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This is to certify that the thesis prepared under my supervision by

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Proteins in Bacillus Subtilis and the νr M Gene Product in Escherichia coli

is approved by me as fulfilling this part of the requirements for the

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One aspect of life that exists in all phyla is the ability to recognize external substances as nutrients (attractants) or toxins (repellents). In bacteria, this process by which the organism migrates to higher concentrations of attractant or lower concentrations of repellent is known as chemotaxis. It is thought that chemotaxis represents the first evolutionary development of a sensory-motor system in nature. For this reason, chemotaxis is well studied from at least three different but related approaches: Physical, genetic and biochemical.

The physical or behavioral basis of chemotaxis is historically the oldest and most well established. Bacteria, under normal conditions, swim (one directional movement) and tumble (Thrash around in several directions in three dimensional space) through the medium. When an attractant is added, the time of swimming towards the attractant is increased while the tumbling interval between swams is reduced. This results in a bias on an otherwise random walk through three dimensional space with the accumulation of much of the bacterial population at high concentrations of attractant (Adler, 1975). During the period of smooth swimming, the flagella rotate exclusively in the counterclockwise direction and exclusively clockwise rotation is observed for tumbling. However, the transience of these responses reflects the postulated existence of an adaptation mechanism, that allows the bacteria to respond to concentration changes rather than absolute chemical concentrations (Parkinson, 1977).

Many of the chemotaxis genes in Escherichia coli have been studied, identified and characterized, but their role in the mechanism of chemotaxis has not been completely elucidated. It has been established that, in E. coli, methionine is continuously required during taxis. Its role is that of methyl donor and it is believed that this methylation is involved in stimulus trans-
duction and sensory adaptation (Parkinson, 1977). The identification of
the methyl sensing chemotaxis protein (MCP) as the receiver of the methyl
group donated by methionine, allowed further work in the role of sensory
adaptation in chemotaxis (Koh, Goy, Larsen and Adler, 1977). It was later
found that when bacteria are stimulated by the addition of attractant, the
amount of methylation increases to a new level and stays there as long as
attractant is present. The amount of methylation was proportional to the
size of the stimulus and upon dilution away of the attractant, the new
methylation level dropped quickly to it's original basal level (Goy, Springer
and Adler, 1977). This information further implies that the role of
methylation of the MCPs in chemotaxis is that of sensory adaptation.

Based on this and other vital information, I would like to discuss a
model for chemotaxis recently proposed by Orval and Fields (1977). In the
presence of high concentrations of attractant, this chemical compound binds
to the membrane chemoreceptor which then changes conformation and binds to a
signaller. This signaller then binds to the MCP which then draws up ligand X -
the controlling parameter - from the inside of the cell. With ligand X bound
to MCPs, it is unlikely for it to bind to the flagellar switch, thus in the
absence of ligand X the flagella rotate counterclockwise and swimming occurs.
Next, a methyl transferase working slowly but at it's $V_{max}$, methylates the
MCPs, causing release of ligand X and a rise in the cytoplasm to it's original
level.

As the attractant is diluted away, it leaves it's chemoreceptor which
leaves the signaller causing it to unbind from the methylated MCP. As this
happens, more of ligand X is released from the MCPs causing a temporary rise
in the cytoplasmic level of ligand X - this allows clockwise rotation of
flagella for tumbling. Next, a 'de-methylase' working quickly at it's $V_{max}$
(Faster than the methyl transferase) cleaves the methyl groups from the NCPs, allowing them to bind ligand X and restore the original level of ligand X in cytoplasm. This finally restores the normal frequency of tumbling and swimming (Ordal and Fields, 1977).

Based on experiments done in this laboratory, it has been postulated that ligand X is Ca\(^{2+}\) ion or possibly a complex of Ca\(^{2+}\) and a small molecular weight protein. It is possible that the NCPs are Ca\(^{2+}\) binding proteins, and are so with less affinity when methylated (Ordal and Fields, 1977).

Much of the work in chemotaxis has been done on the gram-negative bacteria Escherichia coli and Salmonella typhimurium, and it is not known whether the distantly related gram-positive bacteria Bacillus subtilis uses a similar mechanism for chemotaxis. With this information, the isolation and purification of that protein in B. subtilis which binds ligand X was attempted. With a pure batch of this protein, it would be possible to verify that ligand X is indeed Ca\(^{2+}\) ion. It was originally believed that the NCPs directly bound Ca\(^{2+}\) ion, thus I set out to isolate and purify the NCPs from B. subtilis membranes. However, further experiments in our laboratory and in other laboratories indicated that although NCPs are Ca\(^{2+}\) binding proteins, the sensory adaptation mechanism may be working through a complex of NCP, Ca\(^{2+}\) and a small molecular weight protein. This small molecular weight protein has been identified in E. coli as the product of the chemotactic gene cheW. It's molecular weight is 12,000 daltons and it is assumed to bind Ca\(^{2+}\) much like Troponin C does in muscle tissue (Warrick, Taylor and Koshland, 1977). Thus, I reoriented my efforts in the direction of isolating and purifying the cheW gene product in E. coli. Nevertheless, it was maintained that for one aspect in the verification of the 'Ca\(^{2+}\)' as ligand X' Model, that protein which binds Ca\(^{2+}\) ion must be isolated and purified.
Materials and Methods

**Material strains**

Wild type strain of *Bacillus subtilis* 61005 was used for all MTP experiments. Further information concerning the origins of this strain is provided in a paper by Ullah and Ordal (submitted for publication).

**Media**

K-phosphate buffer is 10m potassium phosphate pH 6.5. Various concentrations of Triton X-100 in K-phosphate buffer were used. Tryptone broth is 1% tryptone and 0.5% sodium chloride. L broth is tryptone broth with 0.5% yeast extract. Chemotaxis buffer has been described in a previous paper (Ordal and Goldman, 1973). Protoplast incubation buffer was 50mM potassium phosphate, pH 7, 30mM sodium lactate, 20mM magnesium chloride, 0.1mM EDTA, 50 microgram/ml chloramphenicol and 20% sucrose. Laemmli sample buffer has been described (1970). Low pH sample buffer contains N-acetyl pyridinium chloride in a low pH urine solution.

**in vivo methylation**

This procedure was borrowed from that described in Ullah and Ordal (submitted for publication). Bacteria were inoculated from cultures frozen in glycerol into tryptone broth and grown overnight. In the morning, the culture was diluted 1:50 into 5ml L broth and grown to a Klett of 700. The culture was washed twice with chemotaxis buffer and one with protoplast incubation buffer. Bacteria were suspended in 0.5ml of this buffer at 0.0 at 600nm of 0.75 unit and supplemented with 1mg/ml lysosome. The bacteria were incubated at 37°C with shaking for 20 minutes and then supplemented with 1 mM \((\text{C}^3\text{H}_3)\) methionine (5 µCi/mmole). Incubation was continued 20 minutes until the reaction was stopped by putting on ice.

**Separation of MTP by DEAE and Electrophoresis**

The incubated cell solution was centrifuged at 12,000 rpm for 10 minutes.
The pellet was resuspended with 0.1% Triton X-100 in 0.1M phosphate buffer
to open up the cells. The membrane ghosts were recentrifuged at 15,000 rpm for
20 minutes. The membrane ghost pellet was then solubilized with 100 μl of
10% Triton X-100 (2:1 - Triton:protein) and homogenized every 25 minutes for
1 - 2 hours. The solution was ultracentrifuged at 40,000 rpm for 1 hour in a
Beckman 65Ti rotor at 4°C. The supernatant was then applied to a DEAE bini-
gel A column (5cm x 2cm) and washed with several volumes of phosphate buffer
containing 0.1% Triton X-100 before a 0 - to 7.0mM KCl linear salt gradient was
developed. Forty fractions containing one ml each were collected and counted
for radioactivity (dpm) in a scintillation counter. Approximately 6 - 7
fractions containing the highest amount of radioactivity were then pooled and
applied to a carboxy-methyl sephadex column followed by elution with a 0 -
0.4M KCl linear salt gradient. Again, forty fractions containing one ml each
were collected and counted for dpm in the scintillation counter. The
procedure of pooling the few fractions containing the highest dpm was re-
peated for application to the hydrophobic phenyl sepharose column. Prior to
addition to the column, the pooled fractions were adjusted to 1.0M (NH₄)₂ SO₄
since the elution off the column was accomplished with a high to low gradient
(1.0M (NH₄)₂ SO₄ - 0M) and low to high salt gradient.

Following each running of column chromatography, an SDS gel electrophoresis
(method use in C.W. Ordal's laboratory) slab or tube was developed. Tube
gels were cut into 0.5mm slices and counted for radioactivity in the scintillation
counter. Slab gels were stained for protein with a coomassie blue stain.
The initial step in the purification of the mRNA was to take the labeled
ammonium sulfate fractionation of the homogenate and chromatograph the
protein onto the DEAE Bio Gel G column. Methylated membrane proteins can be
detected by counting the radioactivity (dpm) of the DEAE fractions (see figure 1).
The same results were virtually repeated each time - 80 to 90% of the radio-
activity applied to the column was eluted off early, prior to the running of the
0 - 7.0mE KCl linear salt gradient. It is thus seen that the labeled, methylated
membrane proteins do not stick to the position ion exchange column.

The next step in the purification process was to apply the high dpm pooled
fractions off the DEAE column, over a negative ion exchange column - Carboxy-
methy1 sephadex (CM seph). From a representative CM sephadex profile (See figure
2 ) it is assumed that the radioactively labeled methyl proteins accumulate and
bind to the column with the same efficiency. This assumption is made based on
the observation that there is one large peak of label spread over a few
fractions. It appears that some purification has been accomplished, however,
it is not quantitated at this point.

It was important to qualitatively assess the amount of purification that
had been accomplished by the running of two opposite ion exchange columns. Thus,
three fractions were selected from the CM sephadex profile for running low pH
tube gel electrophoresis. This data (See figure 3) indicated several labeled
methyl-protein species at high molecular weight and did not distinguish the
amount of labeled species per peak. It was also unknown what kind of electrophoretic
system would provide us with the maximum amount of information. Thus,
a high pH gel electrophoresis system adopted from the method by Laskali (1970)
was attempted. These results are shown in figure 4 . Comparison of these
results to that found by a low pH gel electrophoresis indicates that the labeled
methyl group is found attached to a low molecular weight protein species at
high pH while at low pH the C^3H_2-MCP is seen at high molecular weight. It appears that a methyl transfer is occurring at pH 8.9 that does not occur at pH 4.5.

At this point in the purification process, a more pictorial view of the amount and kinds of proteins present was needed. It was assumed that the high pH LacZal system was cleaving off the methyl groups from the MCPs. Thus a slab gel, based on dye staining and not radioactive counting, was selected allowing us to stick with the LacZal system regardless of the effect on the methyl groups. This electrophoretic profile indicates the presence of at least 10 major protein species with one of the strong dark bands being the MCP at 68,000 daltons (See Figure 5). There was an indication that the purification of the MCPs was progressing, however, further separation needed to be accomplished.

It was assumed that the proteins that remained together after running through two opposite ion exchange columns must be physically similar. Since these proteins are all membrane proteins, they could differ in their degree of hydrophobicity. Based on this assumption, the next step in the purification process was attempted - application of the high pH pooled fractions from the CM seph column onto a hydrophobic column consisting of phenyl sepharose beads. The phenyl sepharose profile can be seen in Figure 6. Much like the two ion exchange profiles, the majority of the radioactive label appeared early off the column and was concentrated into one major peak spread over a few fractions. Eight fractions from this profile were selected to be run on a high pH gel electrophoresis slab. Although the result is faint, it is quite clear that there are only a few major protein species left (See Figure 7). It appears that considerable purification is accomplished when solubilized membrane solution is passed over these ion exchange columns and one hydrophobic column.
Figure 2
LOW pH GEL ELECTROPHORESIS

Figure 3
HIGH pH GEL ELECTROPHORESIS

Figure 4
CM Sephadex Separation of B. subtilis

Figure 5
Figure 6

Amonium Sulfate
linear gradient
(1.0-5 M)

PHENYL SEPHAROSE Fractions
Phenyl Sepharose separation of *B. subtilis*

**Figure 7**
The isolation and purification of the MCPs from *B. subtilis* membranes is imperative for confirmation of the 'Ca^{++} controlling model' of chemotaxis. A positive ion exchange column was primarily used to achieve some initial separation. It appears that the MCPs are not covered with negative charges on their surfaces since the proteins came off the column much earlier, before the salt gradient was set up (see Figure 1). This result was repeatable and it is thus safe to assume that separation has occurred from those membrane proteins that do stick to the DEAE column. Oddly enough, a similar result is seen for the negative ion exchange column. The radioactively labeled methyl protein species do not stick to the CM Sephadex column (see Figure 2). As with the DEAE, a large peak is observed early, prior to the running of the salt gradient. Since this result is repeatable, it may be safe to assume that the surfaces of the MCPs are not covered with positive charges that would allow binding to the CM Sephadex column. Separation has occurred from those membrane proteins that do stick to a CM Sephadex column.

It was uncertain how many protein species were labeled and coming off the two columns in the large peaks. This was qualitatively assessed by low pH electrophoresis in the gel. The results seem to indicate a few dominate protein species at high molecular weight (see Figure 3). However, the number of different proteins at a certain molecular weight could not be discerned. It was hoped that by using a different electrophoretic system, similar results might be obtained. Following the CM Sephadex column, the high pH peak was run through a high pH electrophoretic system borrowed from Laemmli (1970) (see Figure 4). Surprisingly, the labeled methyl protein species were found in the low molecular weight region rather than the high molecular weight region as was found using a low pH electrophoretic system. This may be explained by cleavage of the labeled methyl group from the high molecular weight.
weight HMP and transferred to a low molecular weight species. It is postulated that during sensory adaptation in chemotaxis the HMPs are demethylated to allow the Ca$^{++}$ to bind to the HMP. It is also believed that the methyl group cleaved by the 'demethylase' is transferred to a low molecular weight protein and not just lost into the cytoplasm of the cell. Possibly this occurs around the high pH of the lasmali system.

There was still information that we could derive from the electrophoresis at the lasmali pH. It was a matter of relating the dependence of the labeled methyl group on the HMPs. A slab or plate gel electrophoretic system was used instead of tube gels and detection of the protein was accomplished by staining with coomassie blue. The high pH slab gel profile indicates that much separation was occurring. Of the hundreds of proteins present in bacterial membranes, the profile indicated the presence of only about ten major protein species of various molecular weights (See Figure 5). After running the membrane lysate over only two columns, a significant amount of separation has occurred. Very few membrane proteins do not stick to either DEAE or Sephadex, but the HMPs fall into this category.

The question remained: If the HMPs do not stick to either positive or negative ion exchange columns, then to what type of column would they stick to? The ten proteins left after the two columns must be somewhat physically similar but they must differ in some respect. Since they are all membrane proteins, it is possible that they would differ in their degree of hydrophobicity. Thus, the next step in the purification process was the application of the high density fractions off DEAE Sephadex onto an hydrophobic column consisting of phenyl sepharose beads. It was encouraging to repeatedly observe that the labeled methyl-protein species stuck to the hydrophobic column (See Figure 6). There were some complications in the phenyl sepharose procedure that should be
mentioned. The protocol called for salting out the proteins from the pooled
CM Sephadex fractions with ammonium sulfate, prior to application onto the
hydrophobic column. However, when this was attempted, a ten-fold decrease in
radioactivity was observed. Thus, upon the next running of this experiment,
the pooled fractions off the CM Sephadex column were adjusted to 1.0 M (NH₄)₂SO₄
and applied to the hydrophobic column. It was with this running that I observed
the ten-fold increase in radioactivity.

As with the earlier columns, several high dpm fractions off the phenyl
spharose column were selected for slab gel electrophoresis. The profile did
not show any dark bands indicating the protein concentration was low when
running the electrophoresis (See Figure 7). However, even with the low
protein concentration, it is clear that there are only a few major proteins
left of which the MCPs are included. The running of the two ion exchange
columns separated the MCPs from the majority of the membrane proteins. The
phenyl spharose column separated the MCPs from at least half of those proteins
remaining that were physically similar to the MCPs. It would require just one
more step in the purification process to completely isolate and purify the
methyl accepting chemotactic proteins. It is that final step I wish to discuss
now.

There are a few reasons why we have purified the MCP this far and then
halted or delayed this process. First, new information within our laboratory
indicated that the MCPs might not bind Ca²⁺ directly but rather work it's
sensory adaptation mechanism through the binding of a small molecular weight
protein - Ca²⁺ complex. Thus, in addition to the importance of isolating and
purifying the MCPs, it became imperative to isolate and purify the small
molecular weight protein, the chem gene product of E. coli. Second, the
final step in the purification process requires some preparatory steps that
have been worked on by other individuals in the laboratory. In the methylation reaction, the MCP works as a substrate for the enzyme methyltransferase. This enzyme could be immobilized on an ultra gel (polyacrylamide and polyagarose beads) column by the cyanogen bromide reaction. This would provide the specific binding of the MCPs to the column and eliminate any remaining protein species. The problem lies in the acquisition of milligram levels of methyltransferase to make the affinity gel column. Methyltransferase has been isolated and purified in our laboratory to the microgram level (Ullah and Ordel, submitted for publication). With this amount, there have been attempts to produce the antibody for methyltransferase from a rabbit. The antibody can be used to isolate milligram levels of methyltransferase by immobilizing the antibody onto the ultra gel column. This project, designed for this summer, will provide the necessary final step in the isolation and purification of the methyl accepting chemotaxis proteins.
Materials and Methods

Bacterial and Viral Strains

Wild type strain of *Escherichia coli*, 159 (ind-), was used for all experiments. A chemotaxis mutant strain, cht2r, was also used. The lysogenic bacteriophage λsA 59 and λ sA 52 were sent to our laboratory by Dr. E. Parkinson.

Media

Modified M9 media contains 0.35% ethydrate Na₂HPO₄, 0.3% KH₂PO₄, 0.1% NaCl, 0.1% NaCl, 0.5% triascine. One liter of this media was adjusted to pH 7.0, sterilized and had the following contents added to it: 1 ml of 3M FeCl₃, 3 ml of 1M MgCl₂, 1 ml of 0.1M CaCl₂, 1 ml Thiamine HCl at 1mg/ml, 5 ml Kiotin at 0.25 mg/ml, Na₂PO₄ to 3x10⁻⁵M, glycerol to 1.0% and maltose to 0.5%. No sulfate was to be added to media used for labeling. Methyl transferase buffer (MTB) is 1% KPO₄, 0.1% NaCl, 0.1% β-mercaptoethanol, 0.1% EDTA pH 7.0. L broth is same as described in materials and methods for MCP purification.

Viral infection of E. coli

The cultures (big and small) were grown and kept separated until after radioactive label incorporating. Overnight culture was inoculated with 159 (ind-) in 170ml of modified M9 containing 2x10⁻⁵M Na₂HPO₄. In the morning the culture was split in two (160ml and 10ml), diluted five fold with modified M9 and inoculated for 30 minutes at 35°C. The cultures were harvested at 30 Klett (1x10 /ml) by spinning down at 10,000 rpm for 5 minutes. Resuspension in 0.01M MgCl₂ was followed by ultraviolet irradiation with constant agitation (380 µwatts/cm²). Each culture was kept in the dark, divided into 0.1 volume aliquots and infected at moi about 10 (1.0x10⁹/ml) with virus. Twenty minutes of adsorption at 35°C was followed by 10 fold increase in volume with modified M9 to the big culture and modified M9 plus carrier
free $^{35}$S-SO$_4$ to the small culture, achieving a final specific activity of 100 mCi/ml. Each culture was incubated for 90 more minutes in the dark at 35°C. During centrifugation at 10,000 rpm for 5 minutes at 4°C the two cell cultures were recombined.

Genetic Analysis

This procedure was patterned after that of Weiss (1976). Centrifuged pellets were washed twice at 23°C with 10mM Tris buffer pH 8 and then suspended in 4ml of 0.1M Tris buffer pH 8 containing 20% (w/v) sucrose. This solution was quickly transferred to a small flask at 37°C where, within one minute, 2.25ml of lysozyme (final concentration = 100µg/ml) was added with continuous stirring for 12 minutes. Prewarmed 0.1M EDTA pH 7 (1:10 v/v EDTA/cells) was added slowly over 5 minutes with continuous stirring. After 8 - 10 minutes incubation at 37°C, the spheroplasts were centrifuged at 12,000 rpm for 20 minutes at 4°C. The supernatant was set aside while the pellet was resuspended with a small volume of H$_2$O, allowing another low speed centrifugation at 19,000 rpm for 20 minutes at 4°C to remove debris. The two supernatants were combined and dialyzed with HEPES buffer (dialysis tubing 3137 - 187 or 137). The dialysate was treated with diigo and diigo for 30 minutes prior to ultracentrifugation at 40,000 rpm for 1 - 2 hours at 4°C in Beckman 75 Ti rotor. The supernatant was dialed in HEPES buffer in preparation for column chromatography.

DEAE anion exchange and SDS Gel Electrophoresis

The use of DEAE Bio Gel A anion exchange chromatography is described in methods and materials for MCF section. The dialysate was applied to the DEAE Bio Gel A column and the proteins were eluted off with 30mls of a 0 - 0.5M NaCl linear salt gradient. The high dpm fractions were selected for gel
electrophoresis. SDS-slab gel electrophoresis and autoradiography is borrowed from the method described by Bonner and Laskey (1974). The procedure for the SDS-micro gel electrophoresis was adopted from Margous and Kenrick (1968).
Results

The procedure to accomplish the isolation and purification of \( 	ext{chol} \) was attempted with the aid of the transducing \( F^+ \text{Fla}^+ \), which contained a piece of bacterial DNA that coded for \( 	ext{chol} \) and a few other che products. This was to be compared with the infection with another virus that contained the same piece of \( E. \text{coli} \) DNA with the \( 	ext{chol} \) gene deleted. The culture of \( E. \text{coli} \) was grown overnight, ultraviolet irradiated, virally infected, and incorporated and prepared for somatic lysis. The cell lysate was then cell centrifuged and poured over a DEAE column. Autoradiograms of some of the highest Dpm fractions is seen in Figure 8. The purpose of this UV infection was to selectively turn off bacterial protein synthesis while allowing synthesis of those proteins coded for by the viral piece of DNA in \( E. \text{coli} \). The autoradiogram shows the amount of new proteins synthesized indicating that the viral and bacterial DNA were equally not selectively turned off.

Since we were not completely satisfied that only the bacterial proteins coded by the virus were made, an experiment was developed to see what level of UV irradiation would selectively turn off the bacterial protein synthesis. The bacterial cells were exposed to the UV irradiation at different times. In each case, the virally infected cells produced the same amount and kinds of protein as the non-infected cells, regardless of the time of UV irradiation (see Figure 9). This experiment was finally repeated with more refined techniques. It appeared that the non-infected bacterial cells were selectively turned off of protein synthesis (See Figure 10). However, upon closer examination, this hypothesis was refuted. First, the virally infected cells were producing an amount of different proteins beyond what would be expected had bacterial protein synthesis been selectively turned off (See slots 1, 3, 5 and 7). Secondly, there were bacterial proteins produced in the control that can be found in the virally infected cells (See slot 2 with...
1, 3, 5 and 7). With this and other complications, it seemed unwise to continue these attempts to selectively turn off bacterial and viral protein synthesis in favor of the synthesis of those proteins coded for by bacterial DNA incorporated into the virus.

At this time, a new method was developed where by cell lysates of shak mutant cells and wild type (X-type) cells, prepared by osmotic lysis, were separately run over the DEAE column and compared to each other, fraction by fraction using micro-gel electrophoresis system. The micro-gel electrophoresis system allowed running of the gel in shorter time so that fraction by fraction comparisons could be made. From the optical density and conductivity measurements, fractions off the DEAE column could be lined up for comparisons. The expectation was to detect the disappearance of a band when going from X-type to shak mutant. After several trials with the micro-gel system, one profile was finally established (See Figure 12). The appearance of the doublet around 60,000M (See slots 1, 3, 5, 7 and 9) for the X-type is observed. Secondly, upon comparison of the X-type with the shak (Odd slots vs. even slots since slot 6), it is noticed that the two bacterial strains are producing different types and numbers of proteins. Thirdly, the band for the standard (xma 64, C1D) slot 6 is quite diffuse indicating some error in the system. Fourthly, the thickened dye front prevented any separation in the low molecular weight region. Much information is provided by this electrophoretic profile with respect to what future experiments can be done to achieve the isolation and purification of the shak gene product.
Figure 11

NaCl linear salt gradient (0-0.5 M)
DEAE separation of 157λ (ind-)

Figure 8
Time Dependence of UV irradiation, 1st run

Figure 9
Time Dependence of UV irradiation, 2nd run

Figure 10
The technique for the isolation of the chev gene product in E. coli is a
most sophisticated one in theory. In practice, however, it remained obstinate
and unyielding. An E. coli transducing phage, λ fla 52, containing a piece of
bacterial DNA was sent to our laboratory by Dr. E. Parkinson. The bacterial
DNA incorporated into the phage, was shown to code for the N-7, gthA, cheX
and cheV gene products of E. coli. We were also sent a phage, λ fla 52 Δ 32,
which contained the same piece of bacterial DNA as above except with the
dehV gene deleted. By selective protein synthesis of the bacterial DNA incor-
porated into the phage and comparison by electrophoresis the cheV gene product
could be isolated. The obstinate portion of this procedure was selective
protein synthesis of the bacterial DNA incorporated into the virus. Normal
bacterial protein synthesis was to be turned off by ultra violet irradiation.
UV has been known to cause thymine dimers in DNA if exposed properly. Normal
viral protein synthesis was to be inhibited by an ind minus repressor present
in the bacteria. The ind minus repressor should make the viral DNA uninduc-
able, thus disallowing viral transcription but it should not affect the
bacterial DNA incorporated into the virus. Initially, the results seemed
encouraging. Newly synthesized proteins, seen by 35S incorporation, came off
a DEAE column in two large peaks with only one sticking to the column (See
figure 11). However, the autoradiogram of the DEAE separation of the 35S
proteins indicated the presence of multiple newly synthesized proteins. This
was not expected of a small piece of DNA with only a few genes on it. The
viral and host bacterial DNA were not being inhibited from transcribing for
proteins. (See figure 8).

It was imperative to develop experiments that would establish under what
conditions does the viral piece of bacterial DNA be transcribed while normal
bacterial and viral transcription be inhibited. Various time intervals of UV
irradiation exposed to the bacterial cells was attempted. The first trial at
this experiment led us thinking that selective protein synthesis was unachievable. The autoradiogram indicates the synthesis of many proteins regardless of the time of UV irradiation (See figure 9). It was suspected that the estimated amount of UV required to cause thymine dimer formation was not reaching the bacterial cells. The protocol had called for a 15 watt germicidal lamp at a distance of 25 cm (380 micro-watts/cm²). We found that in order for the bacterial cells to be exposed to sufficient UV irradiation, the distance must be decreased to about 2 cm. At this new distance, the time interval of UV irradiation experiment was repeated. Again, we were struck with premature excitement. The autoradiogram seems to indicate selective bacterial protein synthesis since the non-virally infected cells, exposed to over 5 minutes of UV irradiation, produced no proteins (See slots 4, 6 and 8 of figure 10). However, upon closer examination, it can be seen that the virally infected cells (at any time of UV exposure) were producing proteins found in the non-virally infected, low time of UV exposure, bacterial cells (compare slot 8 with 1, 3, 5 and 7 of figure 10). It also seems that the viral proteins were being produced since a large number of different kinds and amounts of proteins were observed in all the virally infected cells (slots 1, 3, 5 and 7 of figure 10). It appeared that this highly sophisticated procedure would require a considerable amount of refinement and change before it could be gotten to work. Other complications in this procedure became evident. The original deletion phage stock was not producing viable phage. It would thus take much time to acquire new stock of deletion phage.

At this time a new method for isolation of the *plak* gene product in *E. coli* became available. It seemed imminent that with the original procedure failing us, this new one might be fruitful. Unlike the previous one, the theory behind this new procedure was simple. Wild type (X-type) cells and
should mutant cells be grown and osmotically adjusted so that the cytoplasms could be prepared for application onto the DNA column. Electrophoresis of various fractions off the DEAE cellulose would ensue. The disappearance of a protein band around 20,000 daltons, specific to each, could implicate the presence of an agglutinating antibody. However, electrophoresis is time consuming and since most electrophoresis equipment has only eight slots, comparison of 20 fractions would take between 8 and 10 weeks. It came when it was suggested that we try a micro-gel electrophoresis system. The micro-gel system allows running of the gel, staining, and drying all within three hours. In addition, several gels could be prepared in advance with as many as 30 slots per gel. Thus, comparison between two samples could be made by running a few of slot gels or several short gels, taking advantage of the minimal time required for running the gels.

A few problems quickly became evident upon trials with the micro-gel system. The procedure reported that gels could be made in advance but attempts at this left us with dried out gels. Secondly, the samples did not completely condense to thin bands in the stacking gel as was expected. The result was that the electric field had to be turned off sooner preventing the thickened dye front from running off the gel. Accordingly, the separation of protein bands in the low molecular weight region was minimal (see figure 12). There were too many proteins within this region to accurately measure molecular weights. This is most unfortunate considering that the 12,000 dalton chad gene product migrates to this region. Thirdly, the standards did not come out as expected. This is the result of the standards not being appropriately dissolved in proper sample buffer (see figure 12). Finally, upon comparison of chad with the K-type it is evident that the slots do not contain common proteins (compare slots 1, 3, 5, 7 and 9 with slots 2, 4, 6 and 10). It is
assumed that this is due to improper alignment of fractions off the DEAE column onto the electrophoresis gel. The criterion for doing this has been conductivity and optical density measurements. Apparently, both must be used together rather than reliance on only one. A positive result of this experiment is seen approximately in the 60,000 dalton region - there is a doublet present in the X type that is not seen in phage. The significance of this doublet is not yet clear at this time. Experiments to be conducted later may reveal further information.

It is true that this technique is not a viable one in its present form. However, considering the problems that have been discussed, there is much work that can be done. Experiments must be performed finding out the appropriate percentage polyacrylamide in the stacking gel to get the thin banded dye front. Also, familiarity with the conductivity and optical density measurements for alignment of appropriate fractions must be achieved. Secondly, urea-SDS gels may be used to further enhance the separation in the 12,000 dalton region. This complex of denaturants, urea-SDS, has been known to affect separation in this region (communicated with Dr. Georia). Third, separation by molecular weight can be achieved through the use of a P30 gel filtration column prior to the running of the DEAE column. Separation by molecular weight will narrow down the fractions that phage might be present in. Then the low molecular weight proteins may further be separated by the DEAE column. In addition, if the virus technique is gotten to work, then the two cultures, phage and X-type, when infected with the \( \text{A} \) fla 52 virus will produce an endogenous supply of phage, aiding in its isolation and purification.
References


