffer and 0.5ml toluene was added. The assay buffer consisted of 50mM Tris HCl, 1mM EDTA, 20mM $MgCl_2$, and 10mM $NaHCO_3$, all brought to a pH of 8. The suspension was vortexed slowly for three minutes, then set on ice for ten minutes. The tube was centrifuged as before and after the supernatant was aspirated off the sides of the tube were swabbed to remove residual toluene. We resuspended the bacterial pellet in 0.3ml assay buffer and incubated it at room temperature for ten minutes to activate the Rubisco. The sample at that point was ready to be assayed.

Enzyme Assay

Vials were numbered so that there were quadruplicates for each sample, four for light harvested and four for dark-harvested bacteria, and two blanks. The blanks contained only assay buffer and radioactive bicarbonate. The other eight had assay buffer, RuBP, and radioactive bicarbonate. We capped each vial with a rubber stopper after adding the radioactive bicarbonate and placed all vials in a 27° water bath. Using a stopwatch, the assay was timed to be 60 seconds from when the bacterial solution was injected to when the glacial acetic acid was added. Once each vial had undergone the one minute assay, the rubber stoppers were removed under a flow hood overnight allowing the radioactivity to escape as gaseous CO_2.

The next day the vials were dried in the Savant Speedvac and then we added detergent and scintillation liquid. Each vial was fitted with caps with foil inserts and placed in the mini rack in the scintillation counter. A printout of the

counts per minute was obtained for acid stable radioactivity present as 14-C PGA.

Protein measurement

After the Rubisco assay was performed, the remaining bacterial samples were used to determine the amount of protein present. A standard curve was made using bovine serum albumen (BSA) made up to 2mg/ml. This required 13 numbered test tubes and varying amounts of distilled water and BSA per tube. Next, triplicates were prepared for each bacterial sample using 5 or 10μl of bacterial solution per tube along with 95 or 90μl distilled water. A solution of Biorad and distilled water in a 1:5 ratio was mixed and 3ml of this solution was added to each tube then vortex briefly. Each tube was read in the spectrophotometer at optical density 595 to get the absorbencies; then the BSA standard absorbencies were plotted against μg protein. From the plot, μg protein were read across for the bacterial solutions according to their absorbencies.

Results

Growth patterns for *R.sphaeroides*

We grew cultures on three main media: malate (**Fig2**), butyrate plus bicarbonate (**Fig3**), and butyrate (**Fig4**). Absorbency versus time revealed sigmoid curves with lag, log, and stationary phases. For each graph, two sets of data were plotted and thus two lines resulted, one for a culture grown in constant light conditions, and one in cyclic light/dark conditions. The constant light culture

served as a control and we could use it to compare how a light and dark diurnal cycle affect growth. By obtaining absorbency readings during the dark part of the cycle, we found that the cells multiplied only in the light. They grew logarithmically until they reached a maximum density, then cells precipitated, and absorbency leveled off. They grew fastest on malate, reaching stationary phase around 72 hours into the culture. When grown on butyrate with bicarbonate, stationary phase was reached around 100 hours. However, if the medium consisted of butyrate without the added bicarbonate, the constant light culture topped out at about 30 hours into the culture and the light-dark cycle culture grew very slowly with the highest density reached at about 72 hours. After the culture density topped out without bicarbonate, they dropped down significantly without leveling at a stationary phase. According to Tabita (1995), the cells require bicarbonate added to the butyrate medium in order to grow. We were able to grow the bacteria without bicarbonate, although not as well as with the bicarbonate.

A culture's growth can be monitored and plotted to determine a specific point at which to harvest. For instance, if a sample of log-phase cells is desired, a plot can be drawn and when they reach a high density but the curve still appears to rise without leveling off, then it is ready to harvest. If a stationary phase harvest is desired, the culture can be monitored so that how many days into stationary it is can be determined.

Microscopic appearance of *R. sphaeroides* cells

We took light micrographs of cell suspensions using Normarski optics. We were interested to see the actual cells that we had recently ordered from the American Type Culture Collection. In many cases biochemical researchers do not observe their cultures of bacteria under a microscope. Under a phase contrast microscope, the cells appeared to have different morphologies. However, the light micrographs gave a much different picture. Here, the cells appeared rod-shaped, occasionally with rods linked in chains of two or three. These are the fields that appear in **Figure 5**.

Rubisco assays

Part of my work with *R. sphaeroides* and *R. rubrum* was to determine the best methods for obtaining optimal Rubisco activity.

We experimented with ways to permeabilize the cells for enzyme extraction. When we compared toluene treatment to sonication (Table 1), we found that the toluene did a better job than sonication, which ruptures the cell membrane with high frequency waves. Toluene permeabilizes the cell membrane thus allowing the RuBP to interact in the cell interior and Rubisco.

Table 1: Rubisco activity of *R. sphaeroides* toluene vs. sonication treatments

Treatment	µmol CO2/mg protein/h
Toluene LT	0.102 ± 9.2
Sonicated LT	0.890 ± 5.7
Toluene DK	0.123 ± 16.3
Sonicated DK	0.998 ± 6.0

DK=dark-harvested, LT=light-harvested

Since our data showed that higher activity was achieved with toluene-treated cells, in subsequent experiments we used toluene to prepare bacterial solutions for the enzyme assay. Once we decided to use toluene, we then experimented with amount and length of treatment. We concluded that a ratio of 1:2 bacteria to toluene volumes resulted in the most Rubisco activity, and there was no difference in activity whether we let the cells incubate in toluene for 10 or 30 minutes.

One experiment was to find the most appropriate assay buffer. We compared TE to TEMB buffers and found the TEMB yielded the highest Rubisco activities (Table 2). The difference between the two is TEMB contains MgCl2 and NaHCO3 in addition to the common ingredients of Tris-HCl and EDTA. These data indicate that magnesium and bicarbonate may be necessary to activate Rubisco in bacteria as it is in plants.

Table 2: Rubisco activity of *R. sphaeroides* treated with TE v. TEMB buffers

Treatment	µmol CO2/mg protein/h
TE DARK	0.0822 ± 0.01
TEMB DARK	0.0938 ± 0.008
TE LIGHT	0.0592 ± 0.004
TEMB LIGHT	0.0686 ± 0.011

TE=TrisHCl/EDTA, TEMB=TrisHCl/EDTA+Magnesium+Bicarbonate

Light/Dark regulation of Rubisco in *R. sphaeroides* and *R. rubrum*

Numerous experiments were carried out to test whether inhibition of Rubisco occurs while cells are subjected to dark conditions.

Using *R. rubrum*, we tested whether illumination of dark-harvested cells during the assay of Rubisco would affect the activity as compared to dark cells assayed in the dark. We concluded that illumination did not make a difference in Rubisco activity (Table 3). The "dark + light" sample was dark harvested but illuminated with a bright light during the assay. There appears to be no difference from the samples assayed in total darkness.

Table 3: *R. rubrum* Rubisco Activity: stationary phase, butyrate + HCO3

Sample	Rubisco Activity µmol CO2/mg protein/h
Light	0.5115 ± 0.071
Dark	0.4615 ± 0.053
Dark + Light	0.4100 ± 0.021

Another variable that we experimented with was the phase of growth at the time of harvest. In the past, other students had been using samples that were all harvested when the cultures had grown well into stationary phase. This practice

was questioned when we looked at growth curves that showed precipitation of cells and drop off of growth after entering stationary phase. Table 4 shows Rubisco activity of the same species on the same substrate, but the cells had been harvested in late log phase or stationary phase.

Table 4: *R. sphaeroides* Rubisco Activity, malate late-log v. stationary

Sample	Growth Phase	Rubisco Activity µmol CO2/mg protein/h
Light	Log	1.454 ± 0.0680
	Stationary	0.56 ± 0.0170
Dark	Log	1.86 ± 0.26
	Stationary	0.79 ± 0.125

The log phase cells produced significantly more Rubisco activity than the stationary cells.

Table 5: Rubisco Activity in *R. sphaeroides* and *R. rubrum*, stationary butyrate plus bicarbonate

Species	Sample	Rubisco Activity μmol CO2/mg protein/h
sphaeroides	light	0.549 ± 0.016
	dark	0.384 ± 0.008
rubrum	light	0.6155 ± 0.028
	dark	0.4512 ± 0.003

When the two species were grown on the same medium and assayed at the same time, we compared the Rubisco activity. In this particular experiment (Table 5), there seems to be a difference between the light and dark samples. But there does not seem to be a difference between the two species.

Conclusion

My research has revealed several important ideas. It can be understood from experiments using log phase bacteria that those samples yield higher levels of carbon fixation than stationary phase cells. Future studies of Rubisco should be conducted with log phase samples. Furthermore, after conducting several experiments in which we illuminated dark samples with light during the assay, it seemed that there was no difference in Rubisco activity with the sample harvested and assayed in total darkness. Future assays may be done in the light which may lead to more accurate results.

According to Tabita there is more Rubisco synthesized when cells are grown on butyrate plus bicarbonate than on malate but cells grow faster on

malate (1988, 1995). When comparing the effects of the media on Rubisco activity, my research did show that cultures grow faster on malate (Figures 4 and 5); the malate culture reached stationary phase at about 60-65 hours and the butyrate plus bicarbonate culture reached stationary at about 100-105 hours. But data in tables 3, 4, and 5 indicate that higher activities were found in cells grown on malate, with an average fixation rate of 0.5 μmol CO2/mg protein/hour, than on butyrate plus bicarbonate, which gave an average rate of 0.8 μmol CO2/mg protein/hour.

In my work I did not find evidence of dark inhibition of Rubisco in these species of purple nonsulfur bacteria. In fact, most of the time the activity rates of Rubisco turned out slightly higher in dark harvested samples when compared to light harvested samples from the same culture. This finding contradicts the work of previous students. More experiments will need to be conducted on *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* before conclusions can be drawn regarding regulation of Rubisco in purple nonsulfur bacteria.

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Figure 1: Rubisco activation in plants

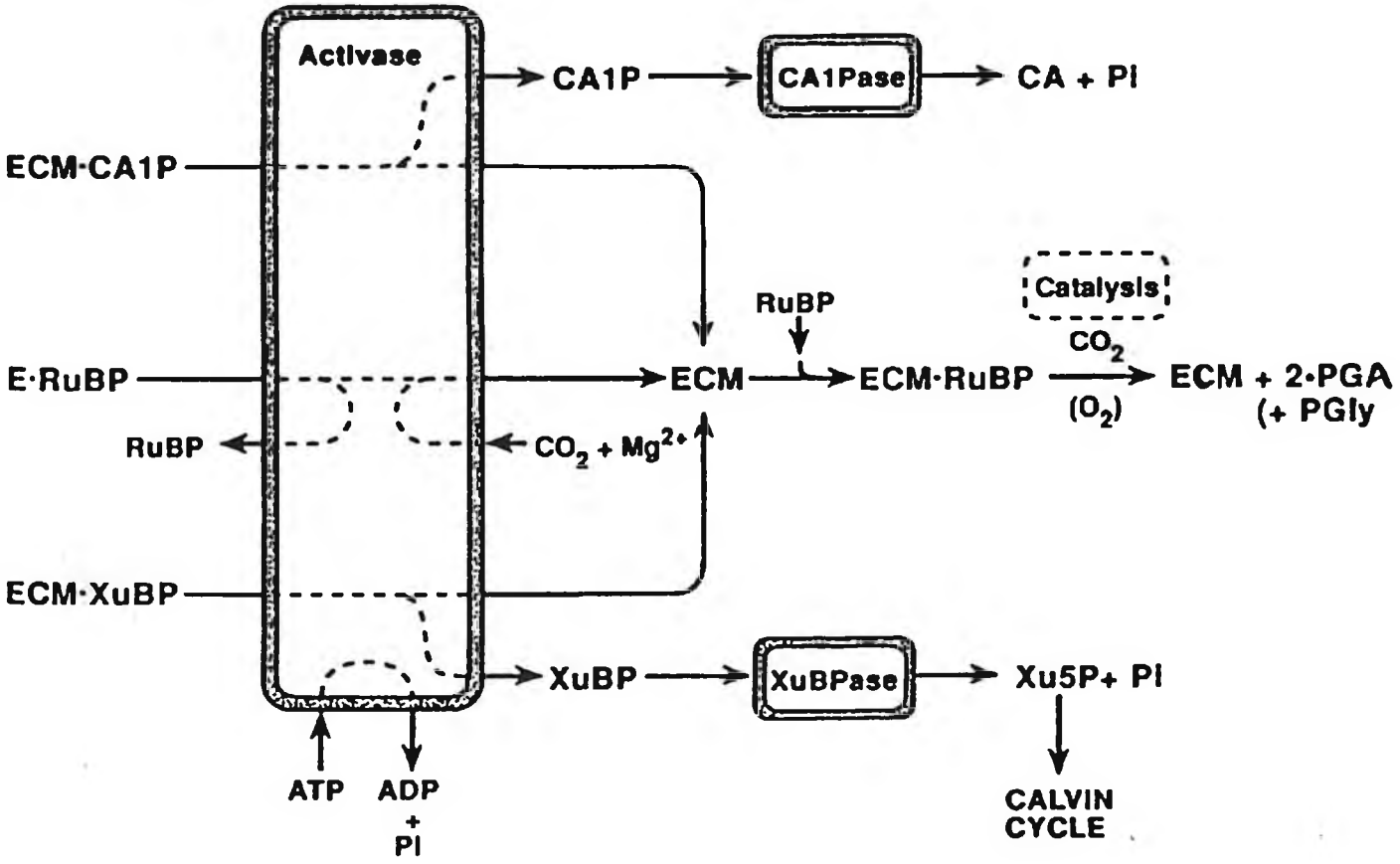

Figure 2: *R. sphaeroides* 1L malate cultures
Constant light vs. light-dark 11/16-11/18/98

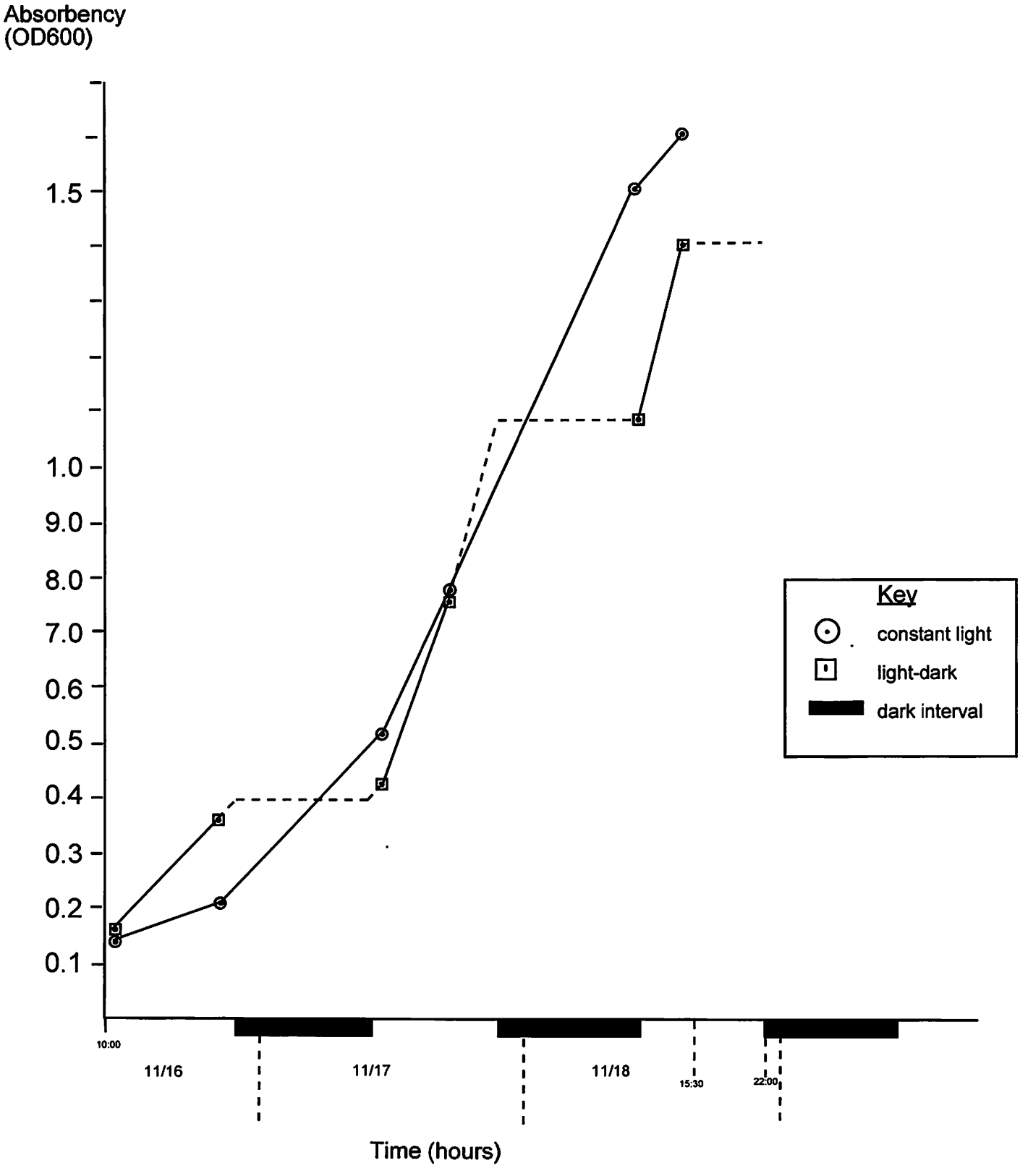

Figure 3 *Rhodobacter sphaeroides* growth curve: butyrate plus bicarbonate

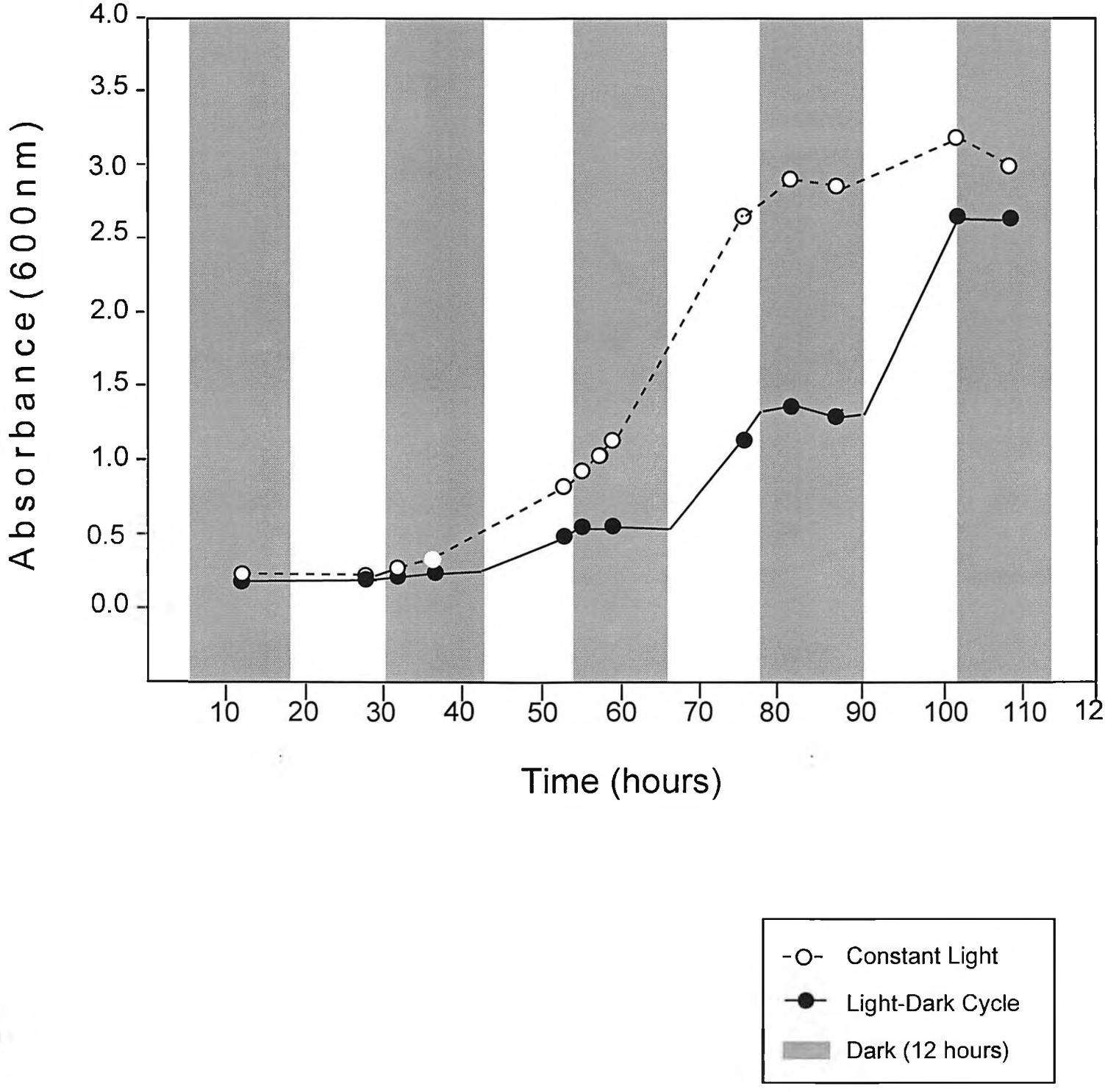

Figure 4 ***Rhodobacter sphaeroides*** **growth curve: butyrate without bicarbonate**

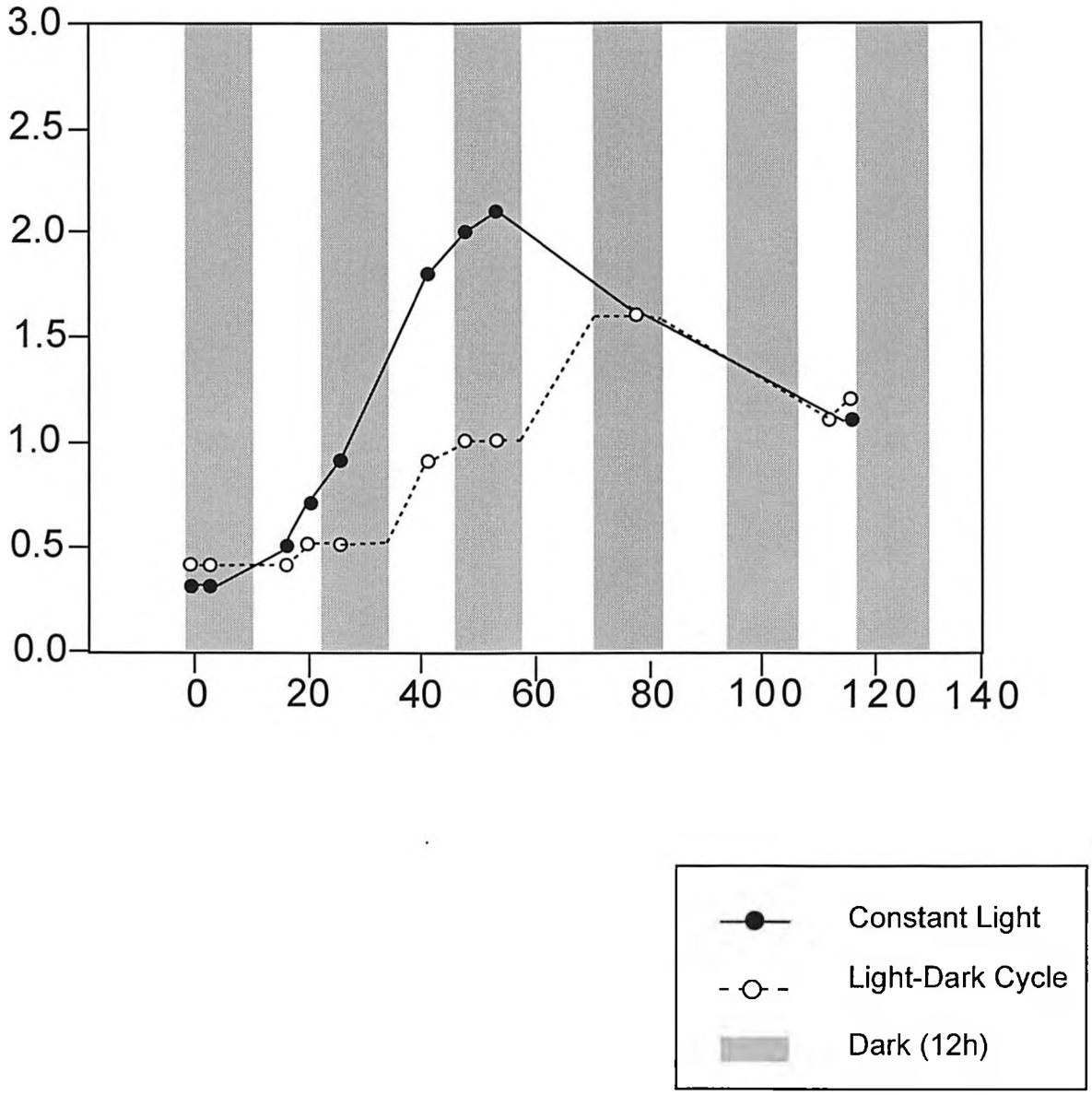

Figure 5: Light Micrograph of Rhodobacter sphaeroides