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Effects of Nitric Oxide Inhibitors and Scavengers on Gravitropism in *Arabidopsis thaliana*

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Plant roots are gravitropic meaning they detect and respond to changes in orientation with respect to gravity. Pagnussat et al.(2003)[1] suggests that this response is initiated by nitric oxide and results in cGMP production. Here we investigate the effects of the removal of nitric oxide (NO) or the inhibition of nitric oxide-sensitive guanylate cyclase to show that such a cGMP producing cycle exists in plants. We also want to determine the exact effects of NO on gravitropism in *Arabidopsis thaliana*. Removal of NO with the NO scavenger 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) showed unexpected results. Reorientation was expected to be delayed, however it occurred within the same time period as the controls. Inhibition of NO synthesis using 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) behaved as expected. It delayed the degree of gravitropic bending, indicating that NO synthesis was required for the gravitropic response. Although the results from the tests with ODQ support our hypothesis, the results from PTIO contradict it.

Plant growth and development are strongly influenced by gravity. When roots are gravistimulated by horizontal orientation, instead of the usual vertical, they respond by bending downward with respect to gravity. This bending is due to differential growth at the root tip. The differential growth at the tip is probably caused by mechanosensing in the root cap and changes in calcium and pH. This results in relocation of auxin efflux carriers and subsequent downward transport of auxin, thus inducing the differential growth and downward bending [2,3,4] The cycle proposed by Pagnussat et al. suggests that it begins with NO and results in the production of cGMP. Our experiments tested for the existence of this proposed cycle.

NO occurs naturally in *A. thaliana*, as in other plants and animals, and is produced by a species-specific nitric oxide synthase (NOS) complex. Nitric oxide activates the guanylate cyclase. The guanylate cyclase then converts GTP into cGMP causing the transport of auxin. The effects of NO and cGMP donors as well as phosphodiesterase inhibitors was documented by two colleagues, Jacobi and Elmer. Their results show that NO and cGMP are strongly tied to the root reorientation of *A. thaliana*, heavily supporting our hypothesis. This report focuses on the effects of a NO scavenger and a guanylate cyclase inhibitor. In theory, if the NO scavenger, PTIO, was present, it would remove free NO and the guanylate cyclase wouldn't be able to convert GTP to cGMP fast enough thus causing delayed root

reorientation. The guanylate cyclase inhibitor, ODQ, is supposed to inhibit the NO/cGMP pathway at the guanylate cyclase and prevent GTP from forming cGMP. The plants showed a delayed reorientation rate when exposed to ODQ, however we received opposite, unexpected results for PTIO. These results both support and contradict the idea that NO is a major signaling molecule involved with root growth and the idea of a NO/cGMP cycle involved with growth, as proposed by Pagnussat et al. More testing is needed to confirm these results. The proposed cycle can be seen in Figure 1.

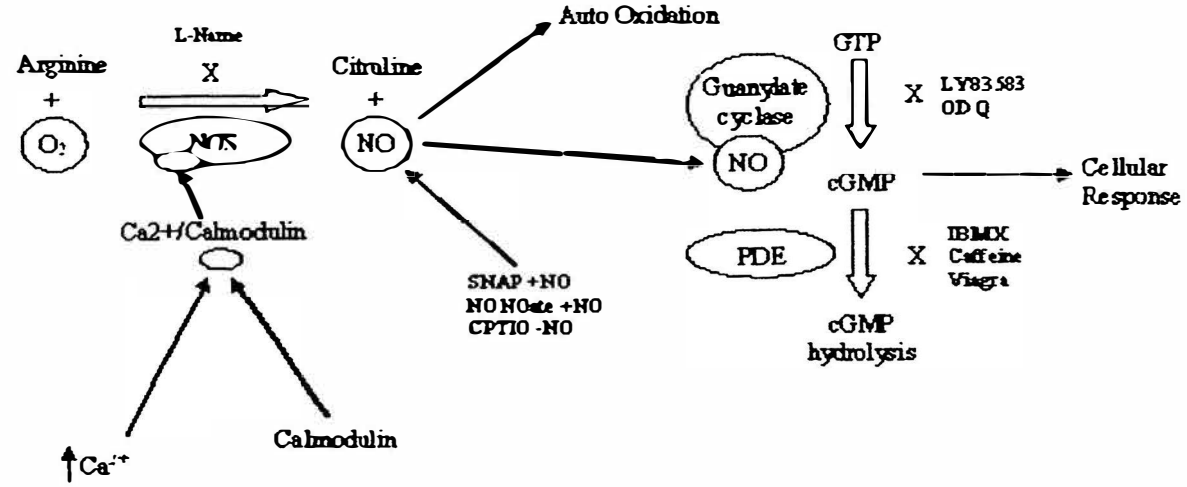

Figure 1. (Above) NO/cGMP Cycle
This pathway is documented in mammals. However, the goal of our project is to prove there is a similar pathway in plants. The NOS is signaled to run when there is an increase in calcium and calmodulin in the cell. The NOS then produces NO which goes on to the guanylate cyclase or is auto-oxidized. The guanylate cyclase then converts GTP into cGMP. The cGMP goes on to produce a cellular response, such as root reorientation. In addition to PTIO and ODQ, L-Name, SNAP, NONOate, LY83583, IBMX, Caffeine, and Viagra are common drugs used to probe this pathway. Their location in the diagram suggests where they inhibit the pathway.

Results

Effects of ODQ (Figures 2 and 6)
1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, known as ODQ, is a selective inhibitor of the NO sensitive, guanylate cyclase. The results from ODQ behaved as expected. The roots had a delayed reorientation time when compared to the controls. See figure 3. As you can see from the graphs, there is no significant difference between the results from the different concentrations. Six day old seedlings were transferred to plates containing 10 μM, 25 μM, and 50 μM of ODQ.

Effects of PTIO (Figures 3 and 7)
2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, known commonly as PTIO, is a stable radical scavenger for NO. It affects NO without affecting NOS. It has been documented that PTIO has significant inhibitory activity against NO biological actions. This however is not supported by the results we obtained. The seedlings exposed to PTIO actually reoriented faster

than the controls. This result was extremely unexpected. Further testing is needed to confirm these results. Six day old seedlings, germinated on plates without drugs, were transferred to new agar plates containing 50 μM, 100 μM, and 150 μM of PTIO.

Control Plates (Figures 4, 5 & 8)
Two types of controls were used, a 90-degree rotated control and a clinostat control. The clinostat control eliminates the effect of gravity by continuously rotating the seedlings. The 90 degree control roots showed no reorientation response until approximately two hours had passed. The clinostat plants showed slight curvature in two directions, indicating that they had no sense of gravity.

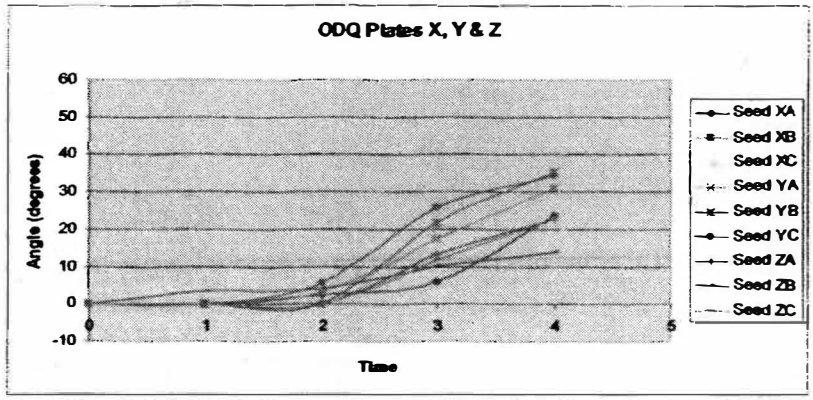

Figure2. ODQ Results
As can be seen from the graph, when exposed to ODQ, the roots didn't really begin to reorient until between two and three hours after being exposed to the drug. And, when compared to the control, the rate and degree of reorientation was much less. Seeds XA, XB, and XC were on plates containing 50μM of ODQ. Seeds YA, YB, and YC were on plates containing 25 μM of ODQ. Seeds ZA, ZB, and ZC were on plates containing 10 μM of ODQ.

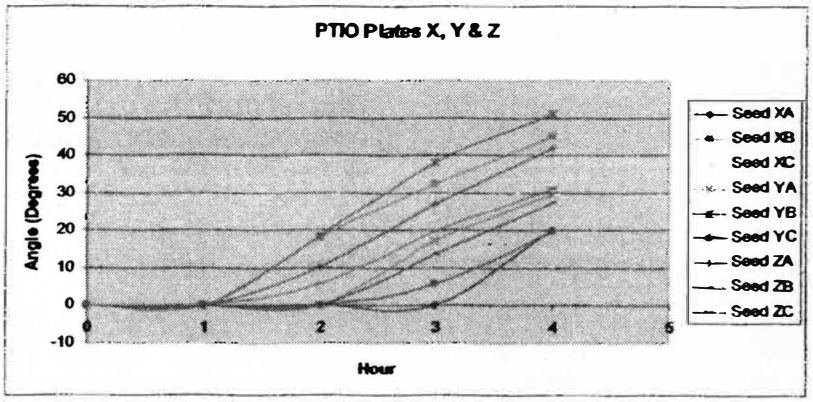

Figure 3. PTIO Results
As can be seen from the graph, the seedlings started to reorient between one and two hours after being exposed to PTIO. Seeds XA, XB, and XC were on plates containing 50 μM of PTIO. Seeds YA, YB, and YC on plates containing 100 μM of PTIO. Seeds ZA, ZB, and ZC were on plates containing 150 μM of PTIO. As you can tell from the graph, there is no significant, consistent difference between the different concentrations.

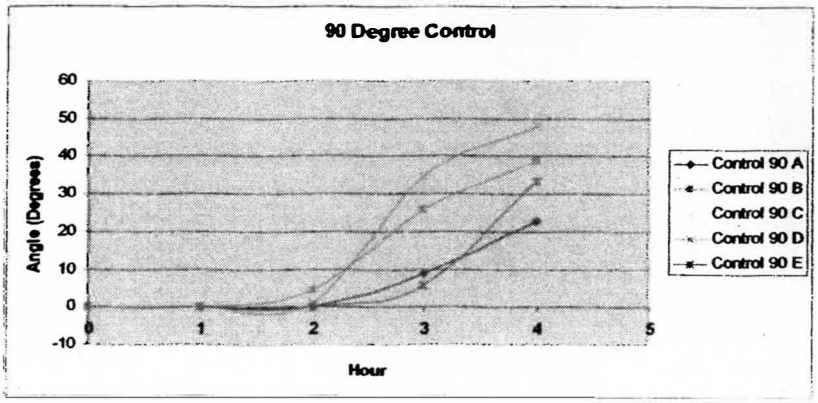

Figure 4. 90 Degree Control
According to the results, and as can be seen in the graph, the seedlings started to reorient downward between two and three hours after being transplanted to new plates.

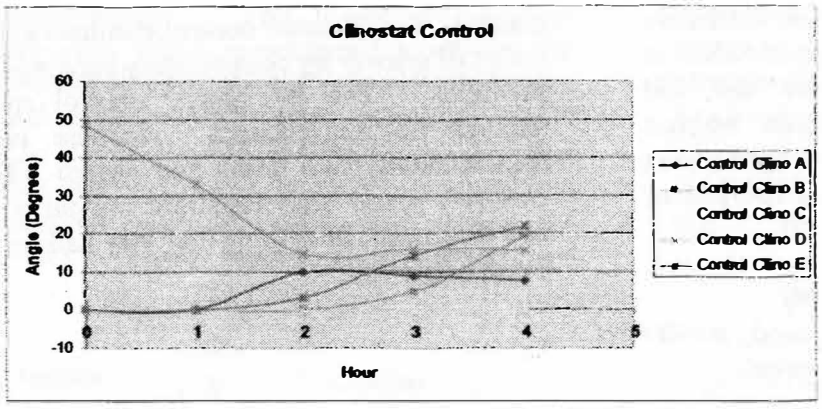

Figure 5. Clinostat Controls
The clinostat control is used to eliminate the effect of gravity. It does this by constantly rotating the seeds at 1 RPM. As can be seen from the graphs, the seedlings had no perception of gravity and grew randomly.

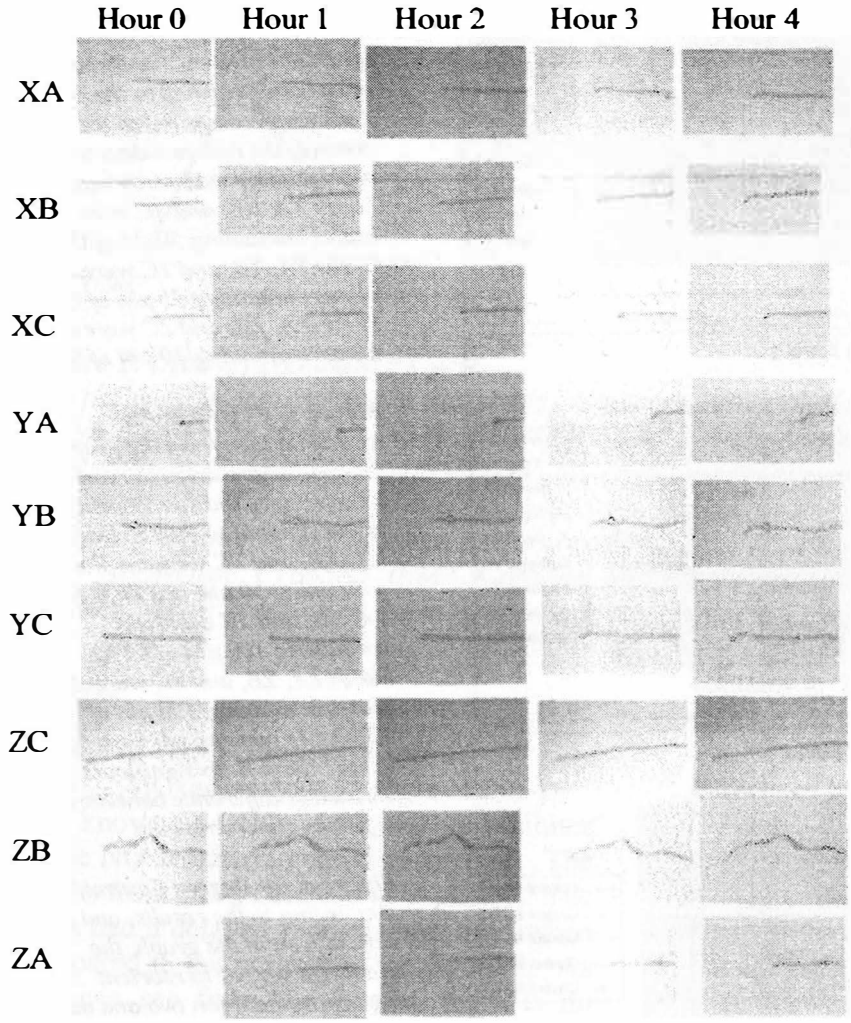

Figure 6. ODQ Images
As can be seen from the images, ODQ has a significant effect on the root reorientation. Seeds XA, XB, and XC were put on plates containing 50μM of ODQ. Seeds YA, YB, and YC were put on plates containing 25 μM of ODQ. Seeds ZA, ZB, and ZC were transferred to plates containing 10 μM of ODQ. The higher concentrations of ODQ definitely affected the roots more.

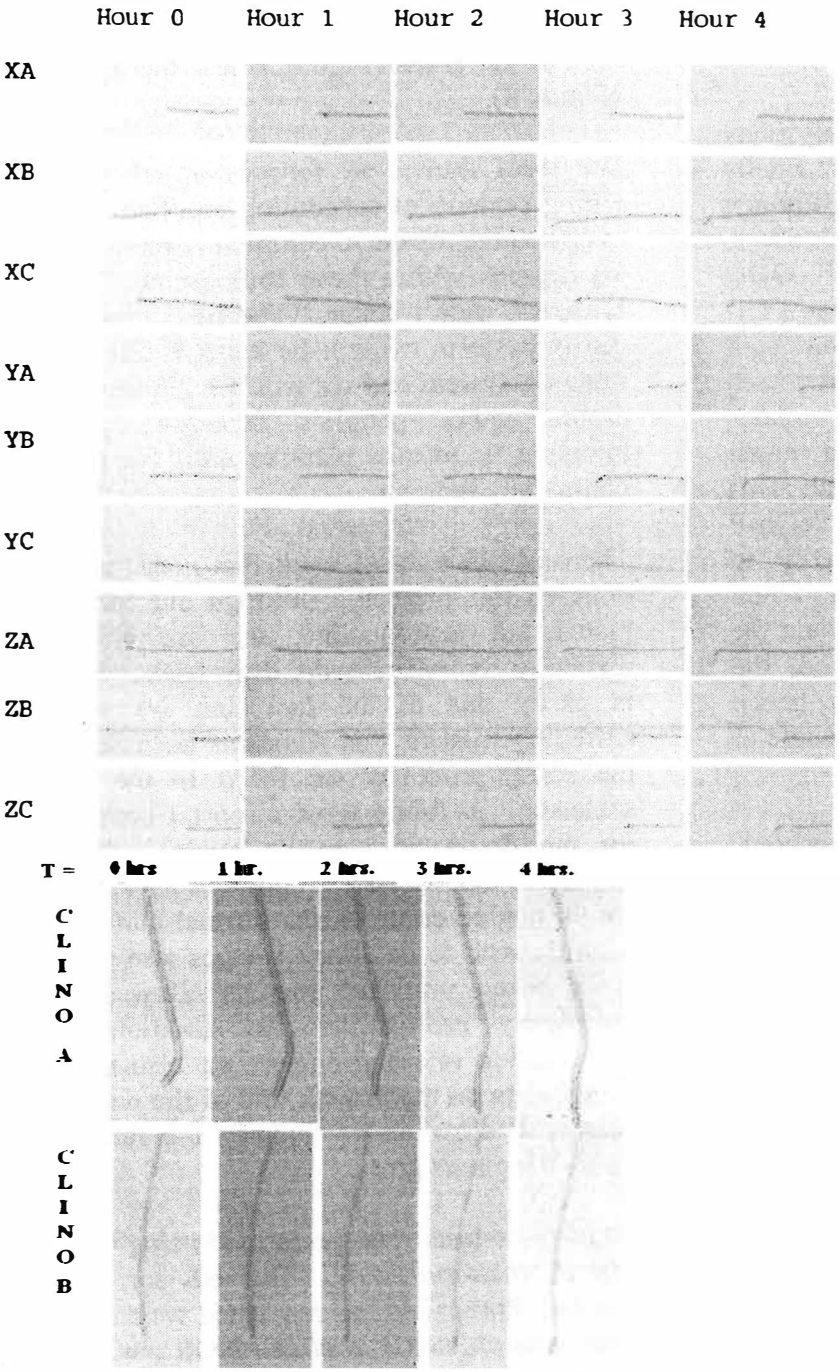

Figure 7. PTIO Images
As can be seen from the images, the roots exposed to PTIO begin to reorient fairly quickly. This was very unexpected. hours after being exposed to PTIO. Seeds XA, XB, and XC were transferred to plates containing 50 μM of PTIO. Seeds YA, YB, and YC were transferred to plates containing 100 μM of PTIO. Seeds ZA, ZB, and ZC were transferred to plates containing 150 μM of PTIO.

Figure8. Clinostat Controls
These images show the reorientation of the clinostat controls.

Plate	Deg/s
ODQ X (50 μM)	0.1151
ODQ Y (25 μM)	0.1485
ODQ Z (10 μM)	0.0831
PTIO X (50 μM)	0.1351
PTIO Y (100 μM)	0.1396
PTIO Z (150 μM)	0.1400

Figure 9. Reorientation Rate
From the table, you can tell the optimal concentration for ODQ was 25 μM. Of the ones tested, the optimal concentration of PTIO was 150 μM. The optimal concentration is defined as the concentration of drug that gives the highest rate of reorientation.

Discussion

In the beginning, this project had many roadblocks and complications. We finally got things underway in January. Although all plants showed a response to both drugs, the results weren't quite as expected. ODQ behaved as predicted but PTIO did not. The control plants and ODQ plants on both started to reorient between hours two and three. However, the control plates reoriented more slowly and at a much smaller angle than the ODQ plants did. This indicates that ODQ inhibited the NO/cGMP pathway at the conversion from GTP to cGMP. This, therefore, limited the amount of cGMP available to be used to reorient the roots. Once all the ODQ was used, the plants were able to reorient quickly, hence the significant jump in reorientation angle at about hour three.

The results for PTIO were not as expected. In mammals, it is documented that PTIO removes the NO that is produced by the NOS. This causes less NO to be available to be sent to the guanylate cyclase and therefore GTP cannot be converted to cGMP. These plants should have showed delayed reorientation like the ODQ trial, but it actually showed a faster reorientation than the controls. These results could possible be from expired drugs or human error. More trials will be preformed next semester to retest PTIO.

The control results worked out to our expectations. Multiple trials were performed on different days, and each time we received similar control results. This was a sign of the reproducibility of the project.

The clinostat controls were as expected. With the elimination of gravity on the roots, they grew multiple directions. This can be seen in the graph (Figure 5) and the images (Figure 8).

We were limited to four hours of photo taking because of scheduling conflicts. We expected the results to completely reorient to 90 degrees within those four hours. This, however, didn't occur. The next time we run these tests we will be using a different imaging system and we won't have to work around other people's schedules, thus allowing us to take pictures until the roots completely reorient.

There is still a lot of work that needs to be done on this project. Although our control plates are reproducible, we are having difficulty reproducing the drug trials. This is partly due to the fact that we were extremely rushed this semester because of the many problems we faced in the fall semester. In future tests, clinostat controls for the drugs need to be made. Also, additional trials for the drug plates as well as the 90 degree controls and normal clinostat controls need to be done. Images also need to be taken until the root is allowed to completely reorient. In addition to reorientation rate and angles, we also need to take data on the growth rate of the plants. This will allow us to quantify the rate at which the plants grow.

All of these tests were performed using wild type *A. thaliana* seeds. Our advisor, Dr. Marshall Porterfield has requested we do the same tests on two *A. thaliana* NOS mutants as well.

Material and Methods

Seed Preparation

All seeds were purchased from the Carolina Seed Company. Prior to planting, seeds were sterilized in a 50% bleach, 45% dH_2O, and 5% Tween20 solution for 10 minutes.

minutes. After 10 minutes in the sterilization solution, they were rinsed in ethanol for one minute followed by a one minute rinse in dH_2O.

Planting

The sterilized seeds were grown on an agar media composed of 1.1g MS+gamborg vitamins, 0.25g MeS, 2.5g sucrose, and 2g phytagel per 500mL of water. The mixture was pHed to 5.75 and then autoclaved for 30 minutes. 50 mL was dispersed into each square Petri dish. The seeds were then planted on the drug-free plates with a modified syringe and allowed to germinate and grow (standing vertically) for approximately six days in a lighted incubation chamber set at 25° C.

Drug Plate Preparation

The process for making the agar was the same above. However, the PTIO and ODQ were added to the liquid agar after autoclaving but before the agar had solidified. After solidifying, the plates were allowed to cool for two hours before the seedlings were transferred.

Transplantation

Seedlings, approximately six days old were transferred from their original drug free plates on to freshly made drug plates using forceps making sure the roots as straight as possible. Three seedling were placed vertically on each drug plate, but then the plate was turned 90 degrees so that the roots could reorient in a new direction, 90 degrees from the original growth direction. Plants were given an hour to adjust to the new plates before the initial 'zero-hour' photo was taken.

Images

All photos were taken using Dr. Anne Maglia's digital camera microscope. Pictures were taken of each seedling at one hour intervals for four hours. During this four-hour time-period, the plants were kept out of the growth chamber.

The camera magnification was 2.1 X . Adobe Photoshop was used to capture the images as well as create Figures 6 and 7. The reorientation angles were measured from the pictures using the software ImageJ. The diagram in Figure 1 was created using Microsoft Word. Graphs in Figures 2 through 5 and Figure 9 were created using Microsoft Excel. The images in Figure 8 were created using Paint.

Acknowledgments

Several people were involved or assisted with this project and many thanks are due to them. First, I'd like to thank our advisor, Dr. Marshall Porterfield and his graduate student Kacey Morris for their guidance and knowledge. I'd also like to thank my project partners Jennifer Jacobi and Jacob Elmer, whom without, late nights in the lab would have been extremely boring. Finally, I'd like to thank Dr. Anne Maglia and her graduate student Barb Banbury for letting us use their camera late to obtain our images.

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