AN ABSTRACT OF THE THESIS OF

Robyn E. ApRoberts for the degree of Master of Science in

Microbiology presented on September 25, 1998.

Title: Replication of Vaccinia Virus in the Presence of

1,10-phenanthroline.

Redacted for Privacy

Abstract approved:_

Dennis E. Hruby

1,10-phenanthroline and its non-chelating isomer, 1,7-

phenanthroline were used to inhibit the replication of

vaccinia virus (VV). Serial passage of VV in the

presence of various concentrations of either 1,10-

phenanthroline or 1,7-phenanthroline was carried out. No

drug resistant mutants were isolated, suggesting that the

observed inhibition was due to a cellular protease as

opposed to the putative viral protease G1L. Cultures

infected in the presence of the inhibitors, were

radiolabeled with ^{35}S-methionine at various time points

post infection, to determine which step of VV replication

was inhibited. Infections in the presence of 1,10-

phenanthroline proceeded only through early gene

transcription, suggesting that the point of inhibition

was uncoating. Finally, cells infected with VV with or

without the inhibitors at time zero and eight hours post infection were used to generate transmission electron microscopic images. Taken together these results indicate that inhibition was occurring at the level of uncoating.

Replication of Vaccinia Virus in the Presence of

1,10-Phenanthroline

By

Robyn E. ApRoberts

A THESIS

submitted to

Oregon State University

In partial fulfillment of

the requirements for the

degree of

Master of Science

Presented September 25, 1998

Commencement June 1999

Master of Science thesis of <u>Robyn E. ApRoberts</u> presented

on <u>September 25, 1998</u>

APPROVED:

Redacted for Privacy

Major Professor, representing Microbiology

Redacted for Privacy

Chair of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the
permanent collection of Oregon State University
libraries. My signature below authorizes release of my
thesis to any reader upon request.

Redacted for Privacy

Robyn E. ApRoberts, Author

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Replication of Vaccinia Virus in the Presence of 1,10-Phenanthroline

Chapter I

Literature Review

BACKGROUND INFORMATION OF VACCINIA VIRUS

Vaccinia virus (VV) is the prototype member of the viral genus *Orthopoxviridae*, whose members infect a wide variety of vertebrate hosts. VV is a large enveloped double-stranded DNA virus with a characteristic brick shaped morphology. The VV genome of approximately 200 kbp encodes more than 250 gene products. Replication takes place entirely within the cytoplasm of an infected cell, largely independent of nuclear involvement (Moss, 1990a). VV does not display a restricted host range characteristic of many eukaryotic DNA viruses. The above features; cytoplasmic site of replication, large genomic coding capacity, and wide host range make VV particularly amenable as a research tool. VV has been utilized in many different fields of biology, including; vaccine development, developmental biology, DNA replication, protein transcription and translation, and virus-host interactions.

VACCINIA VIRUS REPLICATION

The process of virus replication can be divided into

several steps: attachment and penetration, uncoating,

replication, assembly, maturation, and release. (Figure

I.1) Attachment of the virion to the host cell in many

viruses is mediated by means of specific receptor-ligand

interactions, however, no cellular receptor has been

identified for utilization of VV to gain entry into cells.

Next penetration and uncoating occur. Although very little

is known about this process, it is believed that

penetration occurs via a specific receptor initiating

endocytosis of the virus. Through this process, the virus

can begin uncoating due to changes in both environmental pH

as well as three-dimensional structure of the virion

itself. Proteins required for early gene transcription

have been packaged into the infecting virion such as DNA-

dependent RNA polymerase, early transcription factors

(which recognize early promoter sequences), DNA

topoisomerase type 1, and other enzymes involved in mRNA

synthesis (Moss, 1990a). The following step, early gene

translation, provides VV proteins necessary for

intermediate gene expression and DNA replication.

Concurrent with, or directly preceding dissolution of the

structural core components, DNA replication begins. The

newly replicated VV genome is found as a catenated molecule

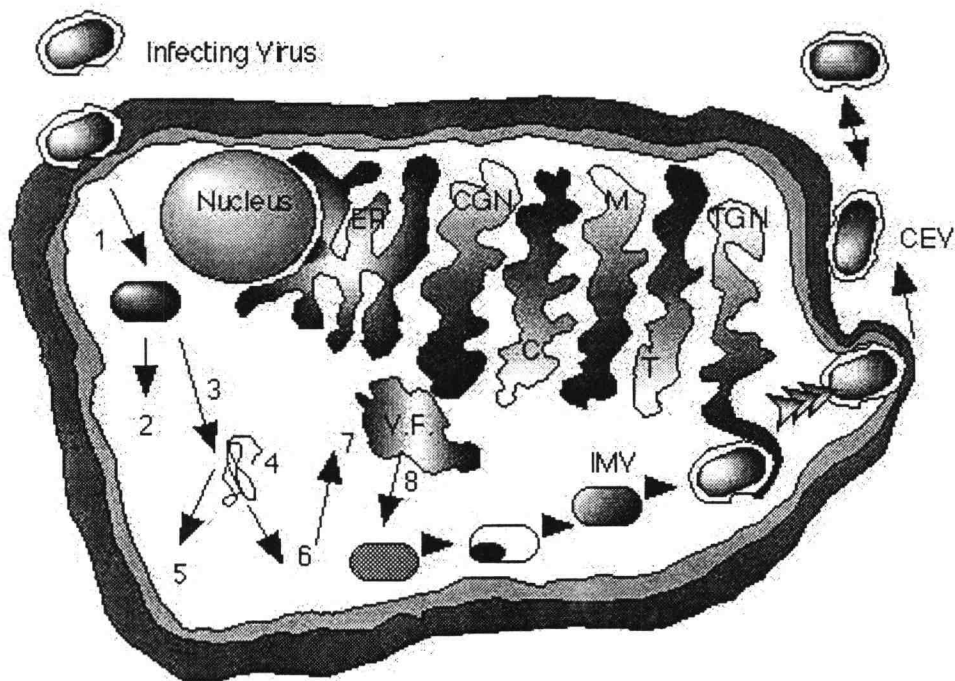within the virus factories in the cytoplasm. Newly

Figure I.1
Vaccinia virus replication cycle. Numbers
represent the major stages of the virus life cycle.
1) Uncoating I. 2) Early gene expression. 3) Uncoating II.
4) DNA Replication. 5) Intermediate gene expression.
6) Late gene expression. 7) Virion assembly.
8) Proteolytic maturation. 9) Release.
(Hansen and Hruby, In press)

replicated DNA is employed as the template for intermediate gene expression, and it encodes a set of late transcription factors (Keck et. al., 1990). The late genes encode the structural proteins, as well as virion enzymes and early gene transcription factors, which are to be packaged into the assembling virion. The cascade of protein expression (early, intermediate, and late) during replication of VV is tightly regulated in a temporal fashion. Assembly of the new virions begins as genomes and structural elements become available. Virions first become infectious following assembly, and proteolysis of the precursor proteins within the assembled virion.

PROTEOLYSIS "LIMITED"

During a productive VV infection, viral proteins undergo a number of post-translational modifications; these include acylation, phosphorylation, glycosylation, ADP-ribosylation, and proteolytic processing (VanSlyke and Hruby, 1990). Enzymes that are provided by the host cell, the virus, or both can perform these protein modifications. Proteolytic processing in general is particularly interesting, for while the specificity of any one reaction differs, it is a mechanism of protein modification that is

conserved in many prokaryotic and eukaryotic systems. Proteolysis is defined as the cleavage of peptide substrates by an enzyme capable of hydrolyzing the susceptible scissile bond. The processing of proteins via proteolysis is ubiquitous among different biological systems, and is essential for sustaining most biosynthetic pathways. In addition, the breakdown process of proteins is unidirectional. There are no known proteins capable of re-ligating the products of a proteolytic reaction, presumably because the free energy required to do so is too high. "Limited proteolysis" first described by Linderstrom-Lang and Ottesen (1949) refers to the selective hydrolysis of peptide bonds within a polypeptide, as opposed to non-specific degradation of proteins. Modifications introduced by limited proteolysis can be used to regulate protein activation, expression, or, as in the case of VV, maturation of the virus.

CLASSIFICATION OF PROTEASES

Proteases are divided into two groups, peptidases and proteinases, based on the particular type of cleavage event (Polgar, 1989). Peptidases, also exopeptidases, are responsible for the cleavage of single or dipeptide units from either the amino or carboxy terminus of a protein.

Proteinases, or endopeptidases are proteases that recognize a specific amino acid sequence or motif within a peptide chain. Proteinases can be further classified into one of four groups according to the amino acids participating in catalysis, and their mechanism of action. Serine proteinases contain a catalytic triad of aspartic acid, histidine, and serine. Cysteine proteinases possess a catalytic dyad of cysteine and histidine. The third type of proteinase, the aspartic proteinases have two aspartic acid residues participating in catalysis. Metalloproteinases, the last class, require the addition of water and a divalent cation into the active site for catalysis. Metalloproteinases contain a structural catalytic triad that contains glutamic acid with two histidine residues. The divalent cation, usually Zn^{2+}, is further stabilized by a downstream glutamic acid distinct from the catalytic pocket (Barrett, 1995).

REQUIREMENTS OF PROTEASES

For catalysis of a specific peptide bond to take place, two structural requirements must be met. First, the region of catalysis must be defined by the amino acids neighboring the susceptible bond. This is required for determining both the primary and secondary specificity of

the proteinase for its substrate. Polgar in 1987, discussed primary and secondary specificity as containing both qualitative and quantitative features. Primary specificity is qualitative in its selection of the scissile bond, and the secondary specificity imparts a quantitative feature by facilitating the cleavage of the selected bond. Some proteinases are promiscuous in either of these features. For example, a well-known proteinase, trypsin cleaves only after basic amino acid residues regardless of the neighboring amino acids. In contrast, recognition of a peptide backbone instead of specific amino acids is characteristic of pancreatic elastase. The second requirement for proteolysis is that the susceptible peptide bond should be accessible to the proteinase in a flexible region. The selected bond should also fit in a three-dimensional conformation into the active site of the proteinase. This second requirement is regarded as conformational specificity of the enzyme for its substrate (Wright, 1997). Proteinases contain a catalytic site and a substrate-binding site. Often these two sites are in close proximity of one another in the folded protein. The active site of the proteinase can be formed either contained within one polypeptide, or when two protein monomers come together to form the catalytic pocket.

FORMATIVE AND MORPHOGENIC

In viral systems, there are two types of limited proteolytic mechanisms, either formative or morphogenic (Hellen and Wimmer, 1992b). Formative proteolysis is the processing of structural and non-structural proteins from a larger precursor polyprotein. Retroviruses and the positive-strand RNA viruses have taken advantage of formative proteolysis as a means of expressing several proteins from a single RNA template. Once cleavage occurs, structural proteins can be targeted to their proper location and non-structural proteins can be activated. There are numerous references in the literature of enzymes that perform formative cleavage, but perhaps the best known is the Human Immunodeficiency Virus (HIV) aspartic acid proteinase. In the case of this enzyme, the catalytic dyad is formed with the dimerization of two monomers. (Loeb 1989, Blundell 1990)

In contrast to formative proteolysis, morphogenic cleavage refers to the processing of assembled viral structural proteins during morphogenesis or maturation of the virus. Associated with the maturation process, morphogenic proteolysis is thought to be required for the acquisition of infectivity of both RNA and DNA viruses, such as VV, HIV, picornaviruses, and bacteriophage T4

(Kraeusslich and Wimmer, 1988; Hellen and Wimmer, 1992b).
In the case of HIV, the cleavage of the polyprotein
precurser is essential for proper genomic RNA dimerization
into immature virions. (Stewart 1990) Activation of HIV
proteinase prematurely, was shown by Kraeusslich (1991) to
abrogate maturation and subsequently the infectivity of the
virus. Bacteriophage T4 structural proteins must be
processed for DNA to be properly packaged. (Hershko and Fry
1975) The proteinase from bacteriophage T4 is activated
after autoproteolytic cleavage, commonly utilized as a
mechanism for regulating proteinases. (Showe 1976)
In general, it is essential that proteinases be accurately
regulated to prevent aberrant proteolysis before assembly.
This is achieved in a number of ways including
compartmentalization of enzymes from their substrates,
proteolytic activation of zymogens, the presence of
inhibitors or co-factors, and the requirement for
contextual presentation of a given substrate (Webster
1993).

PROTEOLYTIC PROCESSING IN VACCINIA VIRUS

The first indication that VV proteins were processed
via proteolysis came in 1967 when Holowczak and Joklik
observed differences in the molecular weights of

radiolableled proteins present in VV-infected cells compared to those found in purified virions. Pulse chase experiments following the above observation, identified the relationship between many protein products and their precursors. A few secreted, non-structural proteins, such as hemagglutinin have been shown to be cleaved during the course of a VV infection (Stroobant 1985, Shida 1986, Kotwal and Moss 1988). More importantly, at least six virion-associated proteins are derived from precursor proteins, namely 4a, 4b, 25K, 23K, 21K, and 17K (VanSlyke 1991, Whitehead and Hruby 1994). Alignment of the amino acid sequences of the precursor proteins at the determined cleavage sites revealed a conserved Ala-Gly-X motif (AG*X). This predicted cleavage motif is conserved in variola major virus, the causative agent of the human disease smallpox (Schelkunov 1993). There is also conservation of the cleavage site of the precursors p25K and p4b when compared to their fowlpox homologues FP5 and F4b respectively (Binns et. al. 1988, 1989). Of the processed proteins, 4a, 4b, and 25K are the three most abundant proteins in the VV particle (Moss and Rosenblum 1973, Silver and Dales 1982, Yang and Bauer 1988). Based on such strict conservation of the AG*X motif, and the processing of the major virion proteins, the importance of this type of modification in

the VV replication cycle readily becomes apparent. The
AG*X motif is represented 82 times in the entire VV genome,
however only nine of these sites, present in five proteins,
have been established as targets of proteolysis.
Therefore, factors other than the required AG*X motif are
also responsible for the specificity of processing. All
five substrates are expressed at late times during VV
replication, and are present for a considerable time before
being processed. Proteins containing the AG*X motif that
are expressed at early times of infection have not been
demonstrated to undergo proteolysis. This suggests not
only the necessity for the AG*X motif, but indicates that
processing of these proteins is constrained temporally as
well. The above also suggests that the responsible
proteinase is of viral origin, as cellular protein
synthesis is shut off early during a viral infection.
Infected cells were blocked for late viral gene expression
in order to determine the gene that was responsible for the
proteolytic activity. The cells were then transfected with
a plasmid copy of a VV-protein substrate, and transcription
factors required for late gene expression. Co-transfection
of cosmid clones representing the entire VV genome were
used in order to determine the region necessary to rescue
proteolytic processing. The only open reading frame

sufficient to rescue processing was the G1L open reading

frame (ORF). (Whitehead and Hruby, 1994).

G1L

The entire sequence of the VV genome has been

published and based on this, the product of the G1L ORF is

predicted to be 591 amino acids, and have a molecular

weight of approximately 68kDa. G1L is expected to be

soluble and have a pI of 6.29, as would be expected for a

cytoplasmic enzyme. Computer analysis of the amino acid

sequence of G1L generated only one motif that resembled any

known proteinase, namely the HEXXH pentapeptide of

metallopeptidases, albeit in the reverse orientation. The

HXXEH motif is followed by a downstream ENE motif, which is

required for stabilization of a divalent cation, presumably

zinc, to the protein. This ENE triplet in G1L provides

both the forward and reverse orientations of the NE doublet

characteristic of the Pitrilysin family of

metallopeptidases. Mutagenesis studies of the glutamate

residue within HXXEH motif, in the transprocessing assay

described earlier, did in fact abolish the processing. In

addition, mutagenesis of the glutamate residue within the

ENE tripeptide abrogated processing (Whitehead and Hruby,

1994). The above data strongly suggest the hypothesis that

the G1L ORF encodes the proteinase responsible for processing and maturation of VV.

METALLOPEPTIDASES CLASSIFICATION

Metalloproteinases are the most diverse group of catalytic peptidases, comprising 30 families. These enzymes contain the HEXXH motif that has been shown by X-ray crystallography to form part of the binding site for divalent cations, most often Zn^{2++}. The two histidine residues within the motif bind the zinc atom and the glutamic acid (Glu) has a catalytic function. The Glu residue promotes the nucleophilic attack of a water molecule on the carbonyl carbon atom of the peptide bond, forming a tetrahedral intermediate (Weaver et. al., 1977). Metalloproteinases are divided into five "clans" based on the catalytic site containing two zinc ligands as well as a third stabilizing zinc ligand located away from the catalytic pocket. In the first clan, named MA, a glutamic acid residue downstream of the HEXXH motif completes the metal-binding site. Clan MA is the most thoroughly characterized containing, thermolysin and other enzymes from both prokaryotic and eukaryotic organisms. Clan MB has another histidine residue as the third ligand. A completely conserved methionine residue in all members of

this clan indicates that it serves a vital function in the structure of the active sites of the enzymes. This has led to the use of the term "metzincins" for the peptidases of this clan, and the most well known are the human interstitial collagenase, and fibrolase. The three remaining clans have not been named, but the third is comprised of members whose motif is HEXXH, but their third metal ligands are not known. An interesting member of this clan is immune inhibitor A, an endopeptidase from *Bacillus thuringiensis*, a bacterium that is a pathogen of lepidoptera and is used as a bio-agent in the control of these lepidopteran pests in agriculture (Loevgren, et. al. 1990). The fourth clan contains metalloproteinases for which it is known that the essential metal atoms are bound at motifs other than HEXXH. There are five families in this clan with representatives from a diverse array of organisms, such as pitrilysin from E. coli, and human insulin degrading enzyme (IDE) from humans. The fifth and final group contains those families for whom the metal ligands are not known. Members of this clan include membrane dipeptidase, and glutamate carboxypeptidase. The motif found in the G1L ORF shows similarity with the fourth clan of metalloproteinases, and more specifically to the family Pitrilysin. (Figure I.2) The Pitrilysin family

Figure I.2. The amino acid alignment of the catalytic residues of some of the members of the Pitrilysin Family of metalloendopeptidases. The high degree of conservation between divergent organisms within the catalytic pocket is clearly evident, and would suggest the importance of this motif to function of the protein.

Figure I.2

ENZYME	CATALYTIC SEQUENCE
Human IDE	GLS **HFCEH** MLF
Rat IDE	GLS **HFCEH** MLF
Drosophila IDE	GLA **HFCEH** MLF
Protease III	GLA **HYLEH** MSL
orfP gene product	GIS **HFLEH** MFF
pqqF gene product	GLA **HLLEH** LLF
GIL	GIA **HLLEH** LLI

contains two subfamilies of enzymes: Pitrilysin and the Mitochondrial Processing Peptidase subfamilies. Characteristic of these sub-families, is an inverted zinc-binding domain, HXXEH, and a downstream Glu that acts as the third zinc ligand. Site-directed mutagenesis studies have identified the two His residues found at positions 88 and 92 in mature pitrilysin to bind zinc, and Glu-91 to be the catalytic residue. The third zinc ligand in pitrilysin is glutamic acid at position 169. Similarly, the family of IDEs showed a loss in proteolytic activity when either the histidines or the glutamic acid were mutagenized, but interestingly IDE retained its insulin binding characteristics, indicating that the substrate, as expected, binds at a site distal to the catalytic pocket.

The IDEs represent a newly identified superfamily of metallopeptidases typified by an inverted HEXXH motif. Although fundamental questions concerning the biological role of IDEs remain, the high degree of conservation among members of this clan would suggest that it has an important biological significance. Considering G1L may in fact be a member in the same family of metalloendopeptidases, and the first metalloproteinase to be described in a viral system, the overall significance of studies concerning this enzyme are evident.

INHIBITORS OF METALLOENZYMES

Proteinases must be efficiently regulated to ensure
that no damage occurs to proteins other than the intended
target. Organisms have developed a number of interesting
mechanisms to by-pass this inherent feature of peptidases.
Expression of a protein that inhibits a particular
proteinase is a common theme, and protein inhibitors are
present in multiple forms in numerous tissues (Kato and
Laskowski, 1980). Often these protein inhibitors are
specific for a particular proteinase, just as the
proteinase is specific for a particular target. Chemical
inhibitors on the other hand can be rather promiscuous in
their inhibitory effect. For instance, EDTA, a chelator of
divalent cations, shows only specificity for some but not
all peptidases requiring a metal atom for catalysis.
Phosphoramidon, another chemical inhibitor, inhibits all
metalloenzymes from Clan MA but not those from Clan MB,
making it somewhat more specific than EDTA. Finally, there
are more specific chemical inhibitors, these are often
times derived synthetically in the laboratory, and inhibit
only one proteinase. Synthetic chemical inhibitors show
promise in medicine for the treatment of many congenital
diseases, including rheumatoid arthritis. IDE can be

inhibited by a number of chemicals including; 1,10-phenanthroline, 1,7-phenanthroline, bacitracin, and N-ethlymaleimide. In addition to these, IDEs have also been shown to be inhibited by excess amounts of insulin, as well as excess amounts of zinc. The inhibition of insulin degrading activity with these inhibitors was shown with the purified form of IDE. 1,10-phenanthroline is a chelator of divalent cations, and 1,7-phenanthroline its non-chelating isomer, which with purified IDE shows only a minor inhibitory effect. The antibiotic bacitracin is a chemical inhibitor that is known to form a complex with zinc, and has been shown to inhibit pitrilysin, a member of the same family as IDE. N-ethylmaleimide (NEM) has been used in cancer research and is thought to have an anti-mitotic effect. NEM has also been used as an inhibitor of IDEs, and shows greater inhibitory effect than 1,10-phenanthroline (Chang and Bai, 1996).

It was of interest to determine if these chemical inhibitors could inhibit VV at the level of proteolytic maturation. It would be expected that if G1L were the proteinase responsible for the maturation process, and if it were a metalloproteinase as indicated by previous experiments, then the process of maturation would be suspended when an infection was subjected to treatment with

an inhibitor. This thesis outlines the experiments carried

out to investigate the effect these chemical inhibitors

have on VV replication, and at what step in the process of

VV replication inhibition is observed.

Chapter II

INTRODUCTION

Vaccinia virus (VV), a member of *Orthopoxviridae* is a large enveloped DNA virus that replicates entirely within the cytoplasm of an infected host cell. The 200 kbp genome of VV encodes for over 250 gene products. During a productive VV infection, a number of proteins undergo post-translational modifications, which include acylation, phosphorylation, glycosylation, ADP-ribosylation, and proteolytic processing (VanSlyke and Hruby, 1990). Modification of proteins is believed to be essential for correct sub-cellular localization of both structural and non-structural VV proteins. In addition, maturation and acquisition of infectivity is thought to be dependent on proteolysis of structural proteins. A number of VV proteins are processed from higher molecular weight species, and these are 4a, 4b, 25K, 23K, 21K, and 17K. Cleavage of each of these proteins has been shown to occur at a common motif, namely, at an AG*X site, where (*) indicates the site of cleavage. Based on conservation of this motif within VV as well as between virus species, it is possible that a single enzyme is responsible for processing. The processed proteins are expressed only at

late times during infection, and proteins containing the
AG*X motif expressed earlier in replication are not
cleaved. This indicates that the specificity of the
protease is regulated not only at a structural level, but
also temporally with respect to replication. Rescue of
proteolytic processing during a trans-processing assay was
achieved with the G1L ORF. Sequence comparison of the G1L
ORF with all other known proteolytic enzymes revealed
homology of the catalytic motif of a family of enzymes
known as the insulin degrading enzymes (IDE). As the name
might imply, this family of proteases is responsible for
the clearance of the circulating hormone, insulin. The
catalytic motif, HXXEH, is a direct inversion of the motif
found in most known metalloproteinases. In addition, found
within the G1L ORF is a downstream ENE triplet, this
provides both the forward and reverse orientation of the
obligate NE dipeptide found in the family of IDEs. The
glutamate of the dipeptide is required for stabilization of
the zinc atom during catalysis. Mutagenesis of the
proposed catalytic motif, in the transprocessing assay
abrogated processing, indicating further the possibility
that G1L is the responsible proteinase, as mutagenesis
studies with IDE showed similar results. The strict
conservation of the HXXEH motif as well as the downstream

ENE tripeptide led to the working hypothesis that G1L is a metalloproteinase involved in the proteolytic maturation of VV during a productive infection.

Proteinases are ubiquitous in nature and so are the protein inhibitors associated with them. It is imperative that a biological system designed to degrade proteins is properly regulated, and one way of achieving this goal is proper expression of inhibitors. With the exception of macroglobulins, which "inhibit" proteinases of all classes, individual protein inhibitors inhibit only proteinases belonging to a single mechanistic class (Kato and Laskowski, 1980). As proteinases show specificity, so do their inhibitor counterparts. Chemical inhibitors as opposed to protein inhibitors also show in many cases a high degree of specificity, although often times they can inhibit an entire class of proteinases. An example of this would be phosphoramidon, which inhibits Clan MA of metalloendopeptidases but not those of Clan MB.
Based on the homology between IDE and G1L it was of interest to determine if G1L could be inhibited by the same chemicals that have been shown to inhibit the IDEs. Some inhibitors of the IDEs are, I,10-phenanthroline which is a divalent cation chelator, and N-ethylmaleimde. 1,7-phenanthroline is the non-chelating isomer of 1,10-

phenantholine. In the absence of the ability to purify the protein product of the G1L ORF, our attention turned to the possibility that inhibitors of the IDEs when added to a VV infection would inhibit replication at the step of proteolytic morphogenesis. I report here the results of testing the ability of various metalloproteinase inhibitors to inhibit VV replication.

Chapter III

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney BSC-40 cells, a derivative
of BSC-1 cells selected for their ability to grow at 40°C,
were grown in monolayers. These cells were maintained in
Eagle minimum essential medium (MEM) (Sigma), and were
supplemented with 10% heat inactivated fetal calf-serum, 2
mM L-glutamine, and 10ug/ml of gentamycin sulfate.
Vaccinia virus (strain WR) Western Reserve was obtained
from the American Type Culture Collection, and was
propagated by low-multiplicity passages and periodic plaque
purification. Infection, purification and titration of
cells with VV-WR was carried out as previously described
(Hruby 1979a).

Serial passages

Confluent monolayers of BSC-40 cells (100mm-dishes)
were treated with either 1,10-phenanthroline, 1,7-
phenanthroline, N-ethylmaleamide (NEM), insulin, or British
Biotech inhibitor, at concentrations of 10mM, 1mM, or
0.1mM. The cells were infected with VV-WR at a
multiplicity of infection (moi) of one. These were

incubated at 37°C under 5% CO_2 95% O_2 for 48 hours. The
infected cells were harvested into 5 ml of sterile
phosphate buffered saline, and progeny virions were
liberated from the cells by three consecutive cycles of
freeze thawing. Titers were determined using a portion of
this crude stock, and the remainder was used to infect a
new monolayer of BSC-40 cells. The above steps were
repeated a total of six times.

One-step growth curve

Confluent monolayers of BSC-40 cells (100mm dishes)
were infected at a moi of one and treated with either 0.1mM
1,10-phenanthroline, 1,7-phenanthroline, or no treatment.
Infected cells were harvested at 0, 2, 4, 8, 12, 24, and 48
hours post infection, into 1ml phosphate buffered saline.
Virus could be titered after three freeze thaw cycles,
which liberates the virus from the cells. Titration was
performed in 6-well dishes of confluent monolayers of BSC-
40 cells as previously described.

Metabolic Labeling

Confluent monolayers of BSC-40 cells were infected
with VV at a moi of five with or without drug, in MEM media
deficient for methionine. Radiolabeled ^{35}S-methionine was

added to the cultures at the indicated times post infection, incubated 15 minutes, rinsed, and then harvested. Cells were centrifuged and resuspended in SDS sample buffer for assay by polyacrylamide gel analysis. Following separation of the radiolabeled proteins, the gels were dried and laid against film, and later developed.

Electron Microscopy

A six well plate containing confluent monolayers of BSC-40 cells were subjected to infection with VV WR at an moi of ten, two wells were subjected to 1,10-phenanthroline treatment 0.1mM, two were treated with 1,7-phenanthroline 0.1mM, and two were the control with no treatment. At 0 and 8 hours post infection, the cells were harvested and fixed for transmission electron microscopy, which was carried out by the Electron Microscopy lab at Oregon State University.

Chapter IV

RESULTS

Inhibitors

The ability of inhibitors to inhibit VV replication.
1,10-phenanthroline is a chelator of divalent cations, and
N-ethylmaleimide (NEM) inhibits metallopepetidases of the
insulin degrading enzyme (IDE) class. These chemicals have
both been shown in studies to inhibit the action of
purified IDE from degrading cellular insulin. The studies
described above were carried out with a purified form of
IDE. G1L, the predicted target of inhibition by 1,10-
phenanthroline and NEM, has not been purified. Based on
the sequence homology between the class of IDEs and G1L,
however, G1L might be susceptible to inhibition by both
1,10-phenanthroline, NEM. Included in the inhibition
studies were the non-chelating isomer of 1,10-
phenanthroline, 1,7-phenanthroline, as well as an inhibitor
obtained from British Biotech, NEM, and insulin (Table
IV.1). The inhibitor from British Biotech is a matrix-
metalloproteinase inhibitor, and was chosen in order to
exclude the possibility that G1L encodes a proteinase from
Clan MA of metalloendopeptidases. Insulin was also chosen

Table IV.1 shows the ability of a given chemical to inhibit
replication of VV in BSC-40 cells. Included in this table
is the susceptibility of the cells themselves to possible
toxic effects of each inhibitor.

Table IV.1

Chemical Inhibitor	Inhibitor Concentration	Virus Titer	Cellular Toxicity
1,10-Phenanthroline	1 mM 0.5 mM 0.1 mM	0 0 4 x 10	+ + +/-
1,7-Phenanthroline	1 mM 0.5 mM 0.1 mM	0 8 x 10 2 x 10	+ - -
N-Ethylmaleimide	1 mM 0.5 mM 0.1 mM	0 0 0	+ + +
Bacitracin	1 mM 0.5 mM 0.1 mM	0 0 0	+ + +
BB-British Biotech	1 mM 0.5 mM 0.1 mM	0 0 0	+ + +
Insulin	100 mM	6 x 10	-

as studies have shown that increasing concentrations of insulin are inhibitory to the IDEs.

One step growth curve analysis

Figure IV.1 is a graphic representation of a one-step grown curve of either wild-type WR strain alone, or in the presence of either 1,10-phenanthroline or 1,7-phennathroline. As indicated the titer of WR alone at 48 hours was approximately 1×10^9 pfu, and the 1,7-phenanthroline was also on the order of 1×10^9 pfu. The infection with 1,10-phenanthroline, however, did not increase and the final titer was three logs below that of either the WR or 1,7-phenanthroline. Of interest also is the 1,7-phenanthroline curve that shows about a 2-hour lag compared to the WR only.
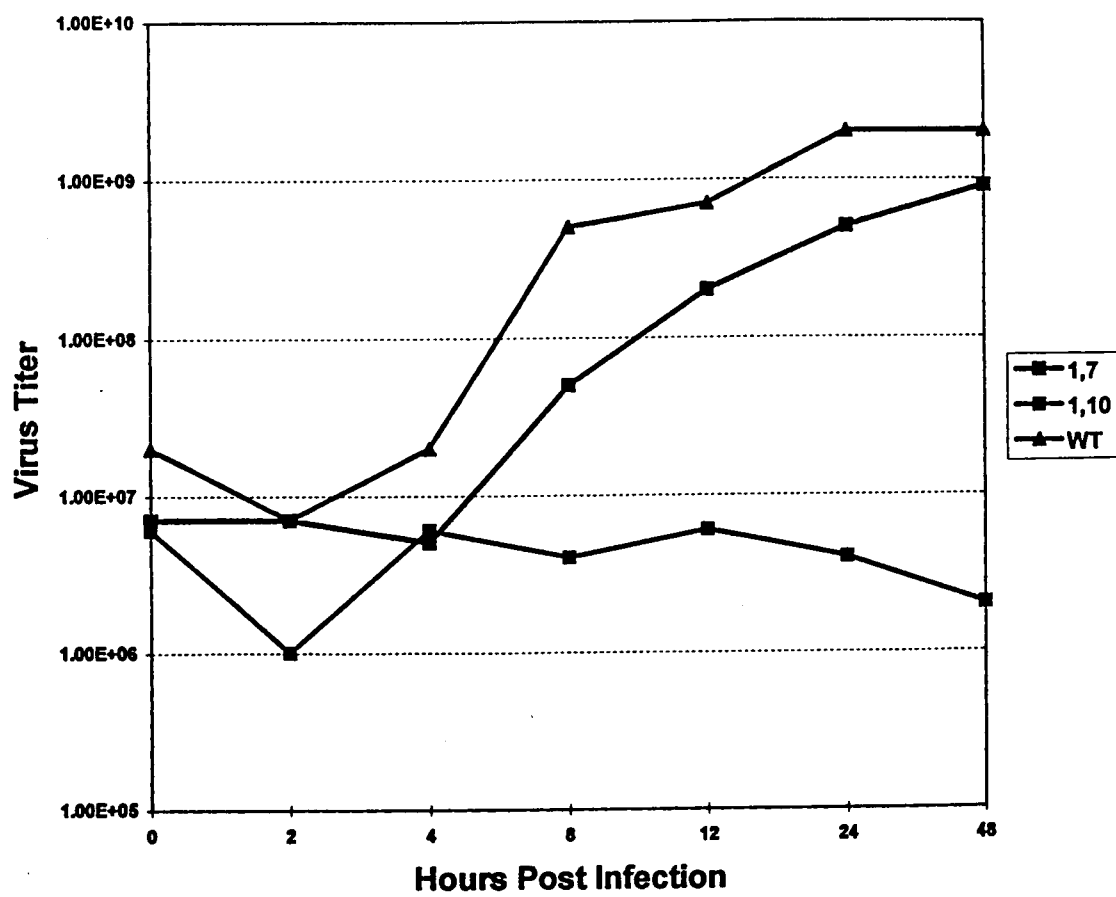
Serial passage in the presence of inhibitors

The acquisition of drug resistant VV mutants in the past has been accomplished often, by serial passage of the virus in the presence of a given chemical. Following the addition of a chemical to an infection of VV, there is a notable drop in viral titer, due to virus that is incapable of replicating in the presence of the drug. After a number of passages those virus resistant to the drug are capable

Figure IV.1. Graphic representation of a one-step growth
analysis of VV in the presence or absence of drug.

Figure IV.1

One Step Growth Curve of VV (+/- Inhibitor)

of replicating in the presence of the drugs and an increase in titer is seen. Serial passage of VV in the presence of 1,10-phenanthroline, did not produce any drug resistant mutants, indicating that the target of the inhibitor was likely of cellular origin. (Table IV.2)


^{35}S-methionine labeling

Pulse labeling during a viral infection can be used to gather information about what proteins are being made or processed at specific times. It was of interest following the one step growth curve to analyze the protein profile of those proteins expressed in the presence or absence of drugs. Figure IV.2 shows the results of a pulse labeling experiment at different times post infection in the presence or absence of 1,7-phenanthroline, or 1,10-phenanthroline. At zero hours post infection all lanes appear to be the same, but at five hours it is clear that the drug treatment has had an effect on viral replication. It appears as though the virus treated with both drugs is incapable of transition from early gene translation to intermediate translation. However, by eight hours post infection the lane with 1,7-phenanthroline treatment looks similar to WR with no drug at five hours. This is in accordance with the one step-growth curve where 1,7-

Table IV.2. Serial passage.

Table IV.2

Virus Titers of Serial Passages

Chemical Inhibitor	First Passage	Third Passage	Fifth Passage
1,10-phenanthroline	4×10^6	7×10^4	<100
1,7-Phenanthroline	8×10^7	4×10^6	1×10^7
Western Reserve No Drug	6×10^9	3×10^7	7×10^8

Figure IV.2. Pulse-chase labeling analysis of VV proteins
in the presence of drug.

Figure IV.2.
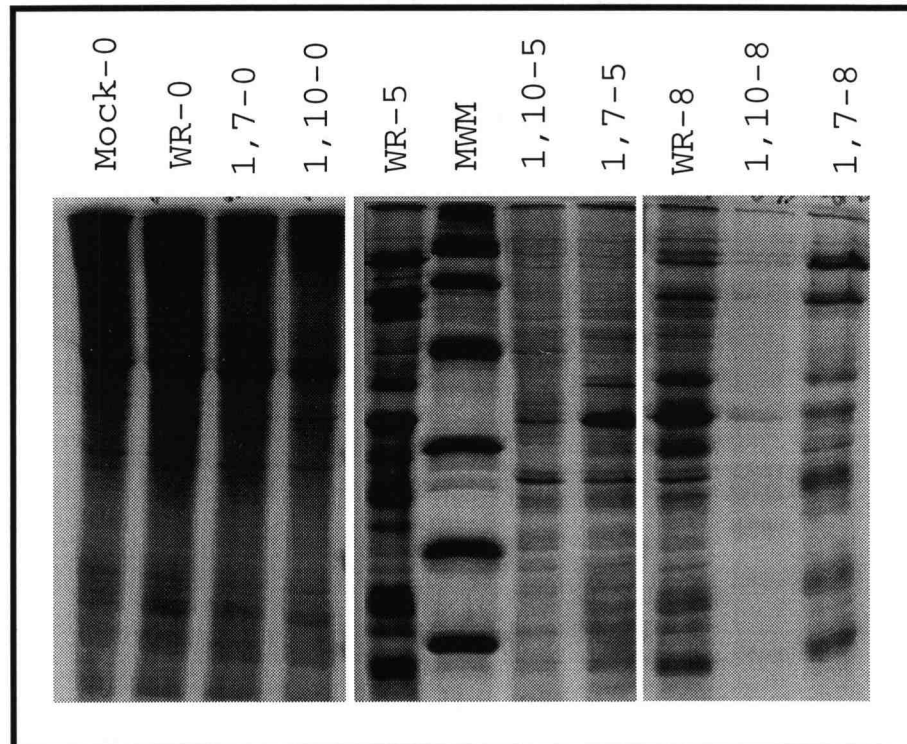
phenanthroline was "behind" the WR only infection by about two hours. The infection with 1,10-phenanthroline, however, did not exit early gene translation as would be expected by eight hours post infection. Longer incubation times did not rescue this deficiency in drug treated infections (data not shown).

Electron Microscopy

Further investigation to determine if the virus was being inhibited at the step of uncoating was done with electron microscopy. The infection without drug showed normal replication as indicated by figure IV.4. Infection of the virus in the presence of 1,7-phenanthroline at eight hours showed a slightly slower progression through replication. The virus is not quite mature at this time point. (Figure IV.5) This is in agreement with the one-step growth curve as well as the pulse labeling experiments. Figure IV.6 shows a virus infection in the presence of 1,10-phenanthroline was very interesting however. It appears as though endocytocis is completed, but that the virus remains within the endocytic vesicle, as indicated by the arrow. There was some cellular toxicity as some cells were amitochondrial when infected in the presence of 1,10-phenanthroline, but cells that were

not deficient in mitochondria, and showed an arrest of VV replication, were found to have these large endocytic vesicles.

Figure IV.4. Electron microscopic analysis of VV
replication Western Reserve. This is an electron
micrograph of a VV at eight hours post infection. Mature
virions are clearly evident, and the magnification is
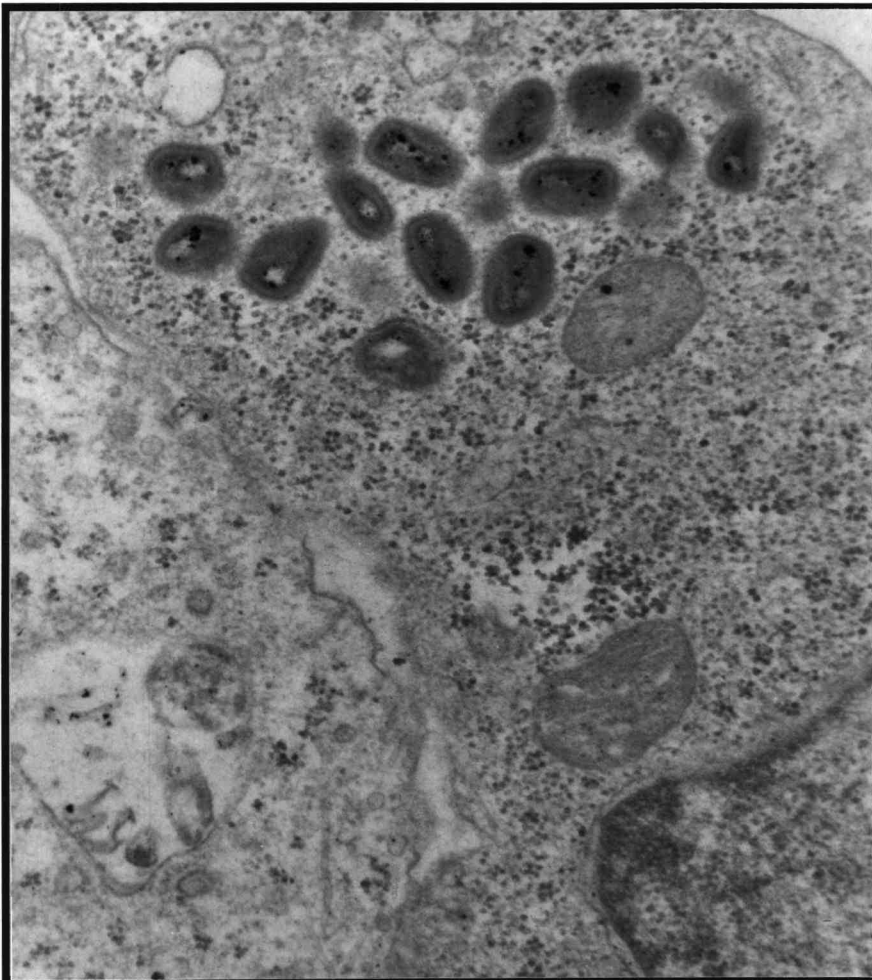28,000x.

Figure IV.3

Figure IV.5. Electron microscopic analysis of VV
replication in the presence of 1,7-phenanthroline. At
eight hours post infection the virions have not matured,
but have been assembled. The magnification of this field
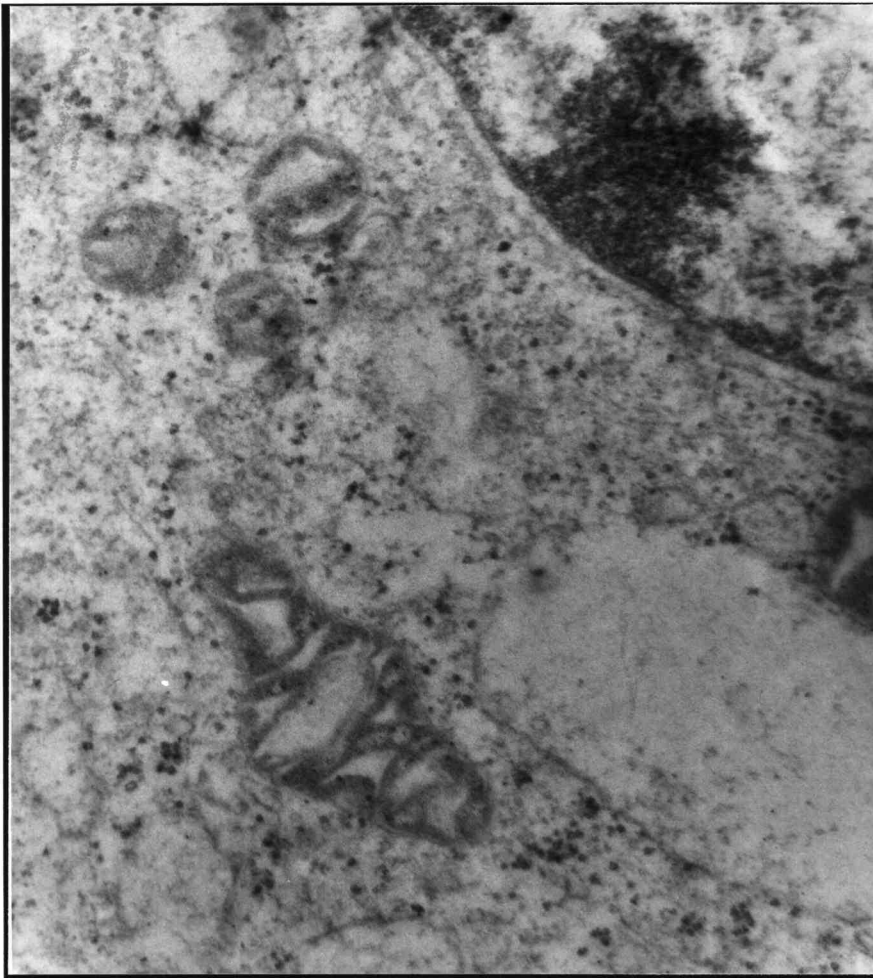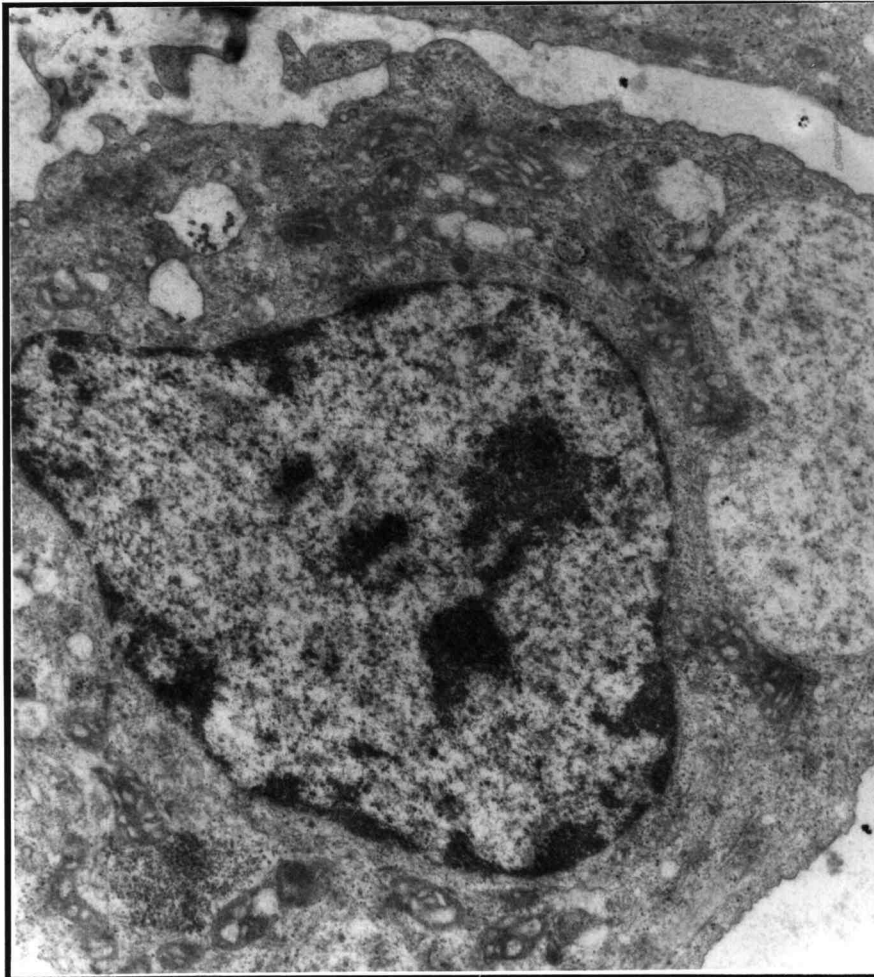is 35,000x.

Figure IV.4

Figure IV.6. Electron microscopic analysis of VV
replication in the presence of 1,10-phenanthroline. This
micrograph is at a magnification of 10,000x, and as
indicated by the arrow, there is an endocytic vessicle with
particles contained within it.

Figure IV.5

Chapter IV

Discussion

Very little is known about the process of uncoating of VV during a productive infection. It is thought that following receptor mediated endocytosis, that there are two successive steps of uncoating. First, the outer envelope is released, and early gene products are shuttled to the cytoplasm and translated. Second, the core is broken down and DNA replication begins. The enzymes and proteins involved in this process of uncoating have not been identified. Utilizing the chemical inhibitor 1,10-phenanthroline, I was able to show that a protein that might be involved in uncoating of the virus is likely to be of cellular origin as opposed to viral. If the protein responsible for uncoating were of viral origin, one might expect to be able to derive a drug resistant mutant, as has been done many times in the past. One-step growth curve analysis showed that replication was being inhibited very effectively. Further, pulse labeling of a viral infection in the presence of the drug indicated that transition from early gene translation to intermediate gene translation was suspended. Finally, electron microscopy indicated that at least part of the time virus was being endocytosed, but

that it was not able to proceed from the endocytic vesicle to the cell even after eight hours. It appears that addition of 1,10-phenanthroline at the time of infection inhibits the viral replication cycle at the level of core uncoating. It is probable that the target of the inhibition is a protein of cellular origin, as no drug resistant mutants were obtained.

The next step to determine whether 1,10-phenanthroline would inhibit G1L, the putative metalloproteinase, would be to add 1,10-phenanthroline two to four hours after a productive VV infection has been established. This would bypass the inhibition at the level of uncoating and perhaps a drug resistant mutant could be acquired.

1,7-Phenanthroline showed a sight inhibition and a time lag in the one-step growth curve, the radiolabeling experiment, and in the electron microscopy experiment. It has been shown previously that 1,7-phenanthroline does exhibit some degree of inhibition against purified human IDE, however, this inhibition is not a chelating action but rather a non-specific, non-competitive type of inhibition. In the above experiment rescue of enzymatic activity of IDE was achieved by addition of Zn^{2+} for 1,10-phenanthroline but not by 1,7-phenanthroline. It is not surprising then that some amount of inhibition was observed in the presence of

the non-chelating chemical 1,7-phenanthroline. It is possible that addition of excess Zn^{2+} following a VV infection in the presence of 1,10-phenanthroline would rescue the inhibition of replication.

It is clear that the replication of VV in the presence of 1,10-phenanthroline inhibited the replication cycle, most likely at the level of uncoating. Perhaps not so clear, is what protein this drug is inhibiting. If the protein involved is a cellular protein as thought based on these experiments, it would be difficult to ascertain which protein it is. It is possible that there was no inhibition to a protein but rather a disruption of various ion channels and a breakdown in the integrity of the cellular membranes. However, work done previously has shown that neither 1,7-, nor 1,10-phenanthroline cause significant abrogation of transport, integrity, or permeability of rat derived epithelial tissue.

Overall, the results of these experiments suggest that in the presence of 1,10-phenanthroline, during a productive infection of VV, the replication cycle is impaired at the level of uncoating. This suggests that one or more cellular metalloproteinases may be involved in liberating the incoming virus from the endocytic vesicle, and in

disrupting the core to allow intermediate gene expression to occur.

Bibliography

Barrett, A. J., (1995). Methods in Enzymology Proteolytic Enzymes: Aspartic and Metallo Peptidases **248**, 183-228.

Binns, M., M. E. G. Boursnell, F. M. Tomley, and J. Campbell. (1989). Analysis of the fowlpox gene encoding the 4b core polypeptide and demonstration that it possesses efficient promoter sequences. *Virology* **170**, 288-291.

Binns, M., T. M. Tomley, J. Campbell, and M. E. G. Boursnell. (1988). Comparison of a conserved region in fowlpox virus and vaccinia virus genomes and the translocation of the fowlpox thymidine kinase gene. *J. Gen. Virolo.* **69**, 1275-1283.

Chang, L. L. and J. P. F., Bai. (1996). Effects of enzyme inhibitors and insulin concentration on transepithelial transport of insulin in rats. *J. Pharm. Pharmacol.* **48**, 1078-1082.

de Groot, R. J., W. r. Hardy, Y. Shirako, and J. H.Strauss. (1990). Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase: evidence for temporal regulation of polyprotein processing in vivo. *EMBO J.* **9**, 2631-2638.

Hansen,S. G. and D. E. Hruby. Biology of Vaccinia Virus Myristylproteins. Recent Research Developments in Microbiology. In press.

Hellesn, C. U. T., and E. Wimmer. (1992). The role of proteolytic processing in the morphogenesis of virus particles. *Experimentia* **48**, 210-215.

Hershko, A. and M. Fry. (1975). Post-translational cleavage of polypeptide chains: Role in assembly. *Ann. Rev. Biochem.* **44**, 775-797.

Keck, J. G., C. J. Baldick, and B. Moss. (1990). Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. *Cell* **61**, 801-809.

Kotwal, G. J., and B. Moss. (1988). Vaccinia virus encodes

a secretory polypeptide structurally related to complement control proteins. *Nature* **335**, 176-178.

Kraeusslich, H. G., and E. Wimmer. (1988). Viral proteinases. *Annu. Rev. Biochem.* **57**, 701-754.

Kraeusslich, H. G., (1991). Human immunodeficiency virus proteinase dimer as component of the viral polyprotein prevents particle assembly and viral infectivity. *Proc. Natl. Acad. Sci. USA* **88**, 3213-3217.

Lovgran, A., M. Zhang, A. Engstrom, G. Dalhammar, R. Landen. (1980). Molecular characterization of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis. Mol. Microbiol.* **4**, 2137-2146.

Moss, B., and E. N. Rosenblum. (1973). Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. *J. Mol. Biol.* **81**, 267-269.

Polgar. L., and P. Halasz. (1982) Current problems in mechanistic studies of serine and cysteine proteinases. *Biochem. J.* **207**, 1-10.

Schelkunov, S. N., S. M. Relenchuk, A. V. Totmenin, V. M. Blinov, S. S. Marennikova, and L. S. Sandakhchiev. (1993) Comparison of the genetic maps of variola and vaccinia viruses. *FEBS-Lett.* **327**, 321-324.

Shida, H. (1986). Nucleotide sequence of the vaccinia virus hemagglutinin gene. *Virology* **150**, 451-462.

Silver. M, and S. Dales. (1982). Biogenesis of vaccinia: interrelationship between post-translational cleavage, virus assembly, and maturation. *Virology* **117**, 341-356.

Stewart, L., G. Schatz, and V. M. Vogt. (1990) Properties of avian retroviruses particles defective in viral protease. *J. Virol.* **64,** 5076-5092.

Stroobant, P., A. P. Rice, W. J. Gullick, D. J. Cheng, I. M. Kerr, and M. D. Waterfield. (1985). Purification and characterization of vaccinia virus growth factor. *Cell* **42**, 383-393.

VanSlyke, J. K., and D. E. Hruby. (1990). Post translational modification of vaccinia virus proteins. In Current Topics in Microbiology and Immunology. Poxviruses, Vol. 163, Moyer, R. W., and P. C. Turner (Eds.). Springer-Verlag, New York.

VanSlyke, J. K., C. A. Franke, and D. E. Hruby. (1991). Proteolytic maturation of vaccinia virus core proteins: Identification of a conserved motif at the N-termini of the 4b and 25K virion proteins. *J. Gen. Virol.* **72**, 411-416.

VanSlyke, J. K., S. S. Whitehead, E. M. Wilson, and D. E. Hruby. (1991). The multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein: a degenerate conserved cleavage motif within core proteins. *Virology.* **183**, 467-478.

Weaver, L. H. and W. R. Dester, and B. W. Matthews. (1977). A crystallographic study of the complex of phosphoramidon with thermolysin. A model for the presumed catalytic transition state and for the binding of extended substances. *J. Mol. Biol.* **114,** 119-132.

Webster, A., R. T. Hay, and G. Kemp. (1993). The adenovirus protease is activated by a virus-coded disulfide-linked peptide. *Cell,* **72**, 97-104.

Yang, W., and W. R. Bauer. (1988). Purification and characterization of vaccinia virus structural protein VP8. *Virology* **167**, 578-584.