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Purification and Analysis of Protein Synthetic Initiation Factors from *Volvox carteri*

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Abstract:

*Volvox carteri*, a multi-celled green algae, can grow synchronously given a sixteen hour light period followed by an eight hour dark period, a cycle which is repeated for a 48 hour growth cycle total. Near the end of each light period, reproductive cells divide rapidly resulting in the differentiation of cells. When the dark period begins, this differentiation stops and the cells remain dormant with little protein synthesis or differentiation occurring. Immediately after the lights come back on, however, the cells again undergo rapid protein synthesis and complete their differentiation. Previous studies have concluded that *Volvox carteri* discontinue protein synthesis during the dark phase due to regulation at the translational level and not the transcriptional level. Therefore, the inhibition of protein synthesis does not lie in the transfer of the protein coding sequence from DNA to mRNA, but rather in the transfer of this information from the mRNA to the ribosomes. My research examined this translational regulation to determine the factor(s) causing the discontinuation of protein synthesis during the dark phase. Evidence from other research further suggests that the control of translation lies in the initiation step rather than the elongation step. Eukaryotic initiation factors aid in the binding of the ribosomal subunits to the mRNA to initiate protein synthesis. It is known that initiation factors can be modified by phosphorylation, regulating their activity. Therefore, my study focused upon isolating some of these initiation factors in order to determine whether or not such modifications are responsible for the inhibition of dark phase protein synthesis in *Volvox carteri*.
Introduction:

*Volvox carteri* is a multi-celled green algae that is placed in the order of Volvocales, with other flagellated organisms such as Chlamydomonas and a number of other green algae. (Kirk 1988). They can be found naturally in wet environments, ponds and lakes, but can also be grown with moderate difficulty in laboratories.

*Volvox carteri* exhibit a complete division of labor between two fully differentiated cell types, the somatic cells and the reproductive cells, also referred to as gonidia (Kirk and Harper 1986). Approximately 2,000 somatic cells encompass 16 larger gonidia cells in a healthy, adult *Volvox* organism. Although the organism looks hollow, it contains a gelatinous substance called extra-cellular matrix secreted by the somatic cells. This matrix holds the *Volvox* in its spherical shape.

*Volvox* have somewhat of a confusing structure, for they are actually an organism within itself. The somatic cells enclose gonidia cells in the gelatinous spheroid. As the gonidia divide and cleave, each gives rise to a juvenile which is a miniature adult containing somatic cells and gonidia of its own (Kirk and Harper 1986). Therefore, three generations are present within a single organism: somatic cells (generation 1), juveniles (generation 2), the juvenile’s gonidia (generation 3).
The asexual life-cycle of *Volvox carteri* can be synchronized by a 48 hour light-dark cycle, consisting of 16 hours of light followed by 8 hours of dark, which is repeated (Kirk and Kirk 1983). As shown in the diagram above, toward the end of the light period following hatching, mature gonidia undergo embryogenesis. Embryogenesis consists of a rapid series of cleavage divisions, some of which are asymmetric. All the cells that will be present in the adult form are represented here, but they are relatively undifferentiated and the embryo is inside out with respect to the adult configuration. Late in the dark period, the cells undergo
inversion (lasting about 45 minutes) and then remain dormant for the remainder of the dark period. While the cells are distinct and occupy their ultimate positions, they are not fully differentiated at this point. Completion of differentiation does not occur until the light period begins (Kirk and Kirk 1983). After the lights return, the young *Volvox* matures. Its cells complete differentiation and both the parental spheroid and the juveniles contained within it expand by deposition of extra-cellular matrix. Following expansion, the juveniles hatch and swim away, leaving the “ghost” somatic structure to undergo programmed cell death. The juvenile spheroids continue to expand while their gonidia mature and prepare for a new round of embryogenesis (Kirk 1988).

Much research in the past focused upon the process by which *Volvox* are grown synchronously on a light-dark schedule. As with most research, the findings triggered a refocusing of attention to a specific area of interest related to this light-dark cycle. As mentioned above, by the end of the dark period following cleavage, the cells are dormant until the lights return. The specific location of this seemingly light-triggered resurgence of growth in *Volvox carteri* and its control has been the focus of much recent study.

In 1983, David and Marilyn Kirk, proposed that "light-mediated changes in protein synthesis provide an opportunity for exploring physiological controls over protein synthesis in this species". Their research demonstrated that marked changes in the polypeptide labeling patterns of both somatic cells and embryos (juveniles) occur when the lights go out and when they come back on. They found that the most dramatic light dependent changes occurred in
juvenile spheroids as they experienced the shift from dark to light. This suggests that the protein synthetic patterns of these juveniles has considerable developmental significance.

Because the expression of genetic material is a two-step process involving transcription followed by translation, the Kirks proceeded first with experiments to determine which step contained the regulatory control of the protein synthetic patterns. The first step, transcription, occurs in the nucleus and involves a DNA strand serving as a template for the synthesis of a complementary strand of mRNA. The second step, translation, occurs in the cell’s cytoplasm. Here, the mRNA sequence is translated by ribosomes into the amino acid sequence of a protein. By using actinomycin, a known inhibitor of transcription, and heat shock, the Kirks proceeded to run several complicated experiments examining both transcription and translation in *Volvox carteri* to locate the level of control.

In the first experiment, *Volvox* were pretreated solely with actinomycin. The cells were labeled in the dark and then after the lights resumed. If the control were located on transcriptional levels, no difference in labeling patterns would be seen because no mRNAs would be formed. Results revealed that prior actinomycin treatment had virtually no effect on the protein synthetic activities of cells after the lights returned. There were dramatic differences in the labeled and treated cells from dark-to-light transition suggesting that the mRNA had already been formed prior to the dark cycle and that the control by light was exerted post-transcriptionally (Kirk and Kirk, 1985).
The second experiment sought to substantiate further these findings which located the control at the translational rather than the transcriptional level. Here, the Kirks pretreated *Volvox* with actinomycin followed by heat shock. The results were similar to that described above. They found no effect on the protein synthetic activities of *Volvox* after the lights returned, but there were dramatic differences in the mRNA labeling patterns from light-to-dark transition (Kirk and Kirk, 1985).

A few other experiments were conducted along these same lines to rule out possible effects actinomycin could have on translation events. These experiments came out negative, showing actinomycin to have virtually no effect upon translation (Kirk and Kirk, 1985).

Lastly, mRNAs isolated from both dark and light *Volvox* were used to synthesize proteins, and it was found that the resultant proteins were extremely similar. The results of the series of experiments conducted by the Kirks suggests that changes in protein synthesis in *Volvox carteri*, following a dark-light transition, are entirely controlled by changes in translation, not transcription (1985).

Kirk and Kirk also proved that the changes in the polypeptide labeling patterns in these cells following the dark-to-light transition was indeed directly a result of illumination and not a programmed cellular event. To achieve this, they demonstrated that different labeling patterns are evident if the lights return prematurely, but no different patterns are observed if the return of lights is delayed (1983).
The Kirk's research then turned to finding the exact component(s) in the Volvox cells which was responsible for the light-mediated changes. Their first hypothesis involved an energetic consideration in which the control lay somewhere in the photosynthetic process, more specifically the chloroplast ribosomes, of Volvox. By using two known inhibitors of chloroplast ribosomes, they negated their own hypothesis. Again by polypeptide labeling before and after, they showed instead that the proteins synthesized following the stimulation by light are synthesized on cytoplasmic ribosomes and not chloroplast ribosomes.

Further proof that the differences were not related to any photosynthetic aspect of Volvox was presented in a 1985 paper published by the Kirks. Their study focused upon the protein synthetic pattern of illuminated cultures of Volvox. By using photosynthetic inhibitors and comparing the patterns of labeled polypeptides before and after illumination, they reported that differences between the two were still evident. The Kirks again concluded that the changes seen upon illumination were not due to the "enhanced flow of high energy compounds from the illuminated chloroplast" (1985).

The Kirks continued their search for the direct level of control exhibited in cytoplasmic ribosomes. Back in 1983, the Kirks had proposed the following: "The dark period serves as a 'regrouping' period in which the Volvox accumulate the transcripts that will be required in the next stage of development". After the lights return, the Volvox commence to execute this program in synchrony. In conjunction with this theory and with evidence that the regulation
lies somewhere within cytoplasmic protein synthesis, the Kirks began their first attempt to determine the exact location of translational control.

In paper written in 1986, the Kirks concluded that "discerning the molecular mechanisms linking the reception of light to changes at the level of the ribosomes should be both extremely interesting and extremely challenging". Because Volvox carteri exhibit one of the most unusual and striking examples of translational regulation and this control plays a central role in the development of the organism, there is extreme interest in how this regulation occurs.

By examining translation of mRNA more closely, we note that there are two phases involved, initiation and elongation. The initiation of translation is a three stage process that requires the participation of Protein initiation factors. Intact ribosomes do not bind directly to the mRNA so as to initiate polypeptide synthesis. Rather, initiation is a complex process in which the two ribosomal subunits (40S and 60S) and Met-tRNA_i assemble on a properly aligned mRNA to form a complex that is able to begin elongation. This assembly process also requires the assistance of protein initiation factors that are not permanently attached to the ribosomes (Voet and Voet, 1990). Over 10 eukaryotic initiation factors have been discovered, but not all of their functions and importance are well known.

Three specific initiation factors have been isolated and found to have considerable involvement in the initiation process of other systems. First, eIF-2, with energy from GTP, aids in the binding of the mRNA and the Met-tRNA_i to the 40S ribosomal subunit. Second,
eIF-4F is a cap binding protein and could explain reduced initiation rates of improperly capped mRNAs (Kozak, 1992). Lastly, eIF-4B has also been shown to undergo phosphorylation shifts in mammalian cells (Manzella, 1991).

A wide variety of studies in other organisms, including yeast and mammalian species, show that translational regulation is most often exerted at the initiation step specifically by eIF-2, eIF-4B, and eIF-4F. It seems reasonable that in *Volvox carteri* this would be the case as well. Typically, such control is exerted through modifications of initiation factor protein by phosphorylation which changes their affinity for the ribosomal subunit.

My research has focused on translational control at the level protein synthetic initiation factors of *Volvox carteri*. In light of previous research which concluded that three initiation factors, eIF-2, eIF-4F, and eIF-4B, were chiefly responsible for the blockage of protein synthesis in their relative systems, I narrowed my initial research upon the purification of these three factors.

The purification of the protein synthetic initiation factors involved five steps: harvesting, sonication and centrifugation, ammonium sulfate fractionation, DEAE cellulose columns, and assay/antibody tests. The first and second steps were preparation steps for purification. Harvesting included the growing and collecting of the organisms, and sonication/centrifugation involved breaking open the organisms and retrieving the protein initiation factors for purification.

Proteins are purified by fractionation procedures. In a series of independent steps, the various physiochemical properties of the
protein of interest are utilized to separate it progressively from other substances. The technique used in my research was Ammonium Sulfate Fractionation.

Because different proteins come out of solutions at different concentrations of salts due to their ionic strengths, the precipitation of desired levels of proteins can be facilitated by adjusting the salt concentration in a solution containing a mixture of proteins. The salt concentration of the remaining solution is then raised so as to precipitate the rest of the proteins. Two fractions were produced; a 0-40% fraction and a 40-70% fraction.

Each of the fractions was then run through a DEAE cellulose column for further purification. DEAE cellulose is an ion exchange resin consisting of charged groups covalently attached to support a matrix. The chemical nature of the charged groups determine the types of ions that bind to the ion exchanger and the strength with which they bind. Because different proteins have different affinities for the DEAE, proteins are eluted out of the column by washing the column with different salt gradients and collecting the different fractions.

At this point the purification was halted due to the lack of a functional assay with which to compare our proteins. Currently, the proteins are being analyzed at the lab of JoAnne Ravel, University of Texas at Austin for a possible match with a known assay. It is my hope that with the base established and the initiation factors identified, research can quickly move forward to determine whether or not initiation factors play a major role in the regulation of protein synthesis in Volvox carteri.
Materials and Methods:

Mediums and Buffers

1) Standard Volvox Medium

(Summation of Starr's (1969) modification of Provasoli & Pintner's Medium, as further modified by Starr, Kochert, and Kirk at various times)

For each 1000 ml of Standard Volvox Medium (SVM) required, stock solutions in the amounts indicated were added to approximately 900 ml of high-purity water, pH was adjusted to 8.0 with NaOH, and volume was brought to 1000 ml. Solution was dispensed into desired tubes or flasks, autoclaved, and stored in the cold.

All stocks except Biotin and B$_{12}$ were made fresh at least every two months. The P IV Metals were made fresh every month, and sooner if there was any problem with the cultures that could not be explained. Biotin and B$_{12}$ stocks were kept frozen, the P IV Metals solution was kept at room temperature in the dark, and all other stocks were kept in the refrigerator.

<table>
<thead>
<tr>
<th>ml</th>
<th>Stock Solution</th>
<th>Stock Concentration</th>
<th>mMoles/l of Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ca(NO$_3$)$_2$·4H$_2$O</td>
<td>11.8 g/100 ml</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>MgSO$_4$·7H$_2$O</td>
<td>4.0 g/100 ml</td>
<td>0.16</td>
</tr>
<tr>
<td>1</td>
<td>Na$_2$ glycerophosphate</td>
<td>5.0 g/100 ml</td>
<td>0.23</td>
</tr>
<tr>
<td>1</td>
<td>KCl</td>
<td>5.0 g/100 ml</td>
<td>0.67</td>
</tr>
<tr>
<td>1</td>
<td>Na$_2$CO$_3$</td>
<td>2.0 g/100 ml</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>Urea</td>
<td>3.0 g/100 ml</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>Hepes (preferred)</td>
<td>6.0 g/100 ml</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>(or) Glycylglycine</td>
<td>5.0 g/100 ml</td>
<td>3.8</td>
</tr>
<tr>
<td>0.1</td>
<td>Biotin</td>
<td>250 µg/100 ml</td>
<td>*</td>
</tr>
<tr>
<td>0.1</td>
<td>Vitamin B$_{12}$</td>
<td>150 µg/100 ml</td>
<td>**</td>
</tr>
<tr>
<td>1</td>
<td>Thiamine</td>
<td>100 mg/100 ml</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>P IV Metal Solution</td>
<td>given below</td>
<td>***</td>
</tr>
</tbody>
</table>

*Biotin: made working stock from frozen stock of 2.5 mg/100 ml Biotin, by 1:10 dilution

**B$_{12}$: made working stock from frozen stock of 15 mg/100 ml B$_{12}$, by 1:100 dilution

Aliquot Biotin and B$_{12}$ working stocks into tubes, in volumes appropriate for one time use, and stored in the freezer.
IV Metal solution

To 1000 ml high purity water added 0.75 g Na$_2$EDTA (EDTA was 13.4 µM, final concentration in medium). After this had dissolved, added the following salts, in the order and amounts indicated, dissolving each before the next was added. (Waited 15 minutes after adding the FeCl$_3$ before adding the MnCl$_2$).

<table>
<thead>
<tr>
<th>Salt</th>
<th>mg</th>
<th>µMoles/l of Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeCl$_3$ 6 H$_2$O</td>
<td>97</td>
<td>2.15</td>
</tr>
<tr>
<td>MnCl$_2$ 4 H$_2$O</td>
<td>41</td>
<td>1.24</td>
</tr>
<tr>
<td>ZnCl</td>
<td>5</td>
<td>0.22</td>
</tr>
<tr>
<td>CoCl$_2$ 6 H$_2$O</td>
<td>2</td>
<td>0.05</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$</td>
<td>4</td>
<td>0.10</td>
</tr>
</tbody>
</table>

2) Buffer E: 20 mM HEPES KOH, pH 7.6; 1 mM Mg(OAc)$_2$; 2 mM CaCl$_2$; 6 mM BME; 120 mM KCl; 0.1 mg/ml of STI; and 0.5 mM PMSF from a 50 mM solution of PMSF in isopropanol prepared just prior to use.

3) Buffer B: 20 mM HEPES KOH, pH 7.6; 0.1 mM EDTA; 2 mM CaCl$_2$; 1 mM DTT; 10% glycerol; and KCl as indicated.

Buffers were stored as long as 1 week at 4 or at -20°C for longer periods of time. DTT, BME, PMSF, and STI were added just prior to use.

Abbreviations used are:
- HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
- DTT, dithiothreitol
- EDTA, ethylenediaminetetraacetic acid
- BME, 2-mercaptoethanol
- PMSF, phenylmethylsulfonyl fluoride
- STI, soybean trypsin inhibitor

General Procedures

The purification scheme of *Volvox* included the following five steps: Harvesting, Sonification/Centrifugation, Ammonium Sulfate Fractionation, DEAE Cellulose Column, and Assay/Antibody Tests. By following the purification procedure of the most well studied plant system, Wheat Germ, with only a few variations, I was able to
begin the purification process of *Volvox carteri* protein synthetic initiation factors (Lax et al. 1986).

**Harvesting:** *Volvox* were harvested generally about 1 hour before the lights went out, when the *Volvox* were in their cleavage state. The isolation at the embryo stage reduced the difficulty in purification because little extra-cellular matrix had been deposited. The cells were examined under magnification to ensure that they were synchronous. If they were not synchronous, *Volvox* were sorted and re transferred to a new flask for continued growth.

*Volvox* were collected by filtration on 30 µm Nitex filters. The flask containing media and synchronized *Volvox* was poured through a sterilized filter that collected only the organisms as the media drained through. This was repeated for all the flasks depending on the number transferred (usually between 8-12 flasks).

*Volvox* were collected in 50 ml sterilized plastic tubes that were filled to the 50 ml mark with media once all the organisms were collected. The 50 ml containing media and *Volvox* was then transferred to a Dounce Homogenizer. The Dounce Homogenizer forced organisms to pass through a defined distance between the wall of the Homogenizer and the pestle pressuring the organisms to break open and release the gonidia. 3.5 ml of Percol, a density gradient centrifugation medium, was added to separate the gonidia from the somatic cells and extra-cellular matrix (ECM). This was followed by centrifugation of the mixture at 1,500 rpm for 5 minutes. Because the somatic cells and ECM are less dense than the gonidia cells, the somatic and ECM floated towards the top of the tube while the more dense gonidia pelleted at the bottom.
The somatic cells and ECM were poured off as waste, and the gonidia were resuspended in fresh media and allowed to continue growth.

About 9 hours later, following an hour of illumination, the gonidia were recollected by the same techniques described above. The gonidia were placed in a plastic tube with minimal media (5-10 ml) and centrifuged again at 5,000 rpm for 5 minutes. The excess media was poured off and the pelleted gonidia was stored in the freezer.

Generally between 5-7 harvests were done before moving on to the next step.

All procedures from this point on were carried out at 0-4°C unless otherwise indicated.

**Sonication/Centrifugation:** The 6-8 gonidia pellets collected from the harvests were each resuspended in approximately 5 ml of Buffer E. The resuspended gonidia were then sonicated 5 times each at 30,000 Hz for 15 second bursts using a Fisher Microsonicator. Sonication uses high frequency sound waves to break cell walls. The tubes were kept on ice between sonication bursts. The tubes were checked for consistency, that is no clumps or pieces of cellular material were seen in the green liquid.

The tubes were then combined and centrifuged at 16,000 rpm for 20 minutes. The resulting pellet, containing the cell wall and other high density components, was discarded, and the supernatant was placed in a 30 ml polycarbonate ultracentrifuge bottle. The supernatant underwent ultracentrifugation at 45,000 rpm for 3 hours in a TI 55.2 rotor at 4°C to pellet the ribosomes. The final
supernatant contained presumably only cytoplasmic components of which were protein initiation factors. These components were now ready to be purified.

**Ammonium Sulfate Fractionation:** As with the Wheat Germ purification steps, two fractions were produced: a 0-40% fraction and a 40-70% fraction.

To obtain the 0-40% fraction, 22.6 gm Ammonium Sulfate was added to 100 ml of sample (supernatant from previous step) slowly over a twenty minute period. The mixture was allowed to stir for an additional 15 minutes followed by centrifugation at 15,000 rpm for 15 minutes. The supernatant was poured off to be used in the next steps while the pellet was considered the 0-40% fraction and was resuspended in 5 ml of Buffer B-40.

To get the 40-70% fraction, 18.2 gm of Ammonium Sulfate was added per 100 ml of supernatant. After centrifugation as above, the supernatant was discarded and the remaining pellet, considered the 40-70% fraction, was resuspended in 5 ml Buffer B-40.

Both fractions were dialyzed overnight against Buffer B-40. Samples of the 0-40% and 40-70% fractions were sent to the lab of JoAnne Ravel, University of Texas at Austin, where they are currently being analyzed.

Polyacrylamide Gel electrophoresis (PAGE gels) were run following the Ammonium Sulfate Fractionation. On each prepared gel, four lanes were used. The first lane was loaded with 5 µg of 0-40% fraction. The second lane was loaded with as much protein as possible, with a maximum sample of 24 µL (about 6-8 µg). The third
and fourth lanes were filled with 40-70% fraction in the same fashion (with a maximum of 20-24 µg).

The gels were run at 30 mAmps for 1-1.5 hours and stained in Coomassie Blue for more than 4 hours. They were then destained overnight and viewed.

**DEAE Cellulose Columns:** (DEAE=Diethylaminoethyl) Two separate columns were set up; one for the 0-40% fraction and one for the 40-70% fraction. The Isco UV-4 monitor was set at .05 absorbance unit (full scale), 280 nm wavelength, and the chart speed was 6 cm/hr.

The DEAE cellulose was placed in Buffer B-40 for initial swelling followed by a first wash in 0.2 M HCl and a second wash in 0.2 M NaOH which produced the correct charge (anionic). The column was packed and Buffer B-40 was run through it until the absorbance at 280 nm was stable, establishing a baseline. Using the Beckman fraction recovery system, approximately 50 collection tubes were set up to receive 70 drops or 4 ml per tube.

200 ml more of Buffer B-40 was loaded onto the column. After most of the B-40 had run through, the sample was loaded onto the column. For the 0-40% fraction, approximately 14 ml of sample was loaded which contained 5.5 mg of protein. For the 40-70% fraction, approximately 16 ml of sample was loaded which contained 20.3 mg of protein. After allowing the sample to run into the cellulose, another 200 ml of B-40 was added.

After the first peak came off and the curve returned to near baseline, a gradient of 50 ml Buffer B-40 and 50 ml Buffer B-150
was set up to run through the column. After the second peak came off and the curve again returned to near baseline, 200 ml of B-150 was washed through the column. Finally after the third peak came off the column, the wash was stepped up to 200 ml of Buffer B-300, and the fourth and final peak came off the column.

Fractions were verified by measuring the absorbance at 280 nm. Fractions corresponding to each peak were then pooled together and concentrated through the ammonium sulfate precipitation, as described above.

The proteins were then ready for further purification steps.

Assay/Antibody Tests: At the time of my research, there were no functional assay with which to compare our Volvox proteins. Pooled fractions were assayed for the detection of protein levels in general, however, by the Bio-Rad method.

Results:

After each harvest and initial centrifugation, Volvox gonidia were stored until there was enough material (usually 5-7 harvests) to go on to the next step. A total of three groups of harvests were purified further. After sonication and centrifugation, the protein was separated into two fractions, 0-40% and 40-70%, by ammonium sulfate fractionation.

By running simple PAGE gels, I was able to visualize the proteins present in our two fractions. Moreover, by running both fractions, 0-40% and 40-70%, side by side on a single gel, I was able to show that not only were protein bands present, but that the fractions contained uniquely different proteins from each other.
Because there is currently no assay for the initiation factors of *Volvox carteri*, I could not determine which bands in either of the fractions represented the protein initiation factors, eIF-2, eIF-4F, and eIF-4B.

At this stage in purification, I also completed three separate Bio-Rad Assays, one for each group of harvests. The data for the three graphs can be found in Table 1. The graphs themselves can be found in chronological order (Figures 1-3).

<table>
<thead>
<tr>
<th>µg Protein</th>
<th>Abs. @ 595 nm (11/17/93)</th>
<th>Abs. @ 595 nm (1/6/94)</th>
<th>Abs. @ 595 nm (2/24/94)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
</tr>
<tr>
<td>20.0</td>
<td>.132</td>
<td>.093</td>
<td>.053</td>
</tr>
<tr>
<td>40.0</td>
<td>.240</td>
<td>.166</td>
<td>.129</td>
</tr>
<tr>
<td>60.0</td>
<td>.370</td>
<td>.202</td>
<td>.199</td>
</tr>
<tr>
<td>80.0</td>
<td>.485</td>
<td>.293</td>
<td>.254</td>
</tr>
<tr>
<td>100.0</td>
<td>.725</td>
<td>.323</td>
<td>.300</td>
</tr>
</tbody>
</table>
Figure 1. Standard Plot of Bio Rad Assay
Data for Protein Concentration (11/17/93)

Figure 2. Standard Plot of Bio Rad Assay
Data for Protein Concentration (1/6/94)
For both fractions, the Bio-Rad assay allowed calculations to be done to determine the final level of protein concentration. The results of these assays are presented in Table 2.

Table 2. Purification Chart

<table>
<thead>
<tr>
<th>Protein Concentration</th>
<th>Volume</th>
<th>Total Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-40% Fraction</td>
<td>.397 mg/ml</td>
<td>15.9 ml</td>
</tr>
<tr>
<td>40-70% Fraction</td>
<td>1.27 mg/ml</td>
<td>18.2 ml</td>
</tr>
</tbody>
</table>
Samples of both fractions were sent to the lab of JoAnne Ravel, University of Texas at Austin for analysis. Approximately 1 ml of each fraction was sent, containing .346 mg protein in 0-40% fraction and 1.2 mg protein in the 40-70% fraction. The laboratory will test our proteins against their Wheat Germ initiation factor antibodies and look for any cross reaction. At the time of this writing, we have not received the results.

The next step in purification, involving the DEAE cellulose column, gave us results that very closely resembled that of the Wheat Germ initiation purification (1986). With both the 0-40% and the 40-70% fractions, four separate peaks came of the column precisely as the salt gradient was changed indicating different proteins affinities to salt concentrations. When comparing the peaks in both of the fractions to their counterparts in the Wheat Germ purification, we note significant similarities between the two results. According to the Wheat Germ purification results, eIF-4B is found in the second peak and eIF-4F is found in the third peak of Fraction 0-40%, while eIF-2 can be found in the second peak of 40-70% Fraction. The results of the DEAE cellulose columns and the predicted location of the specific initiation factors can be seen in Figure 4 (0-40% Fraction) and Figure 5 (40-70 Fraction).

The following two pages of this thesis are missing in both paper copies.
Conclusion and Discussion:

The purification of any component of *Volvox carteri* poses particular difficulty because of the presence of the extra-cellular matrix (ECM) which is gelatinous and very sticky. I worked with considerably small amounts of material because of these purification difficulties and some minor problems with the growth of the cultures themselves.

While the ultimate results of my research are still pending the outcome of the analysis of my protein samples in Texas, I can still reflect upon my other findings and give an overview of what should be examined next in light of my conclusions.

First of all, the purification scheme for Wheat Germ initiation factors worked extremely well for *Volvox carteri* thus far and was followed with only slight variations as detailed above in the methods section. Further purification of *Volvox* initiation factors should also follow the procedure presented in Wheat Germ purification scheme until reaching the assay stage.

Because there is currently no functional assay for *Volvox* initiation factors, we have sent our partially purified proteins to Texas for analysis through antibody detection. They are testing our *Volvox* proteins against Wheat Germ antibodies and looking for cross reaction. Assuming positive results, that is good cross reaction with the Wheat Germ initiation factor antibodies, affinity purification columns, similar to the DEAE columns, can be set up in which the specific antibodies bind to their specific initiation factors. Thus, after the samples are added to the column, the initiation factor proteins bind to their specific antibody and form a
link. When salt concentrations are raised to a specific level, the link between the known antibody and its initiation factor will break and the factor will elute out of the column and be collected cleanly.

After this clean separation of *Volvox* initiation factors, more PAGE gels could be run to conclude specifically where each protein band lies and assess purity. From here, complex experiments dealing with polypeptide labeling in dark and light *Volvox* samples could be done to test the phosphorylation states of the initiation factors. Because initiation factors are regulated by phosphorylation, any difference in phosphorylation patterns between the light and dark phase *Volvox* initiation factors would suggest that the factors are being affected by the changing light. The experiments would involve labeling the initiation factors with P-32 both before and after the lights return. Because phosphates change the charge on initiation factors, running two dimensional gels, which detect changes in isoelectric points of proteins, would reveal the changes in phosphorylation patterns. The exact details of the experiments could be set up similar to the Kirk's experiments in the mid 1980's.

If the results of the analysis from Texas are negative and no cross reaction between the Wheat Germ antibodies and the *Volvox* initiation factors are found, further purification would be halted until a functional assay for *Volvox* initiation factors was located. Options could include the development of antibodies specific to *Volvox* initiation factors which could take years, or possibly trying to cross react the proteins with known antibodies from another known system.
Although much today is left unsolved, exciting possibilities are just around the corner with the results of the analysis from the Texas lab. Hopefully, with positive results, the research I have started will come quickly full circle and we will know what role, if any, initiation factors (eIF-2, eIF-4F, and eIF-4B) play in the dark-light transition of protein synthesis in *Volvox carteri*.

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Works Cited:


