STUDIES ON THE GLYCOPROTEIN OF VESICULAR STOMATITIS VIRUS

By

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A THESIS

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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ABSTRACT

The purpose of this study was to investigate some of the structural features in the glycoprotein molecule of vesicular stomatitis virus (VSV) which could be of importance in its biosynthesis. The approach involved

(1) A comparative study of the primary structure of the in vitro synthesized form and the mature form of the glycoproteins of three serotypes of VSV namely Indiana, New Jersey (Concan) and Cocal.

(2) Biochemical characterization of ts Y1, a temperature sensitive mutant of the Cocal serotype of VSV defective in glycosylation.

(3) Investigation of the labeling of the three serotypes of VSV and of Chandipura and Piry viruses, grown in the presence of \([^3H]\)palmitate.

Studies of Irving et. al., (1979) using the Indiana (Toronto) serotype of VSV demonstrated that the in vitro synthesized unglycosylated form of the membrane glycoprotein \(G\), contains at the \(NH_2\)-terminus an extra polypeptide not present in the mature glycoprotein(G). In order to determine the function related properties of the transient region of the protein (signal peptide), the primary structure of the \(NH_2\)-terminus of the \(G\) protein of two other serotypes of VSV, New Jersey (concan) and Cocal which contain variable sizes of the glycoprotein in their virions was determined. A comparison of the partial \(NH_2\)-terminal sequence of the \(G\) and \(G\) proteins of the three
serotypes of VSV revealed that each of the three serotypes have a signal peptide that is made up of a minimum of 16 amino acids with a high proportion of hydrophobic amino acids. There is a striking unrelatedness in the signal region in contrast to the high degree of homology in the NH$_2$-terminal sequences of the mature virion glycoprotein G. In spite of the lack of homology in the signal region there was a similarity in the overall hydrophobicity.

Temperature sensitive (ts) mutants corresponding to the complementation group (V) of VSV Indiana, which include the mutants with specific lesion(s) in the structural gene of G, have been isolated on the basis of characteristics to grow at the two temperatures of 32°C (permissive) and 39°C (nonpermissive) (Lafay, 1974; Lodish and Weiss, 1979; Pringle, 1982). A temperature sensitive mutant of the Cocal serotype of VSV ts$^+$ synthesizes at the permissive temperature a glycoprotein whose size is smaller (molecular weight 68,000) than the wild type (molecular weight 71,000). At the nonpermissive temperature reduced amounts of noninfectious virus-like particles deficient in glycoproteins were produced. The size of the intracellular glycoprotein was further decreased (molecular weight 64,000) at the nonpermissive temperature. Biochemical studies including sugar labeling, tryptic peptide analysis and NH$_2$-terminal sequence analysis of the glycoprotein suggest that at the nonpermissive temperature unglycosylated product blocked in its transport to the cell surface is synthesized while at the permissive temperature a fully functional product with a single oligosaccharide chain is synthesized. In contrast, the wild type VSV Cocal glycoprotein contains two
oligosaccharide chains at both temperatures. In addition, neither the wild type nor the mutant glycoprotein contain covalently bound fatty acid.

Glycoproteins present in a number of enveloped viruses have been shown to contain covalently bound fatty acid residues (Schmidt, 1982a). The glycoproteins of the three serotypes of VSV and of Piry and Chandipura viruses were examined for the presence of fatty acid (palmitic acid). The results showed that while the mature G protein of VSV Indiana (Toronto) serotype and Piry and Chandipura viruses contain palmitic acid residues, the glycoproteins of VSV Cocal and New Jersey serotypes do not contain palmitic acid, suggesting that acylation of the G glycoprotein is not essential for some vesiculoviruses while it is important (if not required) for others.
The studies reported in this thesis have been presented in part, in the following publications:


Acknowledgement

I would like to express my sincere appreciation and gratitude to the members of my supervisory committee Drs. K.B. Freeman, H.P. Ghosh, W.C. Leung and L.A. Prevec for closely reviewing the progress of this work.

I am grateful to Dr. H.P. Ghosh for the use of the facilities and for the helpful discussions.

I would like to thank Dr. G.E. Gerber for advice received on numerous occasions.

I am also indebted to Dr. L.A. Prevec for the serotypes and strains of VSV and Dr. C.R. Pringle for the mutant tsY.

I am extremely grateful to my parents to whom I dedicate this work for the inspiration, comfort and understanding.

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LIST OF ABBREVIATIONS

A

adenosine

$A_{260\text{nm}}$ unit

An amount of nucleic acid that has an absorbance of 1.0 at 260 nm when dissolved in 1 ml water and measured with a 1 cm light path; it is equivalent to 50 μg of double stranded nucleic acid or 37 μg of single stranded nucleic acid/ml.

Amino acids:

<table>
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<tr>
<td>Arg</td>
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</tr>
<tr>
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<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>Asx</td>
<td>asparagine/aspartic acid</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Glx</td>
<td>glutamine/glutamic acid</td>
</tr>
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</tr>
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</tr>
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<td>isoleucine</td>
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<tr>
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<td>leucine</td>
</tr>
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<td>Lys</td>
<td>lysine</td>
</tr>
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<td>Met</td>
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<tr>
<td>Phe</td>
<td>phenylalanine</td>
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<tr>
<td>Pro</td>
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<tr>
<td>Abbreviation</td>
<td>Meaning</td>
</tr>
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</tr>
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<td>tryptophan</td>
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<td>Tyr (Y)</td>
<td>tyrosine</td>
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<tr>
<td>Val (V)</td>
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<td>ATP</td>
<td>adenosine 5' triphosphate</td>
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<td>ATZ</td>
<td>anilinothiazolinone</td>
</tr>
<tr>
<td>AUPS</td>
<td>absorbance units</td>
</tr>
<tr>
<td>BHK cells</td>
<td>baby hamster kidney cells</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>C</td>
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<td>CHO cells</td>
<td>Chinese hamster ovary cells</td>
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<tr>
<td>Cl</td>
<td>Curie</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DEAE</td>
<td>diethylaminoethylcellulose</td>
</tr>
<tr>
<td>DI</td>
<td>defective interfering</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Endo H</td>
<td>endo-β-N-acetylglucosaminidase H</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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(xvii)
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>dFCS</td>
<td>dialyzed fetal calf serum</td>
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<tr>
<td>Fuc</td>
<td>fucose</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<tr>
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<td>glucose</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<tr>
<td>GTP</td>
<td>guanosine 5' triphosphate</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxyethyl piperazine-N'2-ethane sulfor acid</td>
</tr>
<tr>
<td>HFBA</td>
<td>heptafluorobutyric acid</td>
</tr>
<tr>
<td>HI</td>
<td>hydrophobicity index</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>Man</td>
<td>mannose</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>m.o.i.</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MHV</td>
<td>murine hepatitis virus</td>
</tr>
<tr>
<td>NBCS</td>
<td>new born calf serum</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PITC</td>
<td>phenylisothiocyanate</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenyloxazole</td>
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<tr>
<td>PTH</td>
<td>phenylthiohydantoin</td>
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(xviii)
Quadrol
N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine trifluoroacetic acid
RER
rough endoplasmic reticulum
RNA
ribonucleic acid
mRNA
messenger ribonucleic acid
tRNA
transfer ribonucleic acid
RIPA
radio immunoprecipitation assay
RNP
ribonucleoprotein
RSV
Rous sarcoma virus
R.Y.
replicative yield
SA
sialic acid
SDS
sodium dodecyl sulfate
SRP
signal recognition particle
SFV
Semliki Forest virus
T
thymidine
TCA
trichloroacetic acid
temed
N,N,N',N'-tetramethylethylenediamine
TFA
trifluoroacetic acid
THF
tetrahydrofuran
TLC
thin layer chromatography
TPCK
tosylsulfonyl phenylalanyl chloromethyl ketone
Tris
tris(hydroxymethyl)aminomethane
U
uridine
VSV
vesicular stomatitis virus
INTRODUCTION

Viruses have attracted interest primarily because of their ability to cause numerous illnesses, some of which have been recognized for centuries. Nowadays most viral diseases can be prevented by a vaccination program. The emphasis in studying viruses has shifted to gaining an insight into the intricacies of the eucaryotic cellular processes. The reason for the use of viruses for such a purpose is their total dependence on living cells, inside which the replication of their genome and the synthesis of specialized elements that can transfer the viral genome to other cells is brought about using the cellular synthetic machinery (Luria and Darnell, 1967). The relative simplicity of viruses in smaller genome size and correspondingly small number of encoded proteins, plus their ability to replicate to high titers in mammalian cells, allows their genes and gene products to be studied more easily than with the cell itself. Viruses which form host derived envelopes around the nucleoprotein core serve also as models for membrane biosynthesis.

Viruses have been classified (Lenard, 1978), according to the nature of their hosts into animal viruses, bacterial viruses and plant viruses. Animal viruses can be further subdivided into 6 classes depending on the structural relationship between the nucleic acid in the virion and its mRNA (Baltimore, 1971) as follows:

Class I: The double stranded DNA viruses (e.g. Herpes viruses, Pox viruses, Papovaviruses etc.).

Class II: Single stranded DNA viruses (e.g. Adeno-associated viruses.)
Class III: The double stranded RNA viruses (e.g. Reovirus).

Class IV: Positive strand RNA viruses [Togaviruses (SFV, Sindbis), Coronaviruses (MHV), Picornaviruses (Poliol)].

Class V: Negative strand RNA viruses [Parvovirus (NDV, Sendai), Orthomyxoviruses (Influenza), Rhabdoviruses (VSV), Bunyaviruses (Bunyamwera), Arenaviruses (Pichinde)].

Class VI: Retroviruses i.e. RNA viruses that direct the formation of DNA that acts as a template for making their genomic and mRNA e.g. (RSV).

Vesicular stomatitis virus (VSV), a rhabdovirus which was used for this work is the prototype of the negative strand enveloped viruses. Its genome is a single stranded 42S RNA (molecular weight 4 x 10^6) which forms 7% by weight of the virion and serves as a template for the synthesis of two type of RNA products in infected cells. One is a full length positive transcript that is an intermediate in genome replication, while the other comprises the five unique monocistronic, capped and polyadenylated mRNAs (Huang et. al., 1970; Wagner, 1975; Zakowski et. al., 1981) one for each of the five proteins (Kang and Prevec 1969, 1971) (which together form 64% of the virion) and also a short leader (48 nucleotides long). In the virion, the genome is estimated to be bound to approximately 60 molecules of the L protein, 2000 molecules of the M protein and 230 molecules of the NS protein to form the RNP core (Wagner, 1975). This is enclosed by a host membrane derived lipid envelope which accounts for 20% by weight of the virion (Patzer et. al., 1979) within which the G protein is anchored. The matrix protein M (2-4,000 molecules/virion) is bound to the inner
surface of the envelope. The mature virus appears bullet shaped under
electron microscope (Orenstein et al., 1976). In infected CHO cells a
proteolytic product of M protein termed M^ has been identified (Rosen
et al., 1983).

The organization of the VSV genome and the sizes of
proteins and their structural features is summarized in Figure 1.1 and
Table 1.1 compiled from previous reports (Kang and Prevec, 1969;
McGeoch, 1978; Rose et al., 1980; Gallione et al., 1981; Rose and
Gallione, 1981; Pringle, 1982).

Life Cycle of VSV

The production of a large number of virus particles (approx.
1000/cell) from a single virus particle occurs as a result of intense
synthetic activity within the cell and is described in the following
steps for the purpose of simplifying the complex closely related
process.

1. Attachment of virus to the host cell.
2. Entry into the host cell and viral interference.
3. Transcription and replication.
4. Translational, translocation and post-translational
   modifications.
5. Maturation, assembly and budding.

Attachment of the Virus to the Host Cell

Microinjection of viral RNP into suitable host cells has shown
that the RNP itself is capable of giving rise to mature infectious
virus particles (Thornton et al., 1983). However, mature virus is
required for efficient attachment and entry of the virus into the cell,
Fig. 1.1 The Organization of the 42S VSV Genome.

Table 1.1

<table>
<thead>
<tr>
<th>Viral Proteins</th>
<th>Calculated molecular weight (no. of amino acids)</th>
<th>Apparent molecular weight</th>
<th>Structural Features</th>
</tr>
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<tbody>
<tr>
<td>Nucleocapsid protein (N)</td>
<td>47,355 (422 amino acids)</td>
<td>50,000</td>
<td>Tightly binds to RNA</td>
</tr>
<tr>
<td>Core protein (NS)</td>
<td>25,110 (222 amino acids)</td>
<td>40,000 - 52,000</td>
<td>Domain of 18 (→) charged amino acids, phosphorylation giving rise to NS1 and NS2</td>
</tr>
<tr>
<td>Non glycosylated membrane protein (M)</td>
<td>26,064 (229 amino acids)</td>
<td>29,000</td>
<td>Basic protein also phosphorylated</td>
</tr>
<tr>
<td>Glycosylated membrane protein (G)</td>
<td>57,216 (511 amino acids)</td>
<td>69,000</td>
<td>Two glycosylation sites and covalently bound fatty acids</td>
</tr>
<tr>
<td>Large core protein (L)</td>
<td>7</td>
<td>190,000</td>
<td></td>
</tr>
</tbody>
</table>

57,416 is the molecular weight of the protein derived from the amino acid sequence obtained from the mRNA sequence, Rose and Gallione (1981).

It does not take into account any post-translational modification and it includes the molecular weight of the signal peptide (1827) which is removed during the translocation from free ribosomes to the endoplasmic reticulum membrane. If these factors are taken into account the calculated molecular weight will be

\[
57,416 = 1827 + 5082 + 476 + \text{Oligo-fatty chain} + \text{Acyl groups} + \text{Residues}
\]
VSV G protein is necessary for attachment of the virus to a host cell and has been shown to protrude at least 10 nm from the viral envelope (Howatson, 1970). Removal of the G protein by digestion with trypsin results in marked loss in the infectivity (Cartwright et al., 1969; Bishop et al., 1975). A similar effect was observed when pneumococcal endoglycosidase was used (Moyer et al., 1976). Neuraminidase reduces the infectivity of VSV by at least 90% merely by cleaving off the terminal neuraminic acid from the glycoprotein and infectivity can be restored by resialylation of the glycoprotein by sialyl transferase (Schloemer and Wagner, 1975). Attachment of the virus can be prevented by saturation of host cells with purified VSV G (Bishop and Smith, 1977); G protein lipid vesicles have been shown to be effective in eliciting specific anti-G antibodies that neutralize viral infectivity (Miller et al., 1980). However, doubts about the requirement of G for attachment have been raised. Using thermolabile, temperature sensitive G protein mutants of VSV, loss of infectivity was reported to be a result of the inability of the heat treated mutant to internalize in the lysosome and the effect could not be attributed to the prevention of binding (Miller and Lenard, 1980). In spite of this evidence, G protein is said to be essential in the early stages of infection.

If G protein is essential for attachment then the question that follows is: is the portion of the virus interacting with the cell membrane during attachment a part of G protein? An effort to answer this question was made by determining the binding ability of isolated glycoprotein and intact virion. The results suggested that either the
isolated glycoprotein bound to cell surface components was distinct from the virion or that the manner of the purified glycoprotein attachment differed from the in situ G protein in the virion (Thimmig et al., 1980). The conformation that G protein adopts in the intact virion is most likely different from the isolated glycoprotein. The observation that trypsinization of VSV decreases phosphatidylserine binding to the virus and that the binding of phosphatidylserine to VSV also inhibits VSV attachment and infectivity, suggests that G protein in the virion could interact with the phosphatidylserine in the plasma membrane to bring about viral attachment (Schlegel et al., 1983). Other possible sites for attachment on the plasma membrane have been considered. There could be specific binding sites or receptors (Lonberg-Holm, 1981). The best characterised viral receptor is glycoporin, a sialoglycoprotein that constitutes the binding site for influenza virus on erythrocytes (Kathan et al., 1961; Jackson et al., 1973); other less characterized receptors include the receptor for Epstein Barr virus, which also appears to be the same as the membrane receptor for the C3 component of the complement system (Yefenof et al., 1976) and the receptor for SFV includes the human leukocyte antigen (Oldstone et al., 1980). Proteolytic digestion of the host cell surface does not inhibit VSV infection (Schloemer and Wagner, 1975) suggesting that the binding site for VSV is not a membrane protein. This is further confirmed by studies of VSV binding to Vero cells at 4°C (at which binding occurs efficiently but not penetration) rather than at 37°C demonstrating that there are about 4,000 high-affinity binding sites for VSV on the surface of Vero cells.
(Schlegel et al., 1982). The virus binding site thus appears to be a lipid or complex of lipids as suggested earlier (Haywood, 1974; Mooney et al., 1975). It has been shown that when the binding site in the plasma membrane is extracted with the detergent octyl-β-D-glucopyranoside and further purified to give individual lipids only phosphatidylserine was capable of totally inhibiting the high affinity binding and plaque formation of VSV. Liposomes made from phosphatidylserine have been shown to be attached to VSV by EM (Schlegel et al., 1983). It would be interesting to define the domain of G protein that interacts specifically with an anionic phospholipid like phosphatidylserine while other anionic phospholipids such as phosphatidylinositol, phosphatidyl glycerol etc. have no binding capacity for G protein.

The broad host range of VSV suggests that specific phospholipid is a better candidate as a receptor than a membrane protein.

Entry of the Attached Virus into the Host Cell and Viral Interference

Early evidence for the mechanism of viral entry suggested fusion between the cell surface and the virus occurred and the virus released the RNP by simultaneous fusion and entry (Heine and Schnaitman, 1969, 1971). More recent evidence by EM (Simpson et al., 1969; Dahlberg, 1974) and studies using inhibitors of endocytosis such as chloroquine (Schlegel et al., 1982) and interferon (Tann et al., 1982; Wilcox et al., 1983) suggest that endocytosis or engulfment by the cell is involved in the entry of the virus into the cell. Internalization follows entry of the virus into the host cell and
involves an early association of the virus with "coated pit" regions of
the plasma membrane and upon endocytosis the virus is transferred into
cytoplasmic vacuoles termed endosomes or receptosomes as visualized by
electron microscopy (Simpson et. al., 1969; Dahlberg, 1974; Dickson
et. al., 1981; Matlin et. al., 1982). The lysosomal inhibitor
chloroquine, when added with infecting virus completely inhibits VSV
infection at all multiplicities but is only about 50% inhibitory when
added after the initial adsorption period of 1.5 hours post-infection
(Miller and Lenard, 1980) suggesting a definite role for lysosomes in
virus internalization. There is evidence that acidification in
lysosomes uncoats the virus by fusing this virus with the vesicle
membrane and thereby releasing the nucleocapsid into the cell cytoplasm
(Tycko and Manfield, 1982). It is possible that phosphatidylserine has
a role in promoting such membrane fusion events within an acidic
environment (Portis et. al., 1979). VSV G protein can mediate membrane
fusion even at neutral pH (Hughes et. al., 1979) suggesting that an
acidic environment may be important only for induction of fusion
(Eidelman et. al., 1984).

Viral Interference is an important aspect of the viral life
cycle. Virus infections preventing the possibilities of second
infection by other unrelated viruses or in some instances related
viruses have been termed viral interference. Early studies had
predicted that the production of viral products by an initial virus
renders the infected cell incapable of supporting the replication of a
second virus (Schleisinger, 1959). Interference was thought to operate
either at the level of preventing mRNA translation of the second virus
(Marcus and Carver, 1967) or in a few cases preventing proper entry into the cell membrane. There is evidence to substantiate both lines of thought. Temperature sensitive Sindbis virus mutants defective in RNA replication, establish an interference for VSV replication that is maintained even at the nonpermissive temperature (37°C). VSV enters actinomycin D treated cells and VSV mRNA is formed by the virion associated transcriptase. However, since no translation of viral proteins occurs, no infectious virus particles were formed (Hunt and Marcus, 1974). The second type of interference which possibly operates at the level of virus entry occurs between related retroviruses (Stock and Rubin, 1966). In order to distinguish the interference between different types of viruses and those which are a result of subviral particles of the same type the term homologous interference is used.

There are reports about the defective interfering particles or DI particles that prevent normal viral replication in VSV (Palma et al., 1974). These DI particles are shorter than the normal bullet shaped particles and contain only a portion of the viral genomic RNA. Continual passage of a VSV stock rapidly leads to a greater number of DI particles (Stampfer et al., 1971).

Interfering capacity distinct from the virus itself was first found in the allantoic fluid of eggs that had been exposed to irradiated virus (Isaac and Lindeman, 1957). The substance formed in host cells in response to virus infection that mediated this distinct interfering capacity was termed "interferon". The block in virus infection induced by interferon was thought to occur after viral entry (Friedman, 1970; Wiebe and Joklik, 1975). Recent evidence however,
contradicts the earlier observation. Entry of VSV into mouse L cells pretreated with mouse interferon was inhibited, though adsorption of virus was not. Inhibition was dose dependent and only occurred when cells were treated with homologous interferon (Whitaker-Dowling et al., 1983). Interferon affects all subsequent steps of viral replication.

**Transcription and Replication**

The helical nucleocapsid consisting of the (-) stranded genomic RNA tightly complexed with the viral N protein and loosely bound by NS and L proteins is the functional viral genome (Wagner, 1975; Banerjee et al., 1977; Thornton et al., 1983). It contains those enzymatic activities which are absent in the host cell but are necessary for replication through synthesis of a full length complimentary (+) antigenome strand and for transcription of five functional mRNAs and leader RNA.

**Transcription**

The transcription of VSV genome in infected cells gives rise to five capped, poly(A)-containing RNA species that anneal to virion RNA (Huang et al., 1970; Rose and Knipe, 1975). These RNAs representing the five proteins, L, C, N, NS and M have been isolated from polyribosomes and T1 oligonucleotide "finger printing" shows them to be different from one another. The 5' terminal structure on each of the mRNAs appears identical in nucleotide sequence but there is heterogeneity in the methylation of all of the termini (Rose, 1975). The basic structure is m7 G5'ppp5'ApApCpApGpHpHp ApUpCp-3' where N indicates a variable site (Rhodes and Banerjee, 1976; Rose and
Gallione, 1981)? The intergenic junctions consist of a undecamer
(3')-AUACUUUUUUG-U (5') corresponding to the sequence (5')UAUGAAAAAA(3')
at the mRNA poly(A) junction of each mRNA, which may be the signal for
synthesis of the poly(A) tails of the mRNAs possibly by reiterative
copying ("slippage polymerization"). This undecamer is followed by a
dinucleotide (CA or GA) spacer which does not appear in the transcripts
and thus represents the true intergenic junction (Rose, 1980, Ball and
Wertz, 1981). Thus, the five RNAs have characteristics of eucaryotic
mRNAs. Individually, the five RNAs can be translated in vitro to
produce the five viral proteins in cell-free systems thus proving
unequivocably that they are the mRNAs (Both et. al., 1975; Knipe et.
al., 1975).

With the exception of 48 nucleotides at the 3' terminal leader
region, 59 nucleotides at the 5' terminal tail region, 3 nucleotides
between leader: N gene junction and 2 each at the 4 other junctions in
all accounting for 122 nucleotides the rest of the genome encodes the
main body of five mRNAs with no intervening sequences within the genome
(Rose, 1980; McGeoch, 1981). VSV thus encodes all the genetic
information in a (--) non overlapping single strand of RNA.

The enzymatic activities associated with the nucleocapsid and
which are essential for the synthesis of functional mRNAs have been
well established. They include RNA dependent RNA polymerase activity
(Baltimore, 1970) and other mRNA modifying enzymatic activities such as
guanyl transferase that uses GTP to form a cap structure at the 5' end
of the mRNA (Abraham et. al., 1975a), two methyl transferases that
catalyze the transfer of methyl groups from S-adenosyl methionine to
position 7 of the capping guanosine residue 2'-O-ribose moiety of the penultimate adenosine (Abraham et al., 1975b; Testa and Banerjee, 1977) and a poly(A) polymerase that adds approximately 200 A residues to the 3' end of the completed mRNAs (Banerjee and Rhodes, 1973; Villarreal and Holland, 1973). It is not certain whether all these activities are those exhibited by virus specific enzymes. Trace amounts of host cell enzymes could be associated with the nucleocapsid. The proteins L, NS and N are required for the transcriptase activity of the nucleocapsid (Emerson and Yu, 1975). It is possible that L and NS proteins are responsible for the act of polymerization but the RNA must be coated by the N protein to form a functional template. There is evidence that NS a phosphoprotein (Sokol and Clark, 1976) specifically is noncovalently bound by guanosine 5'-diphosphate (De and Banerjee, 1983). The nature of this bond was not a phosphoamide linkage as in other guanyl transferase enzymes such as the one in vaccinia (Venkatesan and Moss, 1982). Thus the enzymatic role of NS in capping is considered doubtful and the search for the origin of enzymatic activities associated with the virion is still going on.

The five monocistrionic mRNAs are transcribed sequentially from the 3' end of the genome. The kinetics of UV-inactivation of gene function in vivo and in vitro in a coupled transcription-translation system indicated that the genes determining the five structural proteins are arranged in the order (5')-L-G-M-NS-N(3') (Abraham and Banerjee, 1976; Ball and White, 1976; Ball et al., 1976). The five mRNAs are present in infected cells in a decreasing order of abundance depending on their distance from the 3' end of the genome (Villareal
et. al., 1976). Thus, non-equimolar synthesis seems to be a result of partial termination of transcription (attenuation), which occurs at or near the intergenic junction and may be a method of regulation of gene expression (Iverson and Rose, 1981).

Transcription begins from the 3' end with the synthesis of a small 48 nucleotide leader RNA. This leader RNA has been suggested to serve as inhibitor of host cellular transcription since only a small portion of the genome (i.e. leader sequence) needs to be transcribed to affect host cell RNA synthesis (Weck et. al., 1979). This suggestion has been supported by many other studies. McGowan et. al. (1982) demonstrated that purified leader RNA could inhibit DNA dependent transcription in an in vitro transcription system. The leader RNA has been shown to be present in the nucleus of infected cells, where an inhibitor of transcription initiation would be expected to act (Kurilla et. al., 1982). The leader RNA has also been shown to be bound by a cellular protein reactive with anti La-lupus antibody; the only other RNAs found to associate with the La protein are the RNA polymerase III products (Kurilla and Keene, 1983) present in the nucleus. The interaction of La protein with leader RNA raises the possibility of involvement of La protein in the viral inhibition of host macromolecular synthesis. The level of leader RNA has been correlated to the kinetics of leader RNA production in cells infected with UV-irradiated VSV (Grinnell and Wagner, 1983). Thus, the leader RNA of VSV appears to be a small nuclear RNA playing a role in transcription or replication possibly as a result of interactions with La protein.

Inspection of the sequences of the leader RNAs of the Indiana
and New Jersey serotypes and those of five other strains (Colombo and Banerjee, 1978a, 1978b; Keene et al., 1980; Giorgi et al., 1983) reveals several regions of interest in terms of potential regulatory sequences. There is remarkable homology shared by the first 18 nucleotides from the 5' end of the VSV wild type leaders and from 5' DI leader (Giorgi et al., 1983). This region (shown below) is thought to be involved in initiation of viral RNA synthesis (transcription and replication). Recent studies indicate that the N protein selectively binds within the first 14 nucleotides having an A residue at every third position from the 5' end of the leader chain (Blumberg et al., 1983).

Consensus sequence of the conserved region of the VSV leader

5'pp AGCAAGACAAAAACCAUU........3'

where X is C in VSV Indiana leader RNA

and A in VSV New Jersey leader RNA

The wild type leader RNAs of VSV contain an AU-rich region of approximately 16 nucleotides beginning 18 nucleotides from the 5' end. This region has been suggested to resemble the TATA Goldberg-Hogness box (Manley, 1980; Baker and Ziff, 1981; Gruss, et al., 1981) and is presumably involved in interaction with RNA polymerase. Secondary structure predictions based on free energies of formation suggest that this region could potentially form a stable stem-loop structure. This was confirmed by digestion with double strand specific RNase from Cobra venom (RNase VI) (Grinnell and Wagner, 1984). In the VSV genome, this AU-rich region appears to bind viral NS protein. This is required for VSV polymerase activity (Keene et al., 1981). Although the
function(s) of the AU rich region in the VSV Indiana leader RNA transcript is unknown, it may play a role in inhibition of transcription directed by RNA polymerase II (McGowan et. al., 1982). The capability of the AU rich region to inhibit adenovirus DNA transcription directed by both RNA polymerase II and III has been demonstrated by Grinnell and Wagner (1984) using a cDNA recombinant of the leader RNA and defined synthetic oligodeoxynucleotides.

Three models have been proposed for the way in which RNA polymerase reads the genomic RNA to produce mRNA. The single RNA polymerase entry site model proposes that the enzyme begins with the synthesis of the plus strand leader RNA that is complementary to the first 47 residues at the 3' end of the genome. A stop-start mechanism allows the template bound enzyme complex to terminate and release completed transcripts before initiating synthesis at the next gene (Banerjee et. al., 1977; Emerson, 1982). An alternative multiple entry model proposes that each gene is independently and simultaneously initiated by elongation and completion of these short transcripts in a sequential manner (Testa et. al., 1980). The third model suggesting processing of mRNA precursors (Banerjee et. al., 1977) lacks experimental support but cannot be entirely ruled out. In vitro studies on kinetics of transcription revealed that transcription is not continuous. There are pauses at or near each intergenic junction (Rose, 1977). This delay is probably indicative of some process such as polyadenylation, capping, or initiation which is slow relative to transcription. The rate of elongation in vitro was apparently 3.7 nucleotide/second within all five genes, but markedly slower at the
intergenic regions. The significant pauses in transcription at or near the intergenic regions has led to the suggestion that the synthesis of the intervening poly(A) occurs by "chattering" of the polymerase i.e. repeated slippage of the nascent strand instead of the advance of the enzyme with respect to the template. The chattering mechanism results in a read through by which poly(A) sequences not encoded in the template are inserted between accurate transcripts of adjacent genes. The template sequence of each of the five intercistronic poly(A) sites is

\[ 3' \ldots \text{AUAC(NU)UAUGUC} \ldots 5' \]

variable nucleotide

The encircled dinucleotide is not present in the sequences of the mature mRNAs although adjacent sequences are transcribed with an additional 200 adenylate residues adjacent to the (UA) sequence.

**Role of M Protein in Transcription**

Numerous studies have suggested that M protein has a role in regulating polymerase activity. The first evidence for the role of M protein on regulation of VSV transcription was a demonstration of an absolute increase in VSV mRNA at the nonpermissive temperature on infection of cells with a temperature sensitive mutant with a lesion in the M protein (Clinton et. al., 1978). The presence of M in *in vitro* transcription carried out at optimal salt concentration does not inhibit transcription to a significant extent. However, reduction of the salt concentration causes a significant inhibition of transcription (Banerjee, 1979). A possible reason for this inhibition occurring at low ionic strength may be due to interaction of M with the RNP
structure (Newcomb and Brown, 1981; Wilson and Lenard, 1981; Newcomb et al., 1982). M protein purified from a hydroxyapatite column (De et al., 1982) and by extraction of the virus with acidic chloroform-methanol (Mikhejeva and Ghedon, 1983) has been shown to bind RNA in vitro with binding ratios of M to RNP of 2:1 and 1:5, respectively. The binding of M to the RNA structure in vitro has been found to strongly inhibit (about 90%) of the RNA synthesis. The purified M protein interacts with RNP that is actively transcribing in an in vitro system at low ionic strength. As seen by EM, the RNP structure is transformed from a loosely coiled and fully extended structure in the absence of M to a compact form in its presence. It is presumed that M does not affect initiation. Labeling with ATP and [α32P]CTP carried out at low ionic strength results in the synthesis of triphosphorylated oligonucleotides. These represent the 5' ends of leader RNA (pppAAC) and mRNAs (pppAACA) (De et al., 1982). Thus under conditions of low ionic strength M interacts with the 42S RNA and this interaction results in a compact complex that is less efficiently transcribed. It is not clear whether the domain of basic amino acids at the NH2-terminus of M is in fact the region of M involved in this interaction with RNA, although it seems to be the most likely region for this interaction. The conversion of M to M' may serve to regulate intracellular levels of M and in turn regulate transcription (Rosen et al., 1984).

Replication

The mechanism for the turning on of replication and suppression of termination is now becoming clear. The development of in vitro cell
free systems that couple translation and replication has enabled the study of factors regulating replication. Recent work has reported the synthesis of full length 42S RNA of both polarities as well as the five proteins (Hill et al., 1981; Ghosh and Ghosh, 1982; Davis and Wertz, 1983; Peluso and Moyer, 1983).

A hypothesis has been proposed suggesting that RNA polymerase begins synthesis at the 3' end of the (-) strand template and regulation of synthesis occurs when the enzyme reaches the N leader gene junction. In transcription, the complex terminates synthesis and presumably reinitiates at the beginning of the N gene, only 4 bases downstream, in replication the same or a modified polymerase complex reads through this junction and continues synthesis until the end of the template is reached (Simpson and Obijeski, 1974; Szilagyi and Pringle, 1975; Perrault et al., 1983).

The selective binding of N protein to the (+) strand leader RNA (Blumberg et al., 1983) may achieve readthrough of the leader N gene junction (Leppert et al., 1979). In vitro studies have revealed that readthrough of leader RNA termination sites to give full length (+) strand copies can be better achieved using an ATP analog (Testa et al., 1980). Smaller size copies are obtained with various base analogs in vitro (Testa and Banerjee, 1978; Herman and Lazzarini, 1981, Chinchar et al., 1982). Characterization of a new type of VSV mutant denoted Pol R, that gives rise to DI particles which appear to efficiently read through the (-) strand leader termination site under standard in vitro conditions (Perrault et al., 1980) shows that the responsible mutation maps within the template N protein (Perrault,
Thus, a direct role for the N protein within the RNP template in regulating the switch from transcription to replication seems very likely. Whether readthrough requires the binding of N protein to the nascent leader RNA as proposed by Leppert et. al. (1979) or it requires the assembled RNP structure (and not just assembled leader) still needs to be resolved.

Besides the N protein, the L protein has also been shown to be essential (Perlman and Huang, 1973; Repik et. al., 1976). Other studies have also implicated NS to be involved along with L (Emerson and Yu, 1975; Hunt et. al., 1976; Naito and Ishihama, 1976).

Protein synthesis has been found to be mandatory for replication to occur in vivo (Perlman and Huang, 1973; Wertz and Levine, 1973) and in vitro (Davis and Wertz, 1982; Hill and Summers, 1982). An examination of protein synthesis requirement for VSV replication using both intracellular and virion derived DI nucleocapsids in the in vitro replication system in conjunction with purified individual mRNAs revealed that N protein can by itself fulfill the requirement for protein synthesis in RNA replication and allow complete replication, i.e. initiation and elongation as well as encapsidation of genome length progeny RNA (Patton et. al., 1984).

After reading through, the RNA polymerase complex (probably a different one from the one involved in transcription) overrides the termination signals and the complete synthesis of the (+) strand 42S RNA occurs (Perrault et. al., 1983). Since the termini of the (-) strand and the (+) strand are complimentary (Keene et. al., 1979) it has been suggested that replication may occur in part on circular
coiled RNA templates. This suggestion is supported by electron microscopic studies (Naeve and Summers, 1980).

Viral infections cause a morphological change in the susceptible host cell (Wagner, 1975). Some of these changes, in cell shape bring about metabolic changes, such as repression of nuclear metabolic activity (Ben-Zeev, 1983). A study of viral replication has shown that VSV is unaffected by cell shape responsive metabolic change as it replicates in the cytoplasm which is relatively unaffected by cell shape responses. Thus, VSV indirectly inhibits host cell replication and eliminates competition in macromolecular synthesis. A DNA virus in contrast is unable to achieve such a preferential replication as it is dependent on host nuclear metabolism.

There is a report that interferon is inhibitory to viral replication (Masters and Sammel, 1983). This report is based on the observation that the extent of interferon inhibition of viral growth cannot be accounted for by its ability to inhibit penetration, translation or glycosylation and hence its major affect is on replication.

Translation, Translocation and Post-Translational Modification

The five monocistronic mRNA species transcribed from the viral genome are translated in infected cells throughout the cycle of infection to give rise to all the five structural proteins of the VSV L, G, N, NS and M (Mudd and Summers, 1970a; Petric and Prevec, 1970; Wagner et al., 1970; Kang and Prevec, 1971). Other studies where polyribosomes from VSV infected cells were isolated and translated in
vitro into authentic viral proteins have also demonstrated that the RNA species found in infected cells have messenger function (Ghosh et. al., 1973; Morrison et. al., 1974; Knipe et. al., 1975; Toneguzzo and Ghosh, 1975). Viral protein synthesis is completely dependent on the cellular protein synthesizing system for the translation of its mRNA. A number of different cell-free systems have been shown to translate viral mRNAs (Both et. al., 1975; Knipe et. al., 1975; Preston and Szilagyi, 1977; Toneguzzo and Ghosh, 1978). It was at one time thought that there were no intracellular virus specific non-structural proteins (Wagner, 1975) and that the minor intracellular protein peaks observed by Mudd and Summers (1970b) and Wagner et. al. (1970) were probably of cellular origin. However, a recent report suggests that there is a protein termed as M present in the infected cells but not in the virus itself (Rosen et. al., 1983). It would also seem that there is specific incorporation of host cell surface proteins into budding VSV particles (Lodish and Porter, 1980).

Viral protein synthesis in VSV-infected cells has been detected 1 hour after infection, and reaches a peak about 4 hours at high multiplicities of infection (Kang and Prevec, 1971). The N protein is synthesized first in the greatest amount (approximately 40% of total viral protein synthesized) and its synthesis remains high throughout the infectious cycle (Wagner et. al., 1970; Kang and Prevec, 1971). The levels of L protein (which accounts for 2-3% of the total viral protein) remains low despite its mRNA representing approximately 30% of the total VSV mRNA synthesized in vitro (Stampfer et. al., 1969; Kang and Prevec, 1971; Stampfer and Baltimore, 1973; Stamminger and
Lazzarini, (W74). This suggests that there is translational control mechanism regulating L protein synthesis. G and M protein have been found to increase gradually in amount throughout the cycle of infection reaching peaks of 25 and 30% of total viral proteins respectively (Wagner, 1975). The amount of NS protein appears to be greater in the early hours after infection than at later stages (Kang and Prevec, 1971). NS is phosphorylated before or shortly after its synthesis (Moyer and Summers, 1974a). These findings have led to the speculation that NS may itself be a regulatory protein involved in control of transcription and/or replication of the VSV genome.

An examination of the intracellular viral RNA species has indicated that a majority of viral mRNA became polysome associated by 3 hours post-infection (Lynch et al., 1981). A substantial amount of the viral RNA (32 - 36% of the total 13S viral mRNAs) is present in the post-ribosomal supernatant, i.e. not polysome associated, although these mRNA species could be translated into authentic viral proteins in cell-free translation systems (Morrison and Lodish, 1975). A probable explanation for the inability of the cell to translate the free mRNA is that the VSV mRNA is produced in excess of the cell's capacity to efficiently translate it. Alternatively, a translational control mechanism may regulate the polysome-bound and free mRNA levels. The low levels of L protein in relation to the amount of L mRNA discussed earlier together with the inability by most workers to efficiently achieve the translation of L mRNA in vitro lends support to the latter hypothesis (Both et al., 1975b; Ball and White, 1976; Breindl and Holland, 1976; Rose et al., 1977).
The translation of the mRNAs encoding L, NS, N and M occurs on free polysomes, while that of G mRNA occurs exclusively on membrane bound polysomes in infected cells (Both et al., 1975b; Grubman et al., 1975; Morrison and Lodish, 1975; Toneguzzo and Ghosh, 1975). An inspection of the codon usage of G, M, N and NS mRNA derived from cDNA sequencing (Rose and Gallione, 1981; Gallione et al., 1981), revealed that there is an overall deficiency of the dinucleotide CG. This shortage cannot be attributed to a relative shortage of cellular tRNAs recognizing codons containing CG, since CG is also deficient in the noncoding region. The reason for such a codon usage is as yet unexplained. The other striking feature in VSV protein synthesis is the cotranslational translocation of the G protein and its subsequent glycosylation and in some cases fatty acylation. Other important modifications include the phosphorylation of M and NS proteins (reviewed pages 24-50).

Inhibition of host cell protein synthesis in infected cells was observed in early studies of intracellular VSV protein synthesis and has been termed "host shut off" (McAllister and Wagner, 1976). Four different mechanisms for shut off have been proposed. Nuss et al. (1975) and Lodish and Porter (1980b) have suggested that VSV mRNA initiates protein synthesis several fold more efficiently than does cellular mRNA and thus out competes the mRNA for ribosomes. In this regard, Stanners et al. (1977) have isolated a mutant of the HR (Winnipeg) wild type of the Indiana serotype of VSV. Infection of L cells with this mutant does not result in rapid and sharp inhibition of protein synthesis observed with the HR wild type strain. They proposed
that a specific viral gene product termed P is required for this inhibition and that the mutant lacks this. The efficiency of initiation of translation on cellular and viral mRNAs is about the same in infected cells. A systematic study correlating accumulation of viral mRNA and the extent of inhibition of translation of cellular mRNA using different VSV strains in different cell lines added support to the conclusion that the competition for ribosomes by a large excess of viral mRNA as the most likely mechanism of translational control (Lodish and Porter, 1981). Recently, a reduction in protein synthesis was accounted for not by mRNA independent mechanism but by the level of eIF2-GTP initiator methionyl-tRNA complex formation. It has been shown that a depletion of eIF2 occurs as a result of viral infection. This regulation of eIF2 level has been suggested to bring about a selective viral mRNA translation in infected cells (Centrella and Lucas-Lenard, 1982).

Post-Translational Modification of NS and M Proteins

The phosphorylation of NS occurs as an early event, either during or immediately after its synthesis, since $^{32}$P-labeled cytoplasmic NS has been detected at all stages of the infectious cycle (Moyer and Summers, 1974a). The phosphorylation of NS is presumed to be brought about by a cytoplasmic or plasma membrane associated protein kinase. This host derived protein kinase has also been found to be associated with VSV (Strand and August, 1971; Imblum and Wagner, 1974; Moyer and Summers, 1974a). This kinase phosphorylates VSV proteins L, G, NS and M as well as casein, phosvitin, protamine and histones in vitro; in vivo only proteins NS and M are phosphorylated (Sokol and
Clark, 1973; Imblum and Wagner, 1974; Moyer and Summers, 1974a; Clinton et al., 1978, 1979; Clinton and Huang, 1981).

The primary sequence of NS indicates the presence of 12 threonine, and 21 serine residues, a total of 33 potential sites of phosphorylation (Gallione et al., 1981). The predicted secondary structure of NS suggests that many potential phosphorylation sites are located at β-turns that also possess basic amino acid residues. In vivo most of these sites are phosphorylated, since chymotrypsin digestion revealed at least 21 phosphorylated sites, 7 out of the 14 phosphopeptides generated contained both phosphoserine and phosphothreonine (Hsu and Kingsbury, 1982). NS can be resolved into at least two major species, called NS1 and NS2 which differ in their degree of phosphorylation (Clinton et al., 1978, 1979; Kingsford and Emerson, 1980). Although both forms of NS are found in VSV infected cells only NS1, the least phosphorylated species, is associated with VSV core particles (Clinton et al., 1978).

Much less is known about the phosphorylation of M as regards the precise stage in its biosynthesis at which it is phosphorylated. The phosphorylation of M protein is heterologous since only 5 – 10% of the protein molecules contain up to 90% of the phosphate (Clinton et al., 1978). The protein kinase involved in the phosphorylation could be an endogenous cellular enzyme or the host-derived VSV associated protein kinase (Clinton et al., 1982).

**Biogenesis of the Membrane Glycoprotein**

Biosynthesis of glycoproteins found in enveloped viruses such as VSV is a process which involves a complex sequence of
cotranslational and post-translational events that are necessary to transfer the protein from its site of synthesis to its ultimate destination within the plasma membrane. G protein is not found free in the cytoplasm but is associated with cellular membranes (Wagner et al., 1970). The "driving force" for its association with membrane resides within discrete regions or domains of the glycoprotein either as topogenic sequences (Blobel, 1980) or as sites for glycosylation (Pless and Lenarz, 1977; Hubbard and Ivatt, 1981) or in a number of cases for fatty acylation (Magee and Schlesinger, 1982). The elucidation of the intracellular pathway of the G protein has been possible through a wide range of studies. Experiments in which short pulses of radioactive precursors were used, showed that the glycoprotein is synthesized on the rough endoplasmic reticulum (ER). Labeling for longer periods followed by a chase of the pulse-labeled precursors, showed that the glycoprotein molecules migrated from the rough endoplasmic reticulum to the smooth membranes and finally appeared on the plasma membranes. Studies of the kinetics of appearance of the labeled glycoprotein of VSV on the various intracellular membrane fractions showed that the glycoprotein is incorporated into the plasma membrane at about 20 minutes after synthesis (Atkinson et al., 1976; Knipe et al., 1977). Newly synthesized glycoproteins of uninfected cells showed a similar delay in incorporation of the glycoprotein into the plasma membrane, a result suggesting that the viral glycoproteins are transported from the site of synthesis to its final destination by normal cellular mechanisms (Atkinson, 1975).
In vitro protein synthesis using polysomes from infected cells showed that viral glycoproteins are synthesized specifically by membrane bound polysomes (Ghosh et al., 1973). In vitro translation of virus specific mRNAs isolated from membrane-bound and from free polysomes further confirmed that the mRNAs for the glycoprotein of the viral envelope are localized exclusively on membrane associated ribosomes (Toneguzzo and Ghosh, 1975).

In an in vitro translation system devoid of membranes, G protein is synthesized in a unglycosylated form G₁, having an apparent molecular weight of 63,000. In the presence of membranes G₂ protein (molecular weight 67,000) is formed (Toneguzzo and Ghosh, 1977, 1978). G₂, but not G₁, has been established as a glycoprotein by affinity chromatography on a concanavalin A-Sepharose column. Concanavalin binds to carbohydrate moieties containing glucosamine and mannose, and provide a useful method for purification of glycoproteins containing these sugars. Digestion of G₂ with exoglycosidase that hydrolyze the branched chain sugars of glycoproteins showed that G₂ is an incompletely glycosylated form of G (Toneguzzo and Ghosh, 1977). The presence of membranes was shown to be essential during the early period of chain elongation since the presence of membranes after the synthesis of a nascent chain of about 80 amino acids or longer resulted in insertion of the nascent polypeptide into the ER membrane (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1978).

This type of binding of the ribosomes to the ER membrane via nascent peptide chains was observed earlier in the case of secretory proteins (Aronson, 1966; Chefurka and Hayashi, 1966; Redman and
Sabatini, 1966) and led to the postulation that the NH\textsubscript{2}-terminal region of the nascent chain was responsible for the binding (Blobel and Sabatini, 1971). In a translation system containing ribosomes, S-100 from ascites tumor cells, and mineral oil-induced plasmacytoma 41 mRNA, Blobel and Dobberstein (1975) showed that immunoglobulin prelight chain was converted to authentic light chain by the addition of microsomal membranes from dog pancreas. These membranes had been previously stripped of ribosomes by EDTA treatment. Processing also occurred only when membranes were added at the start of incubation (see above). In another experiment they also showed that the processed form could be segregated into the microsomal vesicles in a form resistant to the action of exogenously added trypsin. Processed immunoglobulin light chain was protected from the protease, where as the preformed was completely degraded. This data led to the formulation of the signal hypothesis. This hypothesis stated that translation of mRNAs for preproteins containing signal sequences begins on free polysomes. Elongation proceeds on free polysomes for this class of proteins, until 10 to 40 amino acid residues of the nascent chains have been synthesized. The polysomes containing the nascent sequences then bind to ER membranes. It was also suggested that the nascent chain bearing the signal sequence creates a channel or tunnel in the membrane through which the growing polypeptide chain will enter the lumen. During this translocation step, the presequence is removed and the protein enters the lumen of the vesicle. While there are similarities in the translocation process between secretory and membrane proteins, there is also a very important difference. The secretory protein vectorially
discharged across the rough ER was found to be in the lumen (Blobel and Dobberstein, 1975a). However, the G protein of VSV after being discharged remained inserted in the membrane with a portion of its carboxy-terminus exposed to the cytoplasmic side of the membrane and this exposed portion on the cytoplasmic side was sensitive to proteolysis. Thus, the glycosylated protein G₂ that is synthesized in vitro, and not the unglycosylated G₁, is protected from digestion by proteolytic enzymes. The mobility of G₂ when run on SDS gels after proteolysis was higher because of the loss of the carboxy-terminus that is exposed on the cytoplasmic side of the ER membrane (Toneguzzo and Ghosh, 1978). The insertion of nascent polypeptide serves to bring it into contact with the glycosyl transferases and the dolichol phosphate carrying the high mannose oligosaccharide chain in the ER as well as allow the transport of the proteins from its site of synthesis.

The signal hypothesis, naturally enough, led to the search for the existence at the NH₂-terminus of a sequence that would serve the function of transferring secretory or membrane proteins across the ER membrane. Analysis of the primary sequence of the NH₂-terminus of the unglycosylated form synthesized in vitro in the absence of membranes and the mature form of the protein secreted outside the cell revealed the presence of an extra region of 20 amino acids at the NH₂-terminus of the preprotein synthesized in the absence of membrane which was not found in the mature form. It was suggested that after insertion into the ER membrane this region termed the signal sequence is proteolytically removed from the nascent protein (Blobel, 1978). A comparison of these transient sequences from a number of secretory
proteins revealed that there were some common features, such as the predominance of hydrophobic amino acids within the signal region, the presence of a small neutral amino acid at the end of the signal sequence and of proline within the last 5 amino acids of the signal sequence (Austen, 1979).

The G protein of VSV Indiana was the first membrane protein to be examined for the presence of a signal sequence. Amino terminal sequence determinations of the unglycosylated form G₁, the glycosylated product G₂ and the virion G by two groups (Lingappa et al., 1978; Irving et al., 1979) (shown below) revealed that both G₂ and G are generated by proteolytic cleavage of an amino terminal peptide sequence containing 16 amino acids present in G₁. A large number of membrane proteins are now shown to possess signal sequences.

\[
\begin{align*}
G₁ & \quad 1 \text{MKCLIL} - F₁₀ \text{FP} - V₁₅ \text{KFT} - 2₀ \text{FP} - 2₅ \text{KG} - 3₀ \\
G₂ & \quad 4 \text{KF} - 5 \text{IVFPY} - 1₀ \text{KG} - 4₅ \\
G & \quad 1 \text{KF} - 5 \text{IVFPYNKON} - K
\end{align*}
\]

Studies of the in vitro removal of the preprotein resulting in the corresponding secretory form with no heterogeneity at the amino terminus suggested that the enzyme involved in the cleavage of the signal peptide, now termed "signal peptidase", is an endoprotease (Birken et al., 1977; Lingappa et al., 1977). Initial
characterization of the signal peptidase showed that a deoxycholate solubilized extract of pancreatic microsomes cleaved the signal sequence from preproteins in post translation assays (Jackson and Blobel, 1977). More direct studies of the protease activities in detergent solubilized extracts of pancreatic microsomes have used synthetic fluorogenic amino-coumarin peptide substrates, which can be used to differentiate between endopeptidase and exopeptidase activities present in the solubilized mixture. The results showed that the solubilized activity had an endopeptidase action, which was inhibited by ortho-phenanthroline but was unaffected by leupeptin, antipain or elastin whereas chymostatin, an inhibitor of chymotrypsin significantly inhibited cleavage. This data suggested that signal peptidase has an endopeptidase component. The fate of the signal peptide after its cleavage is still unknown, though it is presumably proteolyzed.

Two types of models have been proposed to explain the cotranslational translocation of secretory and membrane proteins into the ER membrane. One suggests that the insertion of the signal peptide is spontaneous and does not involve the presence of specific receptors. This model includes the membrane trigger hypothesis (Wickner, 1979) or the helical hairpin hypothesis (Engleman and Steitz, 1981). The other model is the one which suggests that the process is receptor mediated (Walter and Blobel, 1981; Sabatini et. al., 1982). Since there is strong evidence for the existence of such receptors the other models are much less probable.

During protein translation, translocation to the endoplasmic
reticulum is mediated by signal recognition particle (SRP) (Walter and Blobel, 1980) (Figure 1,2). The signal recognition particle is a complex of molecular weight 250,000 and is made up of 6 polypeptides with molecular weights 72,000, 68,000, 54,000, 19,000, 14,000 and 9,000 (Walter et al., 1981a; Walter and Blobel, 1983a) and a small cytoplasmic RNA molecule designated 7SL RNA (Ullu et al., 1982; Walter and Blobel, 1982). The SRP has been found to be about equally distributed between membrane associated and free ribosomes (Walter and Blobel, 1983b). In the cytoplasm SRP interacts with ribosomes and when these ribosomes get engaged in the synthesis of secretory (Stoffel et al., 1981a; Walter et al., 1981a), lysosomal (Erickson et al., 1983) or certain classes of membrane proteins (Anderson et al., 1982). SRP interacts with their signal sequence as soon as the latter becomes exposed on the surface of the ribosome and arrests the elongation of the nascent polypeptide chains (Walter and Blobel, 1981b). This elongation arrest is reversed once the ribosome interacts through the tightly bound SRP with a specific integral membrane protein of the endoplasmic reticulum with a molecular weight of 72,000, the SRP receptor (Gilmore et al., 1982a, 1982b) also called "docking protein" (Meyer et al., 1982a, 1982b). The ribosome then forms a functional ribosome membrane junction by interacting via the SRP with the ER membrane (Walter and Blobel, 1981) allowing the vectorial translocation of the nascent polypeptide chain across the lipid bilayer (Blobel and Dobberstein, 1975).

An important aspect of the function of SRP as a cellular adapter specific for the translocation of only the secretory and
PROTEIN TRANSLOCATION (Lewin, 1982)

ORIENTATION IN THE ER MEMBRANE

CYTOPLASMIC SIDE

COOH

ER MEMBRANE

LUMEN

NH2
membrane proteins is its interaction with the signal sequence. A major part of the work communicated in this thesis deals with studying the essential features of the signal sequence of VSV glycoprotein necessary for its recognition by the SRP.

**Glycosylation of Glycoprotein Glycosylation Sites**

A prerequisite for glycosylation of the protein inserted into the ER membrane or secreted into the lumen of ER is the occurrence of the acceptor Asn in the tripeptide sequence, -Asn - X - Thr (or Ser) - where (X) can be any of the 20 amino acids except possibly aspartic acid (Marshall, 1974; Struck and Lennarz, 1980). This structural requirement appears to be rather specific; incorporation of 2-hydroxyornvaline (an analogue that differs from threonine by one methylene group) into peptide chains in vitro was found to inhibit cotranslational glycosylation by 30% (Hortin and Boime, 1980). But the fact that only about a third of the known - Asn - X - Thr/Ser sites on eucaryotic proteins are glycosylated and of those some are inefficiently glycosylated suggests that other determinants are also involved.

A search for the other determinants was approached by utilizing membrane preparations from hen oviduct as an enzyme source, endogenous oligosaccharide lipid as donor, and a series of 13 well characterized proteins containing unglycosylated - Asn - X - Thr/Ser sites as potential acceptors. It was found that although none of these proteins acted as an acceptor in its native conformation, 6 of the 13 could be glycosylated in vitro after denaturation (Pless and Lennarz, 1977; Kronquist and Lennarz, 1978). Since no correlation other than the
known tripeptide was found between amino acid sequence and acceptor activity and it was suggested that glycosylation requires exposure of carbohydrate attachment sites by peptide chain unfolding. Further support for this suggestion was provided by the observation that cyanogen bromide treatment of two of the proteins that had remained inactive despite denaturation, generated oligopeptide fragments that were efficient oligosaccharide acceptors (Kronquist and Lennarz, 1978). Studies with membranes from hen oviduct (Struck et al., 1978; Hart et al., 1979) or yeast (Bause and Lehle, 1979) revealed that a variety of small peptides could be glycosylated in vitro if they met just two prerequisites: the presence of an Asn-X-Ser or Asn-X-Thr sequence and blockage of the COOH and NH2-terminus by other amino acids or by chemical modification. These results demonstrate that while other factors such as proximity to charged or hydrophobic groups may exert some effect (Bause and Lehle, 1979) the predominant structural determinant for exogenous polypeptide glycosylation in defined cell free preparations in these two distantly related organisms is the accessibility of the tripeptide acceptor sequence.

The prediction of the three dimensional polypeptide conformation around a carbohydrate attachment site is possible from the amino acid sequence data. Based on the frequencies with which particular amino acids occur in configurations such as β-sheets, α-helices and β-turns in proteins of known three dimensional structure, (Chow and Fasman, 1974; Beeley, 1977) was able to predict that 30 of 31 N-glycosylated sequences he studied were part of turn or loop structures, which in 22 cases were most probably β-turns. In a similar
estimation, out of 28 glycosylated Asn residues examined 19 were present in β-turns. Since such turn conformations often occur at the surfaces of globular proteins in exposed regions between sheet or helical structures (Kuntz, 1972) these prediction further emphasize the importance of accessibility of the glycosylation site. Although these conformational predictions remain to be confirmed, it is noteworthy that the three-dimensional structure of N-glycosylated proteins [Fc fragment of IgG (Huber et al., 1976) and haemagglutinin membrane glycoprotein of influenza virus (Wilson et al., 1982)] reveals that the site is a part of exposed regions such as the β-turn.

In order to determine the number of oligosaccharide side chains per VSV glycoprotein molecule, a detailed analysis on DEAE Sephadex of the tryptic digestion products of the glycoprotein from VSV (Indiana) grown in Hela cell suspension cultures was carried out (Robertson et al., 1976). The study demonstrated that the glycoprotein contains two glycosylation sites per polypeptide, to which greater than 95% of the carbohydrate is attached. An inspection of the sequence of G protein predicted from the nucleotide sequence of the VSV G mRNA reveals the presence of 18 Asn residues, but only two of these (amino acid residues 178 and 335) occur in glycosylation consensus sequences and these have been suggested to be the actual glycosylation sites (Rose and Gallione, 1981). These sites are at fractional distances of 0.35 and 0.66 from the NH$_2$-terminus. Earlier studies had suggested that the first addition occurs when about 38% of the chain has been synthesized and the second occurs when about 70% has been synthesized (Rothman and Lodish, 1977). Thus, this nearly exact correspondence of the positions
of these sites with fraction of G synthesized when glycosylation occurs suggests that the transfer of oligosaccharides to the nascent protein chain occurs when the Asn residue passes through the rough ER into the lumen. This glycosylation in VSV G is a cotranslational event occurring in the lumen of ER. This is consistent with earlier observations that with Asn-linked oligosaccharides in eucaryotic cells begins with the segregation of these molecules within the lumen of the rough ER (Palade, 1975).

Transfer of Oligosaccharide Chain from the Lipid Carrier to the Protein

Biosynthetic studies have suggested that the N-acetyl glucosamine and mannose residues are preassembled by stepwise addition in an activated state bound to the isoprenoid lipid dolichol pyrophosphate (Waechter and Lennarz, 1976) and are transferred en bloc to appropriate Asn residues during translation of the polypeptide chain (Keely et al., 1976; Sefton, 1977). The first seven residues are added directly from the nucleotide sugars UDP-GlcNAc₂-lipid. Glc₃ Man₉GlcNAc₂-lipids is then completed by the addition of four Man and three Glc residues from the monosaccharide-lipids dolichol phosphate mannose (Dol-P-Man) and dolichol phosphate glucose (Dol-P-Glc); monosaccharide lipids are synthesized from Dol-P, UDP-Glc and GDP-Man. Mature oligosaccharides are then transferred as a unit from the lipid carrier to asparagine residues of nascent and newly made polypeptides (Figure 1.3) (Ghosh, 1980; Struck and Lennarz, 1980; Hubbard and Ivatt, 1981; Staneloni and Leloir, 1982; Snider, 1983).

Drugs that inhibit lipid-linked oligosaccharide and as a result
Fig. 1.3
Synthesis of Dolichol Phosphate Sugar Intermediate and Transfer of the Sugar Chain to the Glycosylation Site of the Protein

\[
\text{H} (\text{CH}_2\text{C} = \text{C} - \text{CH}_3)_{n} \text{CH} - \text{CH} - \text{CH} - \text{CH}_2\text{OH} \quad (n=16-24)
\]

Dolichol (Dol) → CTP → CDP → Dol-P

UDP-GlcNAc → UMP → Dol-P-P-GlcNAc

UDP-GlcNAc → UDP → Dol-P-P-GlcNAc_2

GDP-Man → GDP → Dol-P-P-GlcNAc_5Man_5

Dol-P-Man → Dol-P

Dol-P-P-GlcNAc_2Man_9

Dol-P-Glc → Dol-P

Dol-P-P-GlcNAc_2Man_9Glc_3

Protein → Dol-P-P

α₁,₂ Man → Man₁,₂ → Man → Man → Man → GlcNAc → GlcNAc → Asn → Endo H

α₁,₂ Man → Man → Man

Glc → Glc → Man → Man → Man

High Mannose type Glycoprotein
block glycosylation of Asn residues have provided strong evidence that lipid-linked oligosaccharides are the only source of Asn-linked glycans.

The most commonly used is tunicamycin, a glucosamine containing antibiotic which inhibits the formation of GlcNAc-P-P-Dol (Takatsuki et al., 1975; Tkacz and Lampen, 1975) but not its elongation (Lehle and Tanner, 1976; Heifetz et al., 1979; Keller et al., 1979). Tunicamycin was shown to specifically inhibit the glycosylation of VSV, and Sindbis virus glycoprotein resulting in a suggestion that glycosylation of viral glycoproteins may be essential for normal assembly of enveloped viral particles (Leavitt et al., 1977). The other less used drugs include compactin and 25-hydroxycholesterol which suppress dolichol synthesis (Mills and Adamany, 1978 and Carson and Lennarz, 1979), 2-deoxyglucose (2DG) which inhibits formation of Glc-P-Dol, Man-P-Dol, and GlcNAc-P-P-Dol by competing with Dol-P to form 2DG-P-Dol (Schwarz et al., 1978; Datema et al., 1981) and glucosamine probably by blocking an early step of lipid oligosaccharide assembly (Datema and Schwarz, 1979). Studies with temperature sensitive mutant cell lines in which the glycoproteins have a reduced content of N-linked carbohydrate have further strengthened the case for lipid-linked oligosaccharide involvement. Under nonpermissive conditions, these cells appear to synthesize a normal lipid-linked oligosaccharide but transfer it to the protein at a greatly reduced rate (Tenner et al., 1977 and Tenner and Scheffler, 1979).

In a recent study of how transport of sugar residues occurs across the ER during oligosaccharide lipid synthesis and transfer, the
membrane orientation of oligosaccharide-lipid intermediates was examined. Using the lectin concanavalin A, the lipid-linked oligosaccharides \( \text{Man}_{3-5}\text{GlcNAc}_{2} \) were found on the cytoplasmic side of ER derived vesicles in vitro while lipid-linked \( \text{Man}_{6-9}\text{GlcNAc}_{2} \) and \( \text{Glc}_{1-3}\text{Man}_{9}\text{GlcNAc}_{2} \) were found facing the microsomal lumen. These results suggest that \( \text{Man}_{5}\text{GlcNAc}_{2} \)-lipid is assembled on the cytoplasmic side of the ER membrane and then translocated across the microsomal membrane to the luminal face. \( \text{Glc}_{3}\text{Man}_{9}\text{GlcNAc}_{2} \) lipid is then completed on the luminal side of the ER, where it serves as the donor in protein glycosylation (Snider and Roger, 1984). Endo-\( \beta \)-\( N \)-acetylglucosaminidase \( H \) (Endo \( H \)) is an enzyme which cleaves between \( N \)-acetylglucosamine residues in large high mannose asparagine oligosaccharides (Figure 1.3) (Tarentino and Maley, 1974) and has been used as a tool for the analysis of the intracellular forms of the glycoprotein of VSV and Sindbis virus (Robbins et al., 1977).

**Processing of the High Mannose Oligosaccharide**

This involves the removal of all glucose and 6 mannose residues and the addition of terminal sugar.

**Trimming of High Mannose Oligosaccharide**

The first phase of \( N \)-linked oligosaccharide processing involves the removal of glucose residues from the precursor oligosaccharide (Fig. 1.4) immediately after its transfer to the protein. The kinetics of removal of the 3 Glc residues in VSV infected Chinese hamster ovary (CHO) cells indicate that the half time for the removal of the terminal Glc is less than 2 minutes (Kornfield et al., 1978; Hunt, 1980). The second was removed with an apparent half time of 5 minutes and the
Fig. 1.4 Trimming of High Mannose type of Glycoprotein

Man → Man

Man → Man → Man → Glc → Glc → Glc → Man → Man → Man → Glc → Glc → Glc → Man → Man → Man

1. Glucosidase I
2. Glucosidase II-III
3. Man-Assimilation

Man → Man → Man → Man → Man → Man → Glc → Glc → Glc → Man → Man → Man → Glc → Glc → Glc → Asn → Man → Glc → Glc → Glc → Asn

α-2-Mannosidase

Man → Man

Man → Man → Glc → Glc → Glc → Asn

N-acetyl glucosaminyl transferase I

Man → Man → Man → Man → Man → Glc → Glc → Glc → Asn

Man → GlcNAc → GlcNAc → Asn

SWAINSONINE

α-Mannosidase II

Man → Man → GlcNAc → GlcNAc → Asn

GlcNAc → Man → GlcNAc → GlcNAc → Asn

* Kang and Albein, 1983
   Tulsiani et al., 1982
third at a still slower rate). At least two separate enzymes appear to participate in this reaction. Glucosidase I removes only the terminal Glc residue while glucosidase II-III which is active only after the removal of the terminal residue brings about the release of both the remaining two Glc residues. These enzyme activities have been shown to be present in cell free preparations and in purified enzyme preparations from rat liver (Ugalade et al., 1978; Grinna and Robbins, 1980), calf liver (Michael and Kornfield, 1980), calf brain (Scher and Waechter, 1979), hen oviduct (Chen and Lennarz, 1978; Elting et al., 1980), calf thyroid (Spiro et al., 1975), NIL-8 fibroblasts (Turco and Robbins, 1979) and yeast (Lehle, 1980).

Glucosidases I and II-III found in rat liver are the integral membrane proteins apparently localized on the luminal surface of the rough and smooth ER (Grinna and Robbins, 1979). This convenient location supports the assumption that they participate in the processing of newly synthesized glycoproteins that are sequestered within the lumen of the rough ER. In hen oviduct, the specific activity of glucosidase I was highest in the rough microsomal fraction (Elting et al., 1980) while the mannose removing enzymes of the processing pathway are localized exclusively in the Golgi fraction. This suggests that after removal of the glucose residues the glycoprotein is transported to the Golgi where the rest of the processing occurs. This suggestion is further supported by kinetic studies in CHO cells. Man residues are not removed from maturing N-linked glycoproteins for 10 minutes after the transfer of the high mannose oligosaccharide to the protein. The delay cannot be attributed
to the protection of the mannose residues by glucose residues, since substantial amounts of Man$_3$GlcNAc$_2$ appear at least 5 minutes earlier—(Hubbard and Robbins, 1979). Thus, it suggests that the lag is most likely due to the transit time between ER and the Golgi. There is substantial evidence suggesting that the newly synthesized glycoproteins pass through the Golgi apparatus en route to the cell surface. Studies of the kinetics of appearance of pulse-labeled G in the various isolated subcellular membrane fractions from VSV infected cells have shown that the G protein migrates sequentially from ER to a low density smooth membrane (presumed to be derived from the Golgi apparatus) and finally to the plasma membrane (Hunt and Summers, 1976; Knipe et al., 1977). The core oligosaccharide transferred to G protein in ER has been shown to be modified in the smooth membranes by the removal of mannose residues followed by the addition of terminal sugars (Robbins et al., 1977; Hunt et al., 1978). Using a cell free system the glycoprotein was shown to be efficiently transported from ER to the Golgi complex by an energy dependent process resembling intracellular protein transport (Fries and Rothman, 1980). Using indirect immunofluorescence and immunoelectron microscopy of VSV-infected cells, G protein antigenicity was detected in the Golgi apparatus (Bergmann et al., 1981). A direct approach involving light and electron microscope radiography confirmed the intracellular pathway of the glycoprotein transport (Bergeron et al., 1982).

The precise mechanism which can explain the transport of G from the ER to the Golgi is not known. A suggestion that G is transported via two types of clathrin-coated vesicles, one transporting G from ER
to Golgi and another from Golgi to the plasma membrane (Rothman and Fine, 1980) has been questioned by a study using fluorescent electron microscopy for localization of G, which failed to detect the G protein in coated vesicle structures (Wehland et. al., 1982). It was suggested that the G protein containing vesicles observed earlier arose artifactually by the closure of coated pits of the Golgi complex system during cell fractionation.

After entering the Golgi the α1-3 Man residues of linked oligosaccharide containing only Man and GlcNAc are removed in an ordered sequence shown in Figure 1.4 by the enzyme, Mannosidase I (Kornfeld et. al., 1978; Tulsiani et. al., 1977). The Man5GlcNAc2 formed after removal of the α1-2 Man residues is Endo H sensitive and is the smallest high mannose glycan found in appreciable amounts in mature glycoproteins (Tai et. al., 1975; Chapman and Kornfeld, 1979; Cohen and Ballou, 1980).

Synthesis of Complex Oligosaccharide

In this phase, the protein linked Man5GlcNAc2 is converted to Endo H resistant, complex glycans. Although the host cell (i.e. available processing enzymes) may have some effect on the size of the complex glycans, the primary determinant is the oligosaccharide structure of the protein being processed. Several studies have compared the N-linked oligosaccharides of multiple strains of influenza (Nakamura and Campus, 1979; Klenk et. al., 1980) or VSV (Ethichson and Holland, 1974; Hunt et. al., 1983) grown in several host cell types. Thus, a given glycosylation site may carry a complex glycan in one virus strain, while in a distinct but related strain grown in the same
cells the analogous site may carry a high mannose chain or no carbohydrate at all.

The first step in the formation of the complex oligosaccharide is catalyzed by GlcNAc transferase I, which adds a single GlcNAc residue to the Man5GlcNAc2 structure that remains after removal of the α1,2-Man residues from the precursor oligosaccharide (Narasinhman et al., 1977; Tabas and Kornfeld, 1978; Harpaz and Schachter, 1980a) Figure 1.5. A second enzyme "late mannosidase" then releases the two terminal (non core) Man residues (Tabas and Kornfeld, 1978; Harpaz and Schachter, 1980b). The product of these two reactions are rapidly processed and have not been identified as intermediates in intact cells. The GlcNAc2Man3GlcNAc2 is a substrate for at least four enzymes in vitro, (a) GlcNAc transferase II which can convert it to GlcNAc3Man3GlcNAc2 (Narasinhman et al., 1977; Harpaz, N. and Schachter, 1980b), (b) a fucosyl transferase which can add α1-6 linked Fuc to the innermost (reducing terminal) GlcNAc residue (Wilson et al., 1976); (c) a galactosyl transferase, which forms Galβ1-4 linkages to the terminal (non reducing) GlcNAc residues (Schachter, 1978) and (d) a sialyl transferase, which forms sialic acid α2, 3 or α2, 6 linkages to the terminal Gal residues (Beyer et al., 1979). The complex type glycoprotein is resistant to Endo H after the addition of the terminal sugars (Robbins et al., 1977). The heterogeneity in the addition of sialic acid gives the glycoprotein the appearance of a diffuse band on SDS-polyacrylamide gels.

The biological role of the glycosyl moiety of glycoproteins is still an enigma. Glycosylation is not a prerequisite for insertion
Addition of terminal sugars

\[
\text{Man} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{Asn} \]

\[\text{N-acetyl glucosaminyl transferase II}\]

\[
\text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{Asn} \]

\[\text{Fucosyl transferase}\]

\[
\text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{Asn} \]

\[\text{Galactosyl transferase}\]

\[
\text{Gal} \rightarrow \text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{Asn} \]

\[\text{Sialyl transferase}\]

\[
\text{SA} \rightarrow \text{Gal} \rightarrow \text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{Asn} \]

Complex type oligosaccharide

Fig. 1.5
of the G protein into the rough ER (Rothman et. al., 1978) nor is it required for efficient cleavage of the signal sequence (Irving et. al., 1982). Unglycosylated G protein has been shown to reach the plasma membrane (though not in normal amounts) (Gibson et. al., 1978). G protein of VSV is normally found in the basolateral surface of polarized MDCK cells (Rodriguez-Boulan and Sabatini, 1978) and lack of glycosylation has no effect on the positioning of G protein (Green et. al., 1981). However, the conformation of the G protein is affected in the absence of glycosylation. Unglycosylated G protein becomes aggregated at elevated temperatures which results in a decreased production of virus (Gibson et. al.; 1979, 1981).

Experiments with cell mutants and temperature sensitive viral mutants provide a powerful method for unraveling the biosynthetic pathways and for discovering the role of the oligosaccharide moiety of glycoproteins. A study of the temperature sensitive mutants of complementation group (II) of VSV, which corresponds to the structural gene of G demonstrated that the mutations result in blocks at very different stages in the maturation pathway of G protein (Zilberstein et. al., 1980; Lodish and Kong, 1983). One class of mutants is blocked at an early, pre-Golgi step and the other class the maturation of G protein proceeds normally through several Golgi functions, but is blocked at the nonpermissive temperature at a later step i.e. that of transport from Golgi to the cell surface. Such studies provide some clues as to the roles of various post-translational modifications in the intracellular transport and maturation of G protein. The phenotypic characterization of a temperature sensitive mutant of VSV
described in this thesis was carried out with precisely this aim in mind. As it turned out this mutant was affected only in glycosylation and this provided a unique uncomplicated system to study the role of glycosylation and the affect of difference in extent of glycosylation on the overall viral structure and infectivity. Some of the glycosylation mutants described by Zilberstein et al. (1980) are also defective in fatty acylation and could not assess the role of glycosylation in the transit from the Golgi to the plasma membrane.

**Fatty Acid Acylation**

Membrane proteins from a number of enveloped viruses and animal cells have been found to contain small amounts of tightly bound lipid (Magee and Schlesinger, 1982; and Schmidt, 1982). This lipid consists predominantly of one to two molecules of long chain fatty acids and appears to be linked covalently in ester bond directly to hydroxy amino acids (Ser, Thr, Tyr) in the polypeptide (Schmidt and Schlesinger, 1979). The only protein for which the precise location of the fatty acid linkage has been determined is the gag precursor of murine leukemia virus. A myristic acid is linked to the amino-terminal glycine (exposed as a result of removal of the methionine) by an amide bond (Henderson et al., 1983). The fatty of the G protein is localized in its COOH-terminal membrane spanning region (Capone et al., 1982, 1983). Fatty acid binding is an early post-translational event in virus glycoprotein biosynthesis and intracellular membranes associated with the Golgi apparatus are thought to be the site of acylation (Schmidt and Schlesinger, 1980). Fatty acylation of protein is a modification in search of a function. Fatty acid attachment does
not seem to be essential for the intracellular transport of membrane proteins or for directing these proteins to the membrane. Studies using cerulein, an inhibitor of cholesterol biosynthesis and of transfer of fatty acids to proteins suggest that virus budding from infected cells require fatty acid acylated proteins. The study of the palmitate labeling of serotypes of VSV documented in this thesis was carried out to see whether fatty acid acylation of glycoproteins is a general occurrence and what possible role it plays in the life cycle of VSV.

**Kinetics of Glycoprotein Biosynthesis**

Precursor oligosaccharides \((\text{Glc}_3\text{ManGlcNAC}_2)\) are transferred to glycosylation sites of the VSV glycoprotein before it is released from the polysomes (Rothman et. al., 1978). For 15 minutes the protein linked glycoprotein remains sensitive to Endo H (Robbins et. al., 1977; Tabas et. al., 1978) and consists mainly of \(\text{Glc}_3\text{ManGlcNAC}_2\) and \(\text{Man}_9\text{GlcNAC}_2\) (Kornfeld et. al., 1978).

In the subsequent 15 minutes an increasing fraction of the oligosaccharides become resistant to Endo H (Robbins et. al., 1977; Hunt et. al., 1978; Tabas et. al., 1978; Schmidt and Schlesinger, 1980; Bergmann et. al., 1981; Bergeron et. al., 1982) which indicates that all the Glc residues and six out of the nine Man residues have been removed and the addition of the outer sugars GlcNAc, Gal, SA and Fuc has begun. The fatty acid acylation occurs shortly before the oligosaccharides becomes resistant to Endo H (Schmidt and Schlesinger, 1980) approximately around 15 minutes. This suggests that fatty acid attachment occurs while G protein moves through the Golgi complex.
This is supported by the observation that monensin, an ionophore that causes the accumulation of G protein in the Golgi, does not affect fatty acid acylation of the G protein (Johnson and Schlesinger, 1980).

**Maturation Assembly and Budding**

The early stages of maturation of VSV involves the modification of the host cell membrane with G and M proteins. Cell fractionation studies of infected cells pulsed with $[^{35}S]$methionine and chased for different lengths of time reveal that at early times after its synthesis, the M protein is soluble; from this state it is progressively incorporated into membranous structures with the density of virions and then quickly appears in extracellular virions. The G protein is also associated with the membrane in that it is inserted into it (Wagner et al., 1970; Cohen et al., 1971; Lafay, 1974; Lenard and Compans, 1979). Nucleocapsid structures containing the (-) strand genome RNA as well as the viral N, NS and L proteins are assembled in the cytoplasm. The nucleocapsid becomes associated with membranes modified by viral proteins and budding of the progeny virus from the host cell membrane (Wagner et al., 1970; Cohen et al., 1971; Lenard and Compans, 1974).

M protein serves a very important function in the final stages of virus formation. Crosslinking studies and the studies with mixed pseudotypes suggests that M protein serves as the link between the envelope and the nucleocapsid (McSharry et al., 1971; Dubovi and Wagner, 1977). Several mutants of VSV in complementation group III, defective in the structural gene for M, produce at the nonpermissive temperature, noninfectious particles which contain the viral M and G
proteins but less than 10% of the normal proportion of N protein or
 genomic RNA. Thus suggesting that M and G proteins are sufficient for
some virus budding to occur (Schnitzer and Lodish, 1979). Taken
together with studies of temperature sensitive mutants of group V (e.g.
ts045), which showed that particles can be formed lacking G, but
containing M, N, NS and L in normal proportions (Deutsch, 1976; Little
and Huang, 1977; Schnitzer et al., 1979), reinforces the key role of
M protein in virion formation.

Chloroquine, a drug that prevents the expression of G protein at
the cell surface during the final stages of G protein assembly and
transport of complex type Endo H resistant oligosaccharide containing G
protein from the Golgi to the plasma membrane, gives rise to
noninfectious particles lacking G protein (Dillie and Johnson, 1982).
This again emphasizes the absence of an active role for G in viral
assembly and budding. G protein also has no role in the discrimination
of (+) and (-) strand nucleocapsids present in the cytoplasm to be
packaged into the mature virus (Mann and Wertz, 1982).

The glycoprotein of enveloped RNA virus may play a role in
determining the surface in the polarized cells at which the assembly of
the viral components and the budding occurs (Rodriguez-Boulan, 1983).
In cultured epithelial cell lines, such as Mardin-Darby canine kidney
(MDCK) cells (Misfeldt et al., 1976; Cerjeido et al., 1978) the
viruses assemble with a striking polarity; influenza (an
orthomyxovirus), Sendai and simian virus 5 (two paramyxoviruses) bud
from the apical surface while VSV (a rhabdovirus) obtains its envelope
from the basolateral surface (Rodriguez-Boulan and Sabatani, 1978).
Polarized budding is preceded, and presumably determined by the asymmetric surface distribution of viral envelope glycoproteins (Rodriguez-Boulan and Pendergast, 1980). Carbohydrates do not appear to be responsible for the observed asymmetry (Roth et al., 1978; Green et al., 1981). In cells doubly infected with influenza and VSV, polarized budding is preserved and the respective viral glycoproteins hemagglutinin (HA) and G protein can be localized within the same Golgi apparatus, suggesting that the sorting of apical and basolateral proteins may be a post-Golgi event (Rindler et al., 1982). Recent studies to investigate the exact intracellular localization of VSV, albumin and transferrin made use of immunoelectron microscopy at the highest possible resolution (Ger et al., 1983). The study revealed that the membrane protein G of VSV and two secretory proteins are present in the same Golgi compartment and secretory vesicles suggesting that it is unlikely that the polarized distribution is brought about by Golgi-derived vesicles and that this phenomenon is brought about by an as yet undetermined domain of the glycoprotein.

Interferon specifically inhibits viral assembly by blocking the incorporation of either G or M or both of these proteins in the virus. This inhibition of viral assembly by interferon has been inferred from the observation that there is an overall reduction of less than 10 fold in the progeny virion yield in the presence of interferon whereas there is about a 1000 fold decrease in infectivity (Jay et al., 1983). This effect of interferon cannot be accounted for only by the inhibition of glycosylation described earlier (Maheshwari et al., 1980).

The association of the nucleocapsid with the modified plasma
membrane containing G and M triggers the release of viral particles referred to as budding. The budding mechanism involves the envelopment of the nucleocapsid by the modified plasma membrane and the folding out of the nucleocapsid containing modified cellular plasma membrane with its long axis perpendicular to the membrane and the pinching off of the progeny virion from the host cell (Zee et al., 1970).

Infection of cells with VSV results in gross morphological and cytopathological changes which bring about cell death. These changes ultimately resulting in cell lysis are a culmination of the inhibition of cellular macromolecular synthesis (Wagner, 1975 and Ben-Zeev, 1983).
MATERIALS AND METHODS

MATERIALS

(a) Chemicals and Reagents

All chemicals used were of the best available analytical reagent grade. The monosodium salt of GTP, the dipotassium salt of ATP were purchased from P-L Biochemicals; dithiothreitol (Cleland’s reagent), sodium deoxycholate, cerulenin and creatine phosphate were from Calbiochem; Sephadex G-25 (coarse), and concanavalin A covalently linked to Sepharose were from Pharmacia Inc.; Triton X-100, bis-acrylamide and TEMED were obtained from Bio-Rad Laboratories; acrylamide, sodium dodecyl sulphate (SDS) and the sodium salt of ethylene diamine tetraacetic acid (EDTA) were obtained from BDH Chemicals Ltd.; sucrose, was obtained from Bethesda Research Laboratories; bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), tunicamycin, and spermine were obtained from Sigma Chemicals; crude wheat embryo tRNA was obtained from Dr. X. Ghosh, Department of Biochemistry, McMaster University. Tunicamycin was a gift from Eli Lilly Company Ltd., Indianapolis, IN; actinomycin D was a gift from Merck; wheat germ was a gift from General Mills Inc., CA, U.S.A.; soybean trypsin inhibitor was from Miles Laboratories.

HPLC grade benzene, ethyl acetate, butyl chloride, acetonitrile and tetrahydrofuran (THF) were purchased from Burdick and Jackson; methanol was from Caledon; sequence grade phenylisothiocyanate (PITC), hepta fluorobutyric acid (HFBA) and 0.1 M quadrol were obtained from Beckman. Trifluoroacetic acid (TFA) and ethanethiol were from Pierce Biochemicals; polynene and glycylglycine were obtained from Aldrich.
Ultra high purity grade nitrogen was from Canadian Liquid Air.

(b) **Enzymes**

TPCK-treated trypsin, and lysozyme were obtained from Worthington; **Staphylococcus aureus** V8 protease was from Miles Laboratories. Endo H was a generous gift from Dr. P.W. Robbins of M.I.T. Creatine kinase was from Boehringer Mannheim Corporation.

(c) **Radiochemicals**


(d) **Antiserum**

Antiserum specific to VSV Cocal viral proteins was obtained from Dr. H.P. Ghosh.

(e) **Cell Culture**

CEF cells were prepared as described by Vogt (1967). L60 cells were obtained from Dr. L. Prevec, Department of Biology, McMaster University and maintained in suspension culture in Joklik modified MEM. This supplemented with 5% NBCS, 2% NaHCO\(_3\), 1% penicillin, 1% streptomycin and 1% L-glutamine. Monolayer cultures were maintained in MEM containing 7% NBCS, 2% NaHCO\(_3\), 1% Hepes pH 7.3, 2% L-glutamine, and 1% penicillin and 1% streptomycin. BHK and Vero cells were maintained in monolayers in Dulbecco’s minimal essential medium supplemented with 7% NBCS, 2% NaHCO\(_3\), 1% penicillin, 1% streptomycin, 1% L-glutamine and 1% Hepes.
(f) **Virus**

Plaque purified VSV of the Indiana, Cocal, New Jersey (Concan), New Jersey (Missouri), New Jersey (Hazelhurst), serotypes and Chandipura and Piry viruses were obtained from Dr. L.A. Prevec, Department of Biology, McMaster University.(ts^M a mutant of the Cocal serotype of VSV was obtained from Dr. C.R. Pringle, Institute of Virology, Glasgow, Scotland.)
METHODS

Growth of Cells

L cells were grown in suspension culture in Joklik modified MEM containing 5% (v/v) NBCS or 5% (v/v) FBS. Cells were maintained in log phase.

CEF primary cell culture were prepared as described by Vogt (1967). Eggs incubated for 10 to 12 days at 37°C were wiped with 70% alcohol and placed in a plate. The top was cracked with a sterile forceps handle and the shell removed from the top. The chorionallantoic membrane was removed and the embryo detached and placed in a Petri dish containing PBS. The head, wings, legs and internal organs were removed and the tissue chopped finely with scissors. The chopped tissues from about 12 eggs was then placed in a flask containing 50 ml PBS containing 0.25% (v/v) of concentrated trypsin solution (Gibco) and stirred for 1 h at 37°C. The contents were poured through four layers of cheese cloth to remove large clumps. The mixture was centrifuged at 800-1000 rpm for 10 min at room temperature and the cell pellet suspended in the primary growth medium consisting of high glucose Dulbecco medium containing 10% tryptone phosphate broth, 5% calf serum, 1% penicillin, 1% streptomycin, 2% NaHCO₃ and fungizone at a cell concentration of 1.2 x 10⁶ cells/ml.

Growth of Virus

(a) Quantitation of Infectious Virus Particles

The plaque forming titre determined as plaque forming units (PFU) per ml of stock virus preparations was used as an index of infectivity.
To determine the titre, the virus stock was serially diluted. A 0.1 ml aliquot of the diluted virus was placed on a confluent monolayer of 5 x 10^6 L cells in a Falcon Petri dish (60 x 12 mm) containing 0.4 ml of MEM containing 2% NBCS. Adsorbtion was for 40 min at 37°C in an atmosphere of 5% CO₂ in air and 100% humidity. The monolayer was then overlaid with 5 ml of Earle's MEM (Gibco) containing 5% NBCS and 0.9% agar. Plaques were observed after 20-24 h of incubation at 37°C in a water saturated, 5% CO₂ atmosphere. Cells were fixed with Carnoy's solution (methanol:water:glacial acetic acid; 6:3:1; v/v/v) and stained with 0.1% crystal violet.

(b) Growth of Virus Stock

L cells from an exponentially growing culture were harvested by centrifugation at 400xg for 20 min, then resuspended to 10^7 cells/ml in Joklik modified MEM containing 2% NBCS. Cells were infected at a multiplicity of infection (m.o.i.) of 1 PFU/cell. The virus was allowed to adsorb to the cells at 37°C for 30 min and the culture then diluted to 10^6 cells/ml with Joklik modified MEM containing 2% NBCS. Incubation was for 16-18 h, after which time the cellular debris was removed by centrifugation at 7,000xg for 20 min. The supernatant containing the virus was divided into 10 ml aliquots and stored at -90°C.

Preparation of Radioactively Labeled Viral Proteins

(a) Preparation and Purification of Labeled Virions

For the preparation of [³⁵S]methionine labeled virions, infection of L cells was carried out as described above except that the m.o.i. was increased to 30 PFU/cell. At 2.5 to 3 h post-infection the
infected cells were pelleted by centrifugation at 4000xg for 10 min and resuspended in methionine-deficient Joklik modified MEM (Gibco) containing 2% dialyzed fetal calf serum to a concentration of 2 x 10^6 cells/ml. [35S]Methionine (specific activity 1,000 Ci/mmol) was then added to a concentration of 20 μCi/ml. For [3H]sugar and [3H]palmitate labeling, the concentration was the same but the specific activity was 10–20 Ci/mmol. The infection was continued another 16 to 20 h. The cellular debris was then removed as described previously and the labeled virus was recovered from the supernatant by centrifugation in a SW41 rotor at 40,000 rpm on a 2 ml 30% sucrose cushion for 2 h. The pellets were suspended in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA. A 10 ml linear gradient of 10 to 40% sucrose in NTE buffer (0.15 M NaCl, 0.01 M Tris- HCl, pH 7.5, and 1 mM EDTA) was prepared in 12 ml nitrocellulose or polyallomer tubes. The resuspended virus was layered on gradients and centrifuged in a SW41 rotor at 80,000xg for 1 h. The visible virus-containing band was collected from the gradients, diluted with NTE buffer and the virus pelleted by centrifugation at 125,000xg for 30 min. The final pellet was resuspended in NTE buffer and stored at -90°C.

Alternatively, the medium containing the virus was layered on to a 25–40% sucrose gradient in NTE buffer containing 0.1% BSA and centrifuged in a SW 27 rotor at 25,000 rpm for 4 h.

(b) Preparation of Labeled Intracellular Proteins

L cells were infected with VSV, at an m.o.i. of 30 PFU/cell. After an adsorption period of 30 min at 37°C, the suspension was diluted to 1 x 10^6 cells/ml with methionine deficient Joklik modified
NEM containing 2% dFCS. Actinomycin D was added to a concentration of 2 µg/ml to reduce host cell protein synthesis. The cells were incubated at 37°C for 1 h. [35S]methionine was then added to a concentration of 20 µCi/ml and the incubation was continued another 3 h. The infected cells were harvested by centrifugation at 4000g for 10 min, washed twice with PBS, and solubilized in SDS-containing buffer in preparation for polyacrylamide gel electrophoresis.

**Synthesis of Virus-Specific Proteins Labeled with [35S]Methionine**

Confluent L cells (100 mm plates) adapted to 39.5°C were infected for 45 min with Cocal or tsy1 at 32°C. The excess medium was removed and the cells were washed twice with phosphate buffered saline (PBS) suspended in a medium containing 2% dialyzed fetal bovine serum (dFBS) and 2 µg of actinomycin D/ml was added and the cells were incubated at 32°C and 39°C. At the end of 4.5 h the medium was replaced with MEM containing 2% dFCS and [35S]methionine (40 µCi/ml) and incubated at the respective temperatures for 60 min. The cells were then washed twice with PBS, scraped off the plate, lysed in 400 µl of a buffer containing 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate (RIPA buffer) and 100 Kalli Krein activator units of trasylol/100 ml. The lysate was immunoprecipitated with 10 µl of anti-Cocal antiserum and 50 µl of a 10% (v/v) suspension of protein A-Sepharose beads (Pharmacia). The immunoprecipitated radioactive protein was recovered by heating in sample buffer (0.625 M Tris-HCl, pH 6.8, 1.4 M 2-mercaptoethanol, 3% SDS, 10% glycerol and 0.01% bromophenol blue) (Kessler, 1975). The
virus specific proteins were separated on 5-12.5% gradient polyacrylamide gels containing 0.1% SDS and identified by autoradiography (Toneguzzo and Ghosh, 1977, 1978).

**Synthesis of Intracellular Virus Specific Proteins in the Presence of Tunicamycin**

The procedure was essentially the same as described above with the exception that cells were pretreated with tunicamycin at 4 μg/ml for 1 h. The medium used during the post-infection incubation and during the labeling period also contained 4 μg of tunicamycin per ml.

**Radioactive Labeling of Infected Cells with [3H]Sugars**

L cells were cultured in 100 mm Petri dishes until nearly confluent (6 x 10⁶ cells). The cells were infected with Cocal or tsγ at an m.o.i. of 50 and incubated at 32°C. After 45 min, the unadsorbed virus was removed and the cells washed with prewarmed PBS and 5 ml of MEM was added and the cells incubated at 32°C or 39°C for a further 3.75 h. The medium was then removed and cells washed three times with 5 ml of warm PBS. The cells were then labeled with [3H]mannose, [3H]galactose or [3H]glucosamine (25 μCi/ml) for 60 min. At the end of the labeling period, the cells were washed, harvested, disrupted using sample buffer and analysed on a 10% gel or 5-12.5% gradient gels and identified by autoradiography (Toneguzzo and Ghosh, 1977, 1978).

**Tryptic Peptide Analysis of [3H]Mannose Labeled Glycoproteins by HPLC**

L cells were infected with Indiana or Cocal or tsγ virus and labeled with [3H]mannose as described above with the exception that the
amount of radioactivity used was 50 μCi/ml. [³H]Mannose labeled glycoproteins were separated on a SDS-10% polyacrylamide gel. The G protein band was excised from a wet gel and eluted in the presence of 10 mM Tris-HCl, pH 8.0, 1 mM 2-mercaptoethanol and 0.01% SDS and digested with TPCK-treated trypsin (protein to enzyme ratio 2:1) in a buffer containing 50 mM Tris-HCl, pH 8.0 and 10 mM CaCl₂. The incubation was carried out at 37°C for 6 h. The tryptic digest was applied to an Altex column (0.46 x 22 cm) of Ultrasphere octadecyltrimethyl oxysilane previously equilibrated with 100% of solvent A (4.25 mM sodium acetate, pH 5.05 containing 5% tetrahydrofuran) and was eluted with a linear gradient of solvent A to solvent B (10% tetrahydrofuran in acetonitrile) as follows: 0-25% of B in 15 min, 25-50% of B in a further 35 min and 50-100% of B in 10 min at room temperature at a flow rate of 1.2 ml/min. Fractions of 1.2 ml were collected, dried in an oven at 130°C and counted for radioactivity.

[³H]Palmitate Labeling of Virus

Monolayers of L cells in 100 mm plates were infected separately with Indiana, Coquel or tsyl virus at an m.o.i. of 10 at 32°C for 1 h. After adsorption, MEM containing 2% dFCS and [³H]palmitate (25 μCi/ml) was added. Sixteen hours post-infection the virus was harvested and analyzed by SDS-10% polyacrylamide gel electrophoresis. The unfixed gel was fluorographed as described under Polyacrylamide Gel Electrophoresis and exposed to an X-ray film.
In Vitro Protein Synthesis

(a) Isolation of RNA from VSV Infected Cells

VSV specific RNA was prepared from cells infected either with Indiana, Cocal, New Jersey or tsWI virus as described (Toneguzzo and Ghosh, 1975). L cells maintained in suspension culture at 1 x 10^7/ml were infected at an m.o.i. of 30-50 for 1 h to enable virus adsorption. After the adsorption period the cells were centrifuged at 5,000xg for 10 min and suspended in Joklik MEM containing 2% NBCS and actinomycin D at a concentration of 2 μg/ml and a cell density of 1 x 10^6 cells/ml. At 4.5 h post-infection the flask containing the cells were cooled in an ice bath along with addition of frozen MEM for 10 min. The cells were then centrifuged in pre-cooled bottles at 5,000xg for 10 min at 0°C and washed 3 times with washing buffer (35 mM Tris-HCl, pH 7.5, 146 mM NaCl). The pellet was suspended in 2 volumes of swelling buffer (10 mM Hepes-NaOH, pH 7.6, 15 mM KC1, 1.5 mM magnesium acetate, 6mM 2-mercaptoethanol) and the tube containing the cells was placed on ice for 10 min. The swollen cells were lysed using a 15 ml glass Dounce homogenizer placed in an ice bucket. Twenty-five strokes of homogenizer with five strokes at a time with a gap of two min were generally sufficient to bring about the lysis of more than 80% of the cells. The homogenate was collected in a Pyrex or Corex tube and centrifuged at 4,000xg for 10 min at 0°C. The S-4 extract was made 0.5% with respect to SDS and an equal volume of mRNA extraction buffer was added (50 mM Tris-HCl, pH 9.0, 100 mM NaCl, 0.1% SDS, 6 mM 2-mercaptoethanol). The RNA was extracted with an equal volume of chloroform and phenol (saturated with the mRNA extraction buffer) (1:1,
v/v), was added and the tube covered with teflon tape and shaken by several inversions. The tube was then centrifuged at 7,500xg for 10 min at 4°C. The aqueous phase was re-extracted with 1/2 the volume of chloroform-phenol (1:1, v/v) and the chloroform-phenol phase was re-reextracted with 1/2 volume of the mRNA extraction buffer. The combined aqueous phases were made 0.4 M with respect to LiCl and the RNA was precipitated with 2.5 volumes of 95% ethanol at -20°C overnight or -80°C for 1 h. The precipitate was collected by centrifugation at 10,000 rpm for 30 min and washed with 70% ethanol. The final RNA pellet was dried in a desiccator under vacuum and dissolved in 200 μl of sterile distilled water and stored in aliquots at -90°C after freezing in liquid N₂.

(b) Preparation of Cell Free Wheat Germ Ribosomal Extract

Wheat germ extract was prepared according to Roberts and Patterson (1973). Five to 10 g of unprocessed wheat germ stored in a desiccator at 4°C was mixed along with an equal weight of crushed Pasteur-pipette tips and ground in a chilled mortar containing 25 to 30 ml grinding buffer (20 mM Hepes-NaOH, pH 7.2, 100 mM potassium acetate, 1 mM magnesium acetate, 2 mM CaCl₂ and 6 mM 2-mercaptoethanol) for 30 sec with a pestle, after which 10 ml of the grinding buffer was added and grinding was carried out for a further 30 sec. The ground wheat germ was then passed through 4 layers of autoclaved cheese cloth to remove the glass and any germ debris. The extract was collected in a beaker and transferred to a Corex or hard walled Pyrex tube and centrifuged at 30,000xg for 10 min at 4°C. The resulting S₁₀₀₀₀ supernatant was carefully removed avoiding the pellet and the cloudy
surface layer. The S-30 was then passed through a Sephadex G-25
column equilibrated with 20 mM Hepes-NaOH, pH 7.6, 120 mM
potassium acetate, 5 mM magnesium acetate and 5 mM 2-mercaptoethanol.
The column was eluted with the equilibrating buffer and the fractions
having the highest A_260 nm were pooled and stored in 200 μl aliquots in
liquid N_2.

(c) In Vitro Translation of mRNA

The translation of either Cocal or New Jersey viral RNA was
carried out in a reaction mixture containing 20 mM Hepes-NaOH, pH 7.0,
1 mM DTT, 90 mM potassium acetate, 2.5 to 3.5 mM magnesium acetate,
1 mM ATP, 0.02 mM GTP, 10 mM creatine phosphate, 40 μg creatine kinase
per ml, 100 mM spermine, 0.025 mM of each of 19 unlabeled amino acids,
200 μCi of [^3]H]amino acid or 200 μCi[^35S]methionine per ml and 500 μl
of the wheat embryo extract per ml. The amount of total RNA added
depended on the batch and different serotype. For most purposes 50 to
100 μg of RNA per ml was usually found to be optimal for translation.
After incubation at 27°C for 90 min, aliquots were analyzed for hot
TCA-insoluble radioactivity as described by Ghosh and Ghosh (1972).

Automated NH_2 Terminal Sequence Analysis of Proteins

(a) Isolation of Labeled Proteins

Labeled virus specific proteins either from the in vitro
translation reaction mixture or from infected cell extracts were
separated on a SDS - 8.25% polyacrylamide gel and the wet unfixed gel
wrapped in Saran wrap with radioactive marker spots on the corners and
exposed to an XAR-5 X-ray film at 4°C. The autoradiogram is then used
as a template to excise the appropriate bands. In cases requiring the
isolation of \( ^3\text{H} \)-labeled proteins, \(^{35}\text{S}\)methionine labeled samples processed under identical conditions were separated in adjoining lanes, thus enabling the excision of the unvisualized bands of interest, by extrapolating the excision of bands visualized in the \(^{35}\text{S}\)methionine lanes into the lanes containing the corresponding \(^3\text{H}\)amino acid labeled band. The gel slices were washed with sterile deionized water and placed in a sterile capped 5 ml plastic tube containing 10 mM Tris-HCl, pH 8.8, 0.01% SDS, 1 mM 2-mercaptoethanol and BSA as a carrier at 1 mg/ml. The space between the tube and the cap was sealed with parafilm and left for constant shaking at room temperature for 15-18 h. The eluate was then passed through a syringe plugged with siliconized glass wool and dialyzed in spectropore dialysis membrane (pretreated to remove sulphur) against 1000 volumes of sterile water at 4°C for 24 h with at least two changes of the sterile water. The dialyzed protein was lyophilized and suspended in 0.5-0.7 ml of sterile water containing 2 mg of salt free lysozyme. A small aliquot was tested for purity by electrophoresis and if found to contain other bands such as M or N to an extent greater than 5% the entire sample was reprocessed as described above starting from separation by polyacrylamide gel electrophoresis.

\(\text{b) Automated Edman Degradation}\)

The purified protein was subjected to automated Edman degradation in a Beckman 8900 sequencer with a cold trap modification. The Modified Beckman 0.1 M Quadrol program (No. 345801) which excludes nitrogen delivery to the fraction collector during the steps in which it is under vacuum was employed.
Samples were applied to a spinning cup and subjected to the Beckman sample application subroutine program number 345871 for drying the protein in the form of a film on the inner surface of the cup. In some cases the cup was coated with 3-5 mg of polybrene along with 100 nmol of glycylglycine in water and precycled for three complete degradation cycles prior to addition of the sample to facilitate the retention of the protein to the cup and hence minimize losses (Hunkapiller and Hood, 1978). Each of the protein sequencing runs was preceded by a complete cycle with the PITC delivery shut off.

In case of radioactive samples, 50% of each fraction in the form of anilinothiazolinones (ATZ) was transferred to mini scintillation vials, the contents dried in an oven at 80°C, dissolved in dioxane and the radioactivity determined by counting in an aqueous counting scintillant such as Aquasol.

For identification of the amino acid derivatives arising from lysozyme, the remaining 50% of each fraction was dried under nitrogen and converted to phenylthiohydantoin (PTH) derivatives by any of the known methods.

The most commonly used method involved treatment of the ATZ derivative with 0.2 ml of 25% trifluoroacetic acid containing 1 μl of ethanethiol/ml for 10 min at 80°C (Kotwal et al., 1983), after which the sample was dried under nitrogen and suspended in HPLC grade methanol and identified by HPLC. Fifty nmol of the PTH-derivative of norleucine per 50 μl of methanol was added to each sample prior to HPLC separation to serve as an internal control to monitor the retention time and the yield of the unknown amino acid.
(c) **Analysis of the PTH Amino Acid Derivatives**

The Altex model 322 HPLC system used for the separation of PTH amino acid consists of two model 110A pumps, a model 421 microprocessor controller and an Ultrasphere octadecyltrimethyl oxysilane C-18 column maintained at 45°C in a chamber of Varian aerograph series 1400. An LKB fraction collector (7,000 series), 254 nm Altex detector and an Altex C-K1A integrator also formed a part of the system. The column was washed several times in 70% (v/v) methanol in degased, deionized sterile glass distilled water which had been filtered through a Millipore HAWP (0.45 nm) filter and gradually equilibrated with solution A (4.25 mM sodium acetate, pH 5.05, containing 5% THF) by a gradient washing. Twenty μl of the sample was loaded onto the column and the column was eluted with a linear gradient of 40% solution B (10% THF in acetonitrile) for 20 min followed by isocratic elution with 40% B for a further 20 min (Somack, 1980). Nonradioactive PTH amino acids were quantitated with the integrator using the normalization mode.

In case of sample containing a mixture of a protein labeled with more than two different [³H]amino acids the entire sequencer fractions were converted to PTH-derivatives separated on HPLC along with corresponding standard PTH-derivatives and the HPLC fractions were counted for radioactivity to determine which of the labeled amino acid was present in the cycle.

**Polyacrylamide Gel Electrophoresis (Slab Gels)**

SDS-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (1970). Two glass plates, one notched and one
unnotched (dimensions 16 x 18.75 cm for short gels and 16 x 22 cm for long gels) were washed carefully with detergent, rinsed with distilled H₂O left to air dry, and wiped with 95% ethyl alcohol. Plastic spacers (1.5 mm thick) were placed along two sides of the unnotched plate, the second plate was then placed on top, and the two plates were held together by clamps. The separating gel was made as follows: for 40 ml of 10% polyacrylamide separating gels the following ingredients were mixed in a Buchner side arm flask: 16 ml H₂O, 10 ml separating gel buffer 4x (182 g Tris base, H₂O to 1 L, pH adjusted to 8.8 with HCl), 13.3 ml acrylamide-bisacrylamide solution (30 g acrylamide, 0.4 g bisacrylamide, H₂O to 100 ml, filtered), 0.4 ml 10% SDS, 0.4 ml 10% glycerol and 0.01 ml TEMED. The solution was degased, 0.3 ml 10% ammonium persulfate was added and 5 ml of the mixture with 30 μl TEMED was poured and served as a plug after it solidified. The remaining 35 ml was poured between the plates up to 5 cm from the top and layered with distilled water. The gel was left to polymerize at room temperature for approximately 30 min. During this time a 5% polyacrylamide stacking gel was made from the following ingredients: 11.6 ml H₂O, 5 ml stacking gel buffer 4x (61 g Tris base, H₂O to 1 L, pH adjusted to 6.8 with HCl), 3.8 ml of a 30% acrylamide solution containing 0.4% bisacrylamide, 0.2 ml 10% SDS, 0.1 ml 10% glycerol, 0.015 ml TEMED, and degased. After the separating gel had polymerized the cavity was cleaned with separating gel overlay buffer (10 ml separating gel buffer 4x, 0.4 ml 10% SDS, 0.4 ml 10% glycerol, 30 ml H₂O). The overlay buffer was completely removed from the surface of the gel before pouring the stacking gel. Then, 0.125 ml of 10%
ammonium persulfate was added to the degassed stacking gel mixture and the solution was poured on top of the separating gel. A comb was inserted from the top and pushed down until the top of the teeth were at the top of the notched plate. After 15 min polymerization at room temperature, the comb was removed and the wells were washed with stacking gel overlay buffer (10 ml stacking gel buffer 4x, 0.4 ml 10% SDS, 0.2 ml 10% glycerol, 30 ml H₂O). The gel was clamped onto the electrophoresis apparatus with the notched plate facing the apparatus. The bottom reservoir was filled with reservoir buffer (3.02 g Tris base, 14.4 g glycine, 1 g SDS, H₂O to 1 L). To avoid leakage of the upper reservoir buffer, the space where the edge of the notched plate made contact with the upper reservoir chamber was sealed with 2% agar.

Samples were dissolved in an equal volume of twice concentrated sample buffer [0.25 ml stacking gel buffer 4x, 60 mg SDS, 0.4 ml 50% glycerol, 0.25 ml H₂O, 0.1 ml 2-mercaptoethanol and 10 μl of 0.2% bromophenol blue (Laemmli, 1970)]. The wells were then filled with the samples and carefully overlayed with reservoir buffer. After filling the upper chamber with reservoir buffer, an initial voltage of 125 volts was applied until the samples had entered the separating gel. The voltage was then increased to 150 to 175 volts until the end of the electrophoresis (approximately 5 h).

Once the electrophoretic run was completed the gel was removed from the plates and placed in a tray of staining solution (2.5 g Coomassie brilliant blue R250, 500 ml methanol, 100 ml glacial acetic acid, 400 ml H₂O). The gel was stained for 1 h at room temperature with shaking. The staining solution was then removed and replaced with
destaining solution (1.5 L 95% ethanol, 0.6 L glacial acetic acid, 3.9 L H₂O). The destaining solution was changed after the first and second hour and destaining was continued overnight. For detection of [³⁵S] or [¹⁴C] radioactivity the destained gel was placed onto a piece of Whatman #1 chromatography paper and dried using an Eltech Designs drier. The dried gel was exposed to Kodak XAR-5 film (Eastman Kodak Company) for low count gels and XRP-1 for high count gels.

For detection of [³H]-radioactivity the fluorographic method of Bonner and Lasky (1974) was employed. At the end of the electrophoretic run the gel was removed from the plates and the protein was fixed by immersing the gel in destaining solution for at least 1 h. The destaining solution was then removed and replaced by at least 20 gel volumes of dimethyl sulfoxide (DMSO). In order to remove the water from the gel, it was shaken in DMSO for 30 min, after which the solution was replaced with fresh DMSO and the washing was continued another 30 min. The gel was impregnated with PPO by immersion in at least 4 gel volumes of 22.2% (w/v) PPO in DMSO for 3 h. The gel was then washed extensively with water and dried as described above. The dried gel was exposed to Kodak XAR-5 or XRP-1 film at -70°C for an appropriate length of time.

Silver Staining of Polyacrylamide Gels

The procedure followed was essentially similar to the one described by Wray et al. (1981). The gel after electrophoresis was soaked in 50% reagent grade methanol for at least 1 h and then soaked in a freshly prepared solution 0.8 g AgNO₃ in 4 ml of distilled water was added dropwise to 21 ml 0.365 NaOH plus 1.4 ml 14.8 M NH₄OH with
constant shaking. The volume was made to 100 ml by adding 73.6 ml distilled water over 15 min with constant agitation. The gel was then washed in deionized water for 5 min. It is then soaked in a solution of 2.5 ml 1% citric acid plus 0.25 ml of 38% formaldehyde in 500 ml water until the bands appeared. The gel was then washed in water and placed in 50% methanol to stop stain development.

**Cellular Fractionation of Infected Cells Labeled with** $[^{35}S]$Methionine

Cells infected with ts M were labeled with $[^{35}S]$methionine (5 μCi/ml) at 39°C for 50 min (20 min pulse and 30 min chase). The cells were harvested, washed twice with PBS, suspended in 2 volumes of swelling buffer (10 mM Tris-HCl, pH 9.0, 1 mM magnesium acetate, 15 mM KCl and 6 mM 2-mercaptoethanol) and left on ice for 10 min and disrupted in a Dounce homogenizer. The lysate was then centrifuged at 4,000xg and the S-4 extract was layered onto a sucrose step gradient of 2 M sucrose in swelling buffer with 1.5 M sucrose above it and 0.5 M sucrose on the top and centrifuged in a SW41 at 100,000xg for 1 h. The interface between the 2 M and 1.5 M sucrose layers was collected and centrifuged at 150,000xg for 3 h. The pellet from the second centrifugation consists of the membrane bound polysomes and that from the first centrifugation was the free polysomes. A portion of the S-4 was centrifuged in an Eppendorf centrifuge (15,000xg) for 20 min. The supernatant is the S-15 fraction and the pellet termed the P-15 fraction was suspended in the swelling buffer. All the fractions were suspended in 50 mM Tris-HCl, pH 8.0 by addition of a suitable volume of 1.5 M Tris-HCl, pH 8.0 and divided into two equal parts. To one aliquot,
TPCK-trypsin was added to give 50 µg/ml. The other was left as a control. The samples were then incubated at 25°C for 30 min. After incubation, 200 µg of soybean trypsin inhibitor/ml of solution was added and the virus specific proteins were immunoprecipitated and analyzed by SDS-10% polyacrylamide gel electrophoresis. The gel was fixed, fluorographed as described under Polyacrylamide Gel Electrophoresis and exposed to an XAR-5 film. The bands corresponding to the G protein and the N protein were then scanned.
RESULTS

Section 1: NH₂-Terminal Sequencing of Intracellular Glycoproteins and the In Vitro Synthesized Unglycosylated Form of the Envelope Glycoproteins of Cocal and New Jersey Serotype of VSV.

The glycoprotein molecules present on the surface of enveloped animal viruses play an important role in the process of infection, pathogenicity, immunological response, and replication of the virus (Choppin and Scheid, 1980). The antigenic determinants on the single glycoprotein, G, of VSV have been extensively studied to determine the relatedness of the different serotypes of VSV (Kang and Prevec, 1970; Cartwright and Brown, 1972; Kelley et al., 1972; Brown and Prevec, 1978; Doel and Brown, 1978; Burgé and Huang, 1979; Rose and Gallione, 1983). Studies based on complement fixation and virus neutralization showed that the Indiana serotype has common envelope determinants with Cocal but not with the New Jersey serotype (Cartwright and Brown, 1972). Analysis of the peptide patterns generated by proteolytic cleavage of G protein derived from the three serotypes, showed the presence of some common peptides in the G proteins from the serologically related Indiana and Cocal strains (Brown and Prevec, 1978). In contrast, the peptide patterns for G proteins from the serologically distinct Indiana and New Jersey strains were totally different (Brown and Prevec, 1978; Doel and Brown, 1978; Burgé and Huang, 1979). When the RNA's of Indiana, Cocal and New Jersey viruses were compared by hybridization, very little sequence homology was observed (Resik et al., 1974). Oligonucleotide fingerprint analysis of the RNAs of the different serotypes of VSV showed different patterns.
even for serologically related strains (Clewley et al., 1977).

The G protein of the VSV Indiana serotype has been used as a model to study membrane glycoprotein biogenesis (Ghosh, 1980). *In vitro* translation of RNA isolated from cells infected with VSV in the absence of membrane results in the synthesis of an unglycosylated product (G₁), containing an NH₂-terminal signal peptide of 16 amino acids, which is absent from the mature glycosylated G protein (Lingappa et al., 1978; Irving et al., 1979). The presence of a signal peptide of 15-30 amino acids at the NH₂-terminus has been established for a number of other membrane proteins and a large number of secretory proteins (Blobel, 1980; Kreil, 1981). A comparison of the known signal sequences did not show any conserved structure essential for interaction with SRP or ER membrane. The only similarity in the known signal sequences is the presence of a high proportion of hydrophobic amino acids. Thus in order to examine if the different serotypes of VSV, which contain variable sizes of G protein in their virions show any homology, size differences or differences in hydrophobicity in their NH₂-terminal extensions it was decided to obtain a partial amino acid sequence of the signal peptides of two serotypes of VSV and compare them to the known sequence of VSV Indiana. This comparative study was expected to provide useful information on the nature of the recognition of the signal sequence by the SRP and/or the ER membrane and the approach is outlined in Figure 3.1.

The unglycosylated form of G (referred to as G₁) from the Cocal and New Jersey serotypes of VSV was synthesized *in vitro* in the absence of membrane in a wheat germ ribosomal extract and labeled with
**Experimental Approach**

- **L Cells**
- **Cocal**

**Infection**

**Preparation of S-4 Extracts**

**Extraction of Viral RNA**

**Wheat Germ S-27**

**Pilot In Vitro Proteins Synthesis**

**Bulk Synthesis Using Single/Multilabelled Amino Acids**

**Polyacrylamide Gel Electrophoresis and Autoradiography**

**Excision of the G4/G Bands**

**Isolation of Protein**

**Automated Edman Degradation**

**Anilinothiazolinone (ATZ)**

**Cold ATZ**

**Multilabelled ATZ**

**Conversion to PTH Derivative**

**Identification by HPLC**

**Separation by HPLC**

**Radioactivity Measured**

**Fig. 7.1**
\([^{35}\text{S}}\text{methionine or a number of different}^{3\text{H}}\text{amino acids. The intracellular G protein was also labeled with identical radioactive amino acids in VSV Cocal or New Jersey infected L cells. The doubly labeled G}_{1} or G proteins were separated on a SDS-containing polyacrylamide gel. The results are shown in Figure 3.2. The intracellular G proteins of Indiana, Cocal and New Jersey move with different mobilities corresponding to apparent molecular weights on SDS gels of 69,000, 74,000 and 65,000 respectively. The in vitro synthesized G}_{1} protein from the three serotypes showed apparent molecular weights of 65,000, 70,000 and 61,000 respectively. This is in agreement with earlier reports of the molecular weights of the G proteins (Brown and Prevec, 1978).

The isolation of radioactive G}_{1} and G proteins was accomplished by cutting out the appropriate band from the gel and eluting the protein with a buffer containing SDS, 2-mercaptoethanol and lysozyme or BSA. The recoveries by this method were about 80% and the eluted protein was more than 95% pure with no proteolysis (Figure 3.3). Lane a represents the in vitro translation products of New Jersey RNA-and lane b is the eluted G}_{1} protein rerun to check its purity. N and M protein bands were present to an extent of less than 5% (lane b). This does not affect the sequencing because the NH\textsubscript{2}-terminus of these proteins is blocked based on their inability to be sequenced. Lysozyme at 2 mg/ml was incubated in the elution buffer to prevent protein loss and to serve as an internal nonradioactive control during the sequencing. BSA was found to be a more efficient carrier as it was not dialyzed from dialysis bags which had a molecular weight cut
Fig. 3.2 Autoradiogram of a SDS-10% polyacrylamide gel showing the separation of $^{35}S$ methionine-labeled proteins synthesized in an in vitro translation system and in L cells infected separately with the serotypes. Lanes a, c and e represent intracellularly synthesized proteins of VSV Indiana, Cocal and New Jersey, respectively. Lanes b, d and f represent in vitro synthesized proteins of VSV Indiana, Cocal and New Jersey, respectively. The unglycosylated form of the glycoprotein of VSV obtained in an in vitro translation system has been termed G1.
Fig. 3.3 Autoradiogram of a SDS-10% polyacrylamide gel showing the separation of the G1 protein isolated from unfixed SDS-10% polyacrylamide gel. The G1 protein isolated by the procedure described in the methods was analysed along with the [35S]methionine-labeled proteins synthesized in an in vitro translation system. Lane a shows the in vitro translation products of New Jersey mRNA. Lane b shows the [35S]methionine-labeled New Jersey G1 protein.
off of 15,000. Also, it did not form a cloudy suspension in the buffer as does the lysozyme. When BSA was used as a carrier, 2 mg of lysozyme was added just before loading the protein on to the sequencer cup as an internal standard. The inclusion of a reducing agent facilitated recoveries while the SDS insured that no protein losses due to binding to glass occurred. The final product that was subjected to sequence analysis contained approximately 1-2 mg of SDS. SDS has no adverse effect on sequencing reactions, and in fact, aids in the retention of nonpolar peptides or regions in the protein (Bailey et al., 1977). Other methods such as electrophoretic elution or crushing of the gel pieces and eluting into a buffer were also tried but the recovery was poor and the product was less pure. Recovery of proteins from the gel was greatly enhanced if the gel was unfixed and not dried prior to autoradiography.

Automated Edman Degradation and Analysis of the Internal Standard

The method for obtaining protein sequences developed by Edman involves the reactions shown in Figure 3.4. The first reaction involves the coupling of phenylisothiocyanate (PITC) to the free NH₂-terminal α-amino group of the proteins at 50-55°C at pH 9.0-9.5 to form a phenylthiocarbamyl derivative. The derivatized amino acid is then released from the protein chain by cleavage under anhydrous acidic conditions in the form of a 2-anilino-5-thiazolinone (ATZ) derivative. The ATZ derivative is extracted with butylchloride and then converted to the stable phenylthiohydantoin (PTH) derivative and identified by HPLC. The peptide lacking one NH₂-terminal amino acid residue is then
Figure 3.4 Three-Stage Edman Degradation (reproduced from the Beckman 890C Sequencer manual)
subjected to the same cycle starting with PITC. Thus the sequence in which the amino acids are present in the protein is the same as the order in which they are released as PTH-derivatives.

The automation developed for sequence determination based on the foregoing reactions, has through technological advancements made sequence determination of trace amounts possible (Hunkapiller and Hood, 1978). The latest advances using a gas phase sequencer have made it possible to sequence proteins available only in the subpicomole to picomole range (Esch, 1984).

The sequencing work reported here was performed on a Beckman 890C sequencer equipped with a cold trap modification. The degradation was carried out using a 0.1M Quadrol program employing a combined benzene ethyl acetate wash (Brauer et al., 1975).

In order to examine the efficiency and accuracy of the Beckman sequencer, lysozyme a protein of known sequence was degraded along with the radiolabeled protein and selective cycles were examined by HPLC. A prerequisite for obtaining sequence information is the accurate identification of the PTH-amino acid. Until recently, the common methods of identification were TLC, GC and amino acid back hydrolysis (Croft, 1980). But lately, the method of choice for PTH analysis has been HPLC. This method offers a rapid, non-destructive reproducible, quantitative, and extremely sensitive means of detecting all PTH derivatives in a single analysis. In the cases, where UV absorbing impurities arising from the Edman degradation are low the sensitivity is in the subpicomole range. A large number of reversed phase HPLC systems dedicated to PTH-amino acid analysis have been described. All
of these utilize a number of reversed phase supports combined with isocratic or gradient elution and either achieving the conversion manually or modifying the sequencer to automate the conversion (Zimmerman et al., 1977; Margolies and Brauer, 1978; Johnson et al., 1979; Henderson et al., 1980; Somack, 1980; Tarr, 1981; Black and Coon, 1982; Hawke et al., 1982).

The HPLC system used for the identification of PTH-amino acid reported here is described in the Methods. A typical separation of a mixture of PTH amino acids is shown in Figure 3.5. As seen from the figure, a good resolution is obtained with all PTH-derivatives with the exception of the methionine/valeine and the isoleucine/lysine pairs. This can pose a problem in identifying the fractions of unknown cold proteins having these amino acids but does not affect analysis of a protein of known sequence used as an internal control for checking the efficiency of sequencing of radiolabeled proteins. It is thus of interest to add that recently a system has been developed to resolve the two pairs using the two variables of pH and acetate concentration (Hawke et al., 1982), in the gradient system.

A complete cycle in the absence of PITC (sham cycle) was routinely performed for each protein sequenced in order to establish the extent of protein loss. After the fractions were obtained, the PTH derivative of norleucine was included in each sample to serve as an internal standard for quantitative purposes to correct for injection variability and losses during conversion.

The HPLC analysis of the PTH amino acid derivatives of some of the fractions obtained on degradation of 1.50 mg (115 nmol) of hen egg
Fig. 3.5 HPLC analysis of PTH amino acids obtained from the conversion of the product (ATZ amino acid) of automated Edman degradation of hen egg white lysozyme. The top panel shows a typical separation of a mixture of standard PTH amino acids, containing 1-2 nmol of each derivative, by HPLC as described in the Methods section. Detection of the PTH amino acids was at 254 nm. The variance (in min) for the retention time from 12 separate injections obtained for some of the commonly used PTH standards was Val, 0.18; Pro, 0.15; Phe, 0.33; Lys, 0.33 and nLeu, 0.75. The results shown are from the degradation of 120 nmol of lysozyme. The cycles analyzed are indicated by the number at the top left hand corner of each panel and amino acids assigned to the peaks are indicated by a one letter code. The number next to each peak is the retention time. PTH-nLeu was used as the internal standard in all the analyses of PTH amino acids.
white salt free lysozyme is represented in Figure 3.5. The amino acids identified in specific positions of the protein matched with the known sequence of lysozyme (Canfield, 1963, 1965). The yields were calculated from the area of the peaks provided by the integrator as follows:

\[
\text{Yield of unknown PTH-amino acid (nmol)} = \frac{\text{Area of unknown PTH-amino acid}}{\text{Area of nLeu}} \times \text{Amount of nLeu added (nmol)}
\]

The percent yield was calculated as follows:

\[
\text{percent yield (recovery)} = \frac{\text{Observed yield}}{\text{Expected yield}} \times 100
\]

The yields for the lysozyme run are shown in Table 3.1.

The repetitive yields calculated as shown in Table 3.1 were Lys (93%), Leu (97%) and Gly (97%). Repetitive yield is an index of the extent of meaningful sequence information that one can obtain. It is indicative of the conditions of sequencing, e.g. purity of sequencer chemicals, solvents and the vacuum in the cup, efficiency of condensation of reagent and solvent vapours. Any malfunction can greatly reduce the repetitive yield which would mean that the feasibility of identification of fractions derived from amino acids further away from the NH₂-terminus will also be greatly reduced. For instance 100 cycles can be identified with a repetitive yield of 99% (Croft, 1980). An average of repetitive yields obtained from the
TABLE 3.1

Calculation of repetitive yield (R.Y.) from the values of absolute yields of amino acid derivatives of Lys, Gly, and Leu using Fig. 3.5 and the formula given below.

\[
R.Y. = \left( \frac{1}{N_B - N_A} \right) \times 100
\]

A = yield of a particular amino acid

B = yield of the same amino acid in a subsequent position in the sequence

\(N_A\) = position of the amino acid A in the sequence

\(N_B\) = position of the amino acid B in the sequence

<table>
<thead>
<tr>
<th>PTH amino acid identified</th>
<th>Cycle No.</th>
<th>(N_B - N_A)</th>
<th>Yield (nmol)</th>
<th>(B/A)</th>
<th>R.Y.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>1 (A)</td>
<td>12</td>
<td>60</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>13 (B)</td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>4 (A')</td>
<td>12</td>
<td>37</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>16 (B')</td>
<td></td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>8 (A'')</td>
<td>9</td>
<td>29</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>17 (B'')</td>
<td></td>
<td>23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R.Y. can also be determined from the slope of a plot of log % yield against residue number when there are some amino acids in more than two or three positions in the sequence.
sequencing work described in this section was around 94% allowing for an unambiguous identification of 30 residues and occasionally up to 40 residues in cases when the amount of material was adequate and the rise in background noise due to gradual peptide cleavage under acidic conditions of the reaction was minimal. For some unknown reason the yield of lysine was always low in comparison to other amino acids.

The PTH conversions were carried out in 1N aqueous HCl followed by extractions with ethyl acetate. The ethyl acetate used in this conversion procedure does not extract PTH-derivatives of arginine, histidine and cysteic acid since the side chains of the amino acid are ionized. In such cases the aqueous phase must be examined. The analysis of the aqueous phase following conversion of cycle 5 of lysozyme shown in Figure 3.5 reveals the presence of arginine. There is also a peak in cycle 5 corresponding to the retention time of glycine which is a carry over from cycle 4. The presence of glycine in the aqueous phase is indicative of the incomplete extraction with ethyl acetate.

Two other methods were examined to try to find a more reliable method for conversion of ATZ amino acid to its PTH-derivative. These included treatment of the dry ATZ fractions with 1N methanolic HCl for 10 minutes at 55°C (Horn and Bonner, 1977) and 25% TFA for 10 minutes at 80°C. Thus, 5 mg of lysozyme (350 nmol) was sequenced and the residue from each cycle was dried down under N₂ at room temperature, suspended in 150 μl of methanol and split into three 50 μl portions into three different tubes and dried. The dry ATZ-amino acid was then converted to the PTH derivative with the different procedures.
described above.

The HPLC analysis of the lysine residues recovered from cycles 1 and 13 by each of the three procedures is shown in Figure 3.6. The recovery (% yield) in cycle 1 was 39.5%, 39% and 52% of the starting material for aqueous HCl, methanolic HCl and TFA respectively. In cycle 12, the recovery was found to be 19%, 22% and 25% respectively. Thus, the absolute % yield was higher in both the cycles in the case of TFA conversion. In addition, the background in cycle 13 was slightly higher using a methanolic HCl conversion. Because of the higher recoveries, lower background and ease of conversion, TFA was used for all subsequent conversions.

Partial NH₂-terminal Sequence Analysis of G₁ and G from VSV Cocal and VSV New Jersey (Concan)

The isolated G₁ or G proteins of VSV Cocal or VSV New Jersey labeled with [³⁵S]methionine and a single [³H]amino acid in the case of G₁ or a mixture of [³H]amino acids in the case of G were sequenced using the procedure described in Materials and Methods. Generally, the analyses were carried out for 20–26 cycles for G₁ and for 15–cycles for the intracellular G. The amount of radioactivity loaded depended on the approximate frequency of the particular amino acid in the G protein of VSV Indiana whose composition was known (Capone, J., personal communication). It was not always possible to obtain a large enough count for isotopes with low specific activity despite scaling up of the in vitro reaction several fold. But after concentration of the isotope and optimization of the protein synthesizing system it was possible to overcome this problem and obtain at least a 2-fold count difference.
Fig. 3.6 Recovery of PTH-lysine using different methods of conversion of the ATZ to the PTH-derivative. Shown here are the HPLC scans of cycle 1 (a, c and e) and cycle 13 (b, d and e) from sequence degradation of hen egg white lysozyme. The TFA conversions are shown in a and b; methanolic HCl are shown in c and d; aqueous HCl conversions are shown in e and f.
above background for fractions containing the ATZ-labeled amino acid. Any run showing counts less than 2-fold was rejected and the whole procedure repeated.

The results obtained on sequencing the G₁ and G of VSV Cocal are shown in Figure 3.7 and those for VSV New Jersey are shown in Figure 3.8. Radioactivity above background seen in the figure as peaks correspond to the labeled amino acid positions in the protein sequence; In Figure 3.7, the topmost panel represents the analysis of [³H]leucine labeled protein with peaks in cycles 4, 5, 6, 7, 11 and 13. This means that leucine is present in these positions in the portion of the G₁ protein shown below.

\[
\begin{array}{cccccccc}
1 & 5 & 10 & 15 & 20 & 25 \\
VSV Cocal G₁ NH₂ & Leu & Leu & Leu & Leu & Leu & Leu & --- & ---
\end{array}
\]

The partial sequences of G₁ and G proteins of Cocal and New Jersey serotypes of VSV deduced in a similar way from Figures 3.7 and 3.8 respectively are shown in Figure 3.9.

The identity between amino acids in position 19, 21, 23 and 24 of G₁ and those in position 2, 4, 6, and 7 of the G of VSV Cocal suggests the presence of a signal sequence of 17 amino acids in G₁, a peptide which is absent from the glycosylated G. For VSV New Jersey the overlap between the amino acids present in positions 18, 20, 21 and 22 of G₁ and positions 2, 4, 5 and 6 of G indicates the presence of a signal sequence of 16 amino acids for the G protein.
Fig. 3.7 Partial NH₂-terminal sequence analysis of in vitro synthesized form G₁ protein and the G protein synthesized in L cells infected with VSV Cocal were labeled with [³⁵S]methionine and with one other [³H]amino acid, isolated and subjected to automated Edman degradation as described in Methods. The positions for Leu, Lys, Phe, Pro and Ile for G₁ and Phe and Ile for G were derived from proteins labeled individually with respective [³H]amino acids. The ATZ-derivatives were assayed directly for radioactivity. A portion of each cycle was analysed by HPLC to monitor the degradation of the internal standard which in this case was lysozyme. The positions for Val, Lys and Pro were derived from a mixture of G proteins labeled individually with all these amino acids. Analysis of the PTH-derivatives by HPLC was carried out as described in the Methods. [³⁵S]methionine labeled G₁ and G were included in each sample analysed except for G₁ labeled with Lys and Phe.
Fig. 3.8 Partial NH$_2$-terminal sequence analysis of C$_1$ and C from VSV New Jersey. The labeled proteins were analysed as described in Fig 3.7 for VSV Cocal. Assignments for Leu, Pro, Phe, Ile, and Val of C$_1$ and Phe and Ile of C were from individually labeled proteins, while the Val and Lys positions of C were from a protein labeled with both of these amino acids.
Fig. 3.9 NH₂-terminal sequences of \textit{in vitro} synthesized form G₁ and the intracellular form of the G protein of VSV COCAL and New Jersey determined by sequencing radiolabeled proteins.
A comparison of the NH₂-terminal sequences of the G₁ protein of the two serotypes with that of VSV Indiana G₁ (San Juan) (Lingappa et al., 1978) as shown in Table 3.2 reveals that the overall hydrophobicity of the NH₂-terminal extension of the three serotypes (HI 3.4-3.6) is similar. The distribution of hydrophobic amino acids within the signal region is uniform and except for the amino acid preceding the cleavage site, a hydrophobic amino acid may be found in any position.

A comparison of the NH₂-terminal sequences for the G protein of the two serotypes shows that there is considerable homology as far as the six and seven amino acids compared with Indiana G protein. In the positions compared, there was 100% homology in case of VSV Cocal G protein and 83.3% in the case of VSV New Jersey. This is in contrast to the 78.5% and 57% homology in the first 23 amino acids compared in the respective cases.
Table 3.2

Salient features of the \( \text{NH}_2 \)-terminal sequence comparison of the glycoproteins of VSV serotypes.

(1) \( \text{NH}_2 \)-terminal extension

<table>
<thead>
<tr>
<th>VSV Serotype</th>
<th>No. of amino acids</th>
<th>No. of known positions</th>
<th>No. of hydrophobic amino acids</th>
<th>% Hydrophobicity</th>
<th>HI*</th>
<th>% Homology to Indiana</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indiana</td>
<td>16</td>
<td>16</td>
<td>10</td>
<td>62.5%</td>
<td>3.55</td>
<td>50%</td>
</tr>
<tr>
<td>Cocal</td>
<td>17</td>
<td>12</td>
<td>11</td>
<td>64.7%</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>New Jersey</td>
<td>16</td>
<td>11</td>
<td>11</td>
<td>68.75%</td>
<td>3.4</td>
<td>18%</td>
</tr>
</tbody>
</table>

*All non charged amino acid residues have been ranked according to relative hydrophobicity as follows. Trp, + 6.5; Phe, + 5.0; Ile, + 5.0; Tyr, + 4.5; Leu, + 3.5; Val, + 3.0; Met, + 2.5;
Pro, + 1.5; Ala, + 1.0; His, + 1.0; Thr, + 0.5; Gly, 0; Cys, 0; Ser, -0.5; Gln, - 1.0; Asn, -1.5. This hydrophobicity scale is based on free energy of transfer of amino acids from ethanol to water as measured by Noshi and Tanford (1971). Since all amino acid positions are not known the HI shown above is of the hydrophobic amino acids present in all three. The overall HI for Indiana San Juan sequence obtained by Lingappa et. al. (1978) and New Jersey Odgen obtained by Rose et. al. (1983) is 2.63 and 2.59 respectively.

(2) \( \text{NH}_2 \)-terminal sequence of the mature glycoproteins

<table>
<thead>
<tr>
<th>VSV Serotype</th>
<th>No. of amino acids compared to Indiana</th>
<th>No. of similar amino acids</th>
<th>% Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocal</td>
<td>7</td>
<td>7</td>
<td>100%</td>
</tr>
<tr>
<td>New Jersey</td>
<td>6</td>
<td>7</td>
<td>83.3%</td>
</tr>
</tbody>
</table>

Using the sequence obtained for Indiana San Juan and New Jersey Odgen obtained by Rose and Callione (1983) and that of Cocal obtained by Ghosh et. al. (1981) a comparison of the \( \text{NH}_2 \)-terminal 23 amino acid sequence of the mature glycoprotein shows 78.5% and 57% homology respectively.
Section II. Biochemical Characterization of a Temperature Sensitive of the Cocal Serotype of VSV Defective in Glycosylation.

The biosynthesis of glycoproteins found in enveloped viruses such as VSV is a complex process, involving intracellular transport of the molecule and sequence specific post-translational modifications such as glycosylation and fatty acid acylation. The post-translational modifications occur in precise organelles of the host cell such as the endoplasmic reticulum and the Golgi (Robbins et al., 1977; Ghosh, 1980; Bergeron et al., 1981; Magee, 1982).

Temperature sensitive mutants corresponding to complementation group (V) of VSV, (which includes the mutants with specific lesion(s) exclusively in the structural gene for G) have been isolated from chemically mutagenized stocks on the basis of their ability to grow at the two temperatures of 31°C-32°C (permissive) and 39°C (nonpermissive) (Pringle, 1970; LaFay, 1974; Lodish and Weiss, 1979).

The use of glycosylation defective mutants in elucidation of the steps involved in the biosynthesis of glycoproteins and their use in the study of post-translational modifications at different stages in maturation of the viral glycoprotein is well established (Knipe et al., 1977; Zilberstein et al., 1980; Lodish et al., 1981; Lodish and Kong, 1983). Depending on the stage at which the glycoprotein movement is arrested in infected cells, two classes of VSV Indiana mutants have been characterized. One class comprises the mutants that are blocked in the ER or in the pre-Golgi stage (e.g. ts045, tsl513) at the nonpermissive temperature. The other class of mutants e.g. tsl511 which are blocked in the post-Golgi stage, i.e. the class of mutants in
which the G protein transport from Golgi to plasma membrane is blocked at the nonpermissive temperature (Knipe et al., 1977; Zilberstein et al., 1980; Lodish and König, 1983).

This section deals with a temperature sensitive (ts) mutant obtained from mutagenesis of Cocal virus stocks, termed tsY1 (Pringle and Wunner, 1973).

Assay of Infectious Virus Particles

In order to establish the ts nature of the virus, infectivity assays were performed. The viral stocks were suitably diluted and the titre of plaque forming units per ml of the virus stock was determined for both the wild type and the mutant at the permissive (32°C) and nonpermissive (39°C) temperature. The results summarized in Table 4.1 reveal that in the case of tsY1 plaquing efficiency, the ratio between the number of plaques at 39°C and 32°C, was about 10^-2 as compared to 0.75 in the case of the wild type. Plaque size at 39°C was very small (approximately 0.5 mm) for the tsY1 whilst wild type and tsY1 grown at 32°C have a size about 2-3 mm.

Analysis of Virus Particles

Medium from [35S]methionine labeled cells infected with VSV Cocal wild type or tsY1 at 32°C and 39°C was clarified by centrifugation at 3000 rpm for 10 minutes. Then equal volumes were layered on top of a cushion of 30% (w/v) sucrose in TNE buffer and the tube centrifuged at 40,000 rpm in a Beckman SW41 rotor for 2 hours. The pellets were suspended in equal volumes of TNE buffer and the
Table 4.1
Assay of Infectivity of wild type VSV Cocal and ts\(\gamma\)l Viral Particles

<table>
<thead>
<tr>
<th>Virus</th>
<th>Temperature</th>
<th>PFU/ml</th>
<th>Plaques Efficienc (39^\circ)C/ (32^\circ)C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>(32^\circ)C</td>
<td>(2\times10^{10})</td>
<td>-</td>
</tr>
<tr>
<td>Cocal</td>
<td>(39^\circ)C</td>
<td>(1.5\times10^{10})</td>
<td>0.75</td>
</tr>
<tr>
<td>ts(\gamma)l</td>
<td>(32^\circ)C</td>
<td>(6 \times 10^{10})</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(39^\circ)C</td>
<td>(9 \times 10^5)</td>
<td>(1.5\times10^{-4})</td>
</tr>
</tbody>
</table>

L cells were infected with VSV Cocal wild type or ts\(\gamma\)l viruses at either \(32^\circ\)C or \(39^\circ\)C. After adsorption at \(32^\circ\)C the cells were grown at \(32^\circ\)C or \(39^\circ\)C and plaques produced were counted as described in Methods.
TCA-precipitable radioactivity from an aliquot was measured. The radioactivity from the labeled virus preparation is summarized in Table 4.2. In the case of tsy1, the table indicates that the number of virus particles produced at the nonpermissive temperature were about 30% of those formed at the permissive temperature. There was also a 20% reduction in the number of particles released at 39°C in the case of the wild type VSV Cocal as compared to 32°C. Taking into account this reduction in number of particles in the wild type and correcting the counts obtained at 39°C for tsy1, the number of tsy1 virus particles produced at 39°C was 35%. Equivalent fractions of the suspended [35S]methionine labeled viral pellets of tsy1 at 32°C and 39°C in NTE buffer are mixed with [3H]uridine labeled Cocal virus released at 32°C and 39°C respectively. The viral suspension was analyzed on a 10-40% sucrose density gradient and is shown in Figure 4.1. As seen from the figure the wild type VSV Cocal and the mutant tsy1 synthesized at 32°C have the same rate of sedimentation. However, at 39°C the tsy1 particles sedimented at a lower rate than the VSV Cocal virus released at 39°C. Thus, although there is about 65% reduction in the virus particles produced, there is more than 99.9% reduction in infectivity. At the nonpermissive temperature, therefore, a large proportion of the mutant virus produced is noninfectious.. Analysis of the [35S]methionine labeled virus by SDS-gels (Figure 4.2) revealed five proteins whose sizes corresponded to the viral proteins of Cocal and tsy1. No protein of non viral origin was present. The mobilities and the relative proportions of L, N, NS and M were similar in all four lanes to those of labeled virus. The tsy1 G showed a slightly higher
Table 4.2

A comparison of the labeled virus particles produced (expressed as cpm/10μl of the viral suspension) on infection with VSV Cocal or tsyl virus at the permissive and nonpermissive temperatures

<table>
<thead>
<tr>
<th></th>
<th>Cocal 32 °C</th>
<th>Cocal 39 °C</th>
<th>Reduction (%)</th>
<th>tsyl 32 °C</th>
<th>tsyl 39 °C</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>6.9 x 10^4</td>
<td>5.5 x 10^4</td>
<td>20</td>
<td>5.6 x 10^4</td>
<td>1.7 x 10^4</td>
<td>71</td>
</tr>
</tbody>
</table>
Fig. 4.1 Sucrose density gradients of $[^3H]$uridine-labeled wild type VSV Cocal centrifuged at 23,000 rpm in a SW41 rotor for 1 h in the presence of $[^35S]$methionine-labeled tsYl produced at 32°C (A) and 39.5°C (B). The radioactivity obtained by dual label counting procedures is plotted against the fraction number. The wild type VSV Cocal gradient profile is shown by (---) and that of tsYl is shown by (—).
Fig. 4.2 Autoradiogram of $^{35}$methionine-labeled viral proteins released from virus infected cells and virus specific proteins present in infected cells. VSV Cocal and tsyl virus particles released from cells labeled at 32 °C (lanes a and b) or at 39 °C (lanes c and d) were purified through a 30% sucrose cushion by centrifugation of the medium containing the released virus particles at 40,000 rpm for 2 h and separated on a SDS-10% polyacrylamide gel (lanes a and b) or a SDS-5-12.5% polyacrylamide gel (lanes c and d). Lanes a and c represent wild type VSV Cocal viral proteins of particles released at 32 °C and 39 °C respectively, and lanes b and d represent tsyl proteins of particles released at 32 °C and 39 °C respectively. Lanes e-h represent virus-specific proteins from infected cells labeled 4 h post-infection in the presence of $^{35}$S-methionine for 1 h and analyzed on a SDS-5-12.5% polyacrylamide gel. Lanes e and g represent VSV Cocal proteins synthesized in cells infected at 32 °C and 39 °C respectively. Lanes f and h represent tsyl proteins synthesized in cells infected at 32 °C and 39 °C respectively. Lane i represents $^{35}$S-methionine labeled VSV Cocal proteins used as molecular weight markers.
mobility and its characterization by analysis in infected cells will be discussed later. The other striking feature is the absence of G in the ts01 virus particles released at the nonpermissive temperature (lane d), which may explain the shift in the peak on the sucrose density gradient (Figure 4.1).

The role of G in infectivity is well established and the significantly lowered plaquing efficiency in the case of ts01 at 39°C may be attributed to the deficiency of G in the mature virus particles. The relative deficiency of the G protein in the ts01 virus at 39°C is not due to decreased synthesis of G relative to the other intracellular viral proteins. Thus, Figure 4.2 shows that the [35S]methionine labeled virus specific proteins synthesized in cells infected with Cocal or ts01 at 32°C and 39°C. Lanes e and g represent proteins of VSV Cocal at 32°C and 39°C. No difference in the mobilities of the proteins is obvious at the two temperatures. Lane f represents ts01 specific proteins at 32°C. The G protein has a higher mobility than that of Cocal. Lane h shows the ts01 viral proteins labeled at 39°C. The G protein in this latter case has a higher mobility than in the other three cases.

The extent of the differences in apparent molecular weights between the G proteins of Cocal and ts01 at 32°C and 39°C was also investigated by analyzing the [35S]methionine-labeled G proteins synthesized in the presence and in the absence of tunicamycin and in an in vitro translation system. Cells infected with wild type VSV Cocal and the mutant ts01 virus were labeled with [35S]methionine and the virus encoded proteins were immunoprecipitated using antiserum to VSV
Cocal proteins and analyzed on a 5-12.5% gradient polyacrylamide gel (Figures 4.3, lanes b-i).

The G protein synthesized by wild type VSV Cocal infected cells at 32°C or 39°C had an apparent molecular weight of 71,000. In contrast, the G protein synthesized by tsγ1 at 32°C and 39°C had apparent molecular weights of 68,000 and 64,000, respectively. The tsγ1 infected cells at 39°C also showed the presence of a minor amount of protein of molecular weight 68,000 possibly as a result of reversion of the mutation. The decrease in molecular weights of the G protein of the tsγ1 at 32°C and 39°C suggested that either the polypeptide was of a smaller size or that differences in the extent of glycosylation had occurred. The apparent molecular weights were determined from the calculated molecular weights of VSV Indiana proteins.

Two approaches were adopted to determine the molecular weights of the unglycosylated proteins of Cocal and tsγ1. The first method was to label infected cells in the presence of tunicamycin. This antibiotic inhibits glycosylation by preventing the transfer of oligosaccharides to the lipid intermediate dolichol phosphate (Takatsu et al., 1975). Tunicamycin treatment does not affect the translocation of the G protein to the endoplasmic reticulum membrane nor does it hinder the efficient cleavage of the NH₂-terminal extension by the signal peptidase (Irving et al., 1982). Thus the glycoprotein may be visualized in its unglycosylated form devoid of the NH₂-terminal extension after tunicamycin treatment. As shown in Figure 4.3, lanes f-i the proteins synthesized by both the mutant and the wild type at
Fig. 4.3. Autoradiogram of $[^{35}\text{S}]$methionine-labeled virus specific proteins synthesized by VSV Cocal wild type or tsY1-infected cells. A SDS-5-12.5% polyacrylamide gel was used for the separation. Lane a represents VSV Indiana viral proteins labeled with $[^{35}\text{S}]$methionine as molecular weight markers. Lanes b and c, and d and e represent proteins synthesized in the absence of tunicamycin, by cells infected with wild type Cocal at 32 °C and 39 °C and with mutant tsY1 at 32 °C and 39 °C, respectively. Lanes f and g, and h and i represent proteins synthesized in the presence of tunicamycin (4μg/ml) by cells infected with VSV Cocal wild type at 32 °C and 39 °C and tsY1 at 32 °C and 39 °C, respectively. Lanes j and k contain in vitro translation products synthesized in a wheat germ extract programmed with RNA isolated from VSV Cocal wild type and tsY1 infected cells, respectively.
32°C or 39°C in the presence of tunicamycin had the same molecular weight of 64,000. This is the same molecular weight as the G protein of tsY1 synthesized at 39°C in the absence of tunicamycin. The differences in sizes detected in the absence of tunicamycin are possibly due to differences in the extent of glycosylation and not due to a noticeable difference in the protein sizes.

This was further confirmed by in vitro translation of RNA isolated from cells infected with Cocal or tsY1 using a wheat germ system. As shown in Figure 4,3, lanes j and k, the molecular weight of Cocal and tsY1 protein corresponding to the glycoprotein region are the same. Previous studies have shown that the in vitro translation of VSV G mRNA results in the synthesis of the unglycosylated translation product, (Toneguzzo and Ghosh, 1979), which contains an extension of 16 amino acids at the NH$_2$-terminus present in the mature form of the glycoprotein (Lingappa et. al.,1978; Irving et. al.,1979). The identical sizes of the unglycosylated forms of the G protein of VSV Cocal wild type and tsY1 indicates that the differences are not due to a decreased size of the polypeptide. The other inference one can draw is that the in vitro synthesized unglycosylated G protein shows a lesser mobility than the unglycosylated protein synthesized in vivo in the presence of tunicamycin (Figure 4,3, lanes f-i and lanes j and k) indicating that the NH$_2$-terminal extension is efficiently cleaved in the wild type and in the mutant at both temperatures. It also indicates that translocation to the endoplasmic reticulum and its cleavage there, takes place efficiently in both the mutant and wild type at 32°C and 39°C.
In order to demonstrate the proper processing of the signal peptide and to show that the mutant G proteins possess the same NH₂-terminal sequence as the wild type glycoprotein, the partial NH₂-terminal sequences of tsg1 G proteins synthesized in vivo at 32°C and 39°C were determined using [³H]phenylalanine labeled intracellular G proteins. As shown in Figure 4.4, the amino acid phenylalanine is present in the 2nd and 6th position in tsg1 G isolated at 32°C and 39°C and this is in agreement with NH₂-terminal sequence of the intracellular G protein of the wild-type VSV Cocal virus (Figure 4.5). Also when [³H]leucine and [³⁵S]methionine labeled intracellular G of tsg1 at 32°C and 39°C was subjected to automated Edman degradation no counts were detected in the first 20 cycles again indicating that the signal sequence had been removed. Failure to achieve the cleavage, would have yielded peaks in 1st position due to methionine, and in 4, 5, 6, 7, 11 and 13 positions due to leucine.

[³⁵S]Methionine labeled viral glycoproteins of Cocal and tsg1 at 32°C were isolated and subjected to partial proteolysis using V8 protease. The products analyzed on a SDS-18% polyacrylamide gel are shown in Figure 4.6. The pattern is similar in both the cases, lanes b and c. Thus the evidence presented so far indicates that the apparent size differences in mobility are not a result of differences in protein sizes but a result of different extents of glycosylation. This has been confirmed by [³H] sugar-labeling and tryptic glycopeptide analysis.
Fig. 4.4 Partial NH₂-terminal sequence analysis of the mature
glycoprotein of ts VI at 32 °C and of the unglycosylated protein
synthesized at 39 °C. Intracellular glycoproteins from ts VI
infected L cells labeled with [³⁵S]methionine and [³H]phenylalanine
were separated on a SDS-10% polyacrylamide gel, isolated from an
unfixed wet gel, purified by dialysis and subjected to automated
sequence analysis as described in the Methods. A major portion
of each of the fractions was assayed for radioactivity and the
remaining portion of each cycle was analyzed by HPLC to monitor
the degradation of the internal standard lysozyme. The repetitive
yields of the PTH amino acid derivatives were around 95%.
NH2- TERMINAL SEQUENCES OF COCAL AND tsY1 GLYCOPROTEINS

a. COCAL G1 1 MetLysPheLeuLeuLeuLeuPheIle - LeuProLeu - - - - - Phe - Ile - PhePro - 15 20 25
b. COCAL G 5 LysPhe - IleValPhePro - 1
C. tsY16 32°C 1 Phe - - - Phe - -
d. tsY16 39°C 1 Phe - - - Phe - -

Fig. 4.5 A comparison of the partial NH2-terminal sequences of the in vitro synthesized unglycosylated form G1 and the intracellular glycoprotein of Cocal (Deduced from fig. 3.8) with those of tsY1 intracellular glycoprotein synthesized at the permissive and non permissive temperature.
Fig. 4.6 Autoradiogram of a SDS-18% polyacrylamide gel showing the separation of products of partial proteolysis obtained on treatment of [35S]methionine-labeled glycoproteins isolated from a SDS-10% polyacrylamide gel with the enzyme V8 protease at 20 µg/ml as described by Cleveland et al. (1977) and by Metzöl and Reichmann (1981). Lanes a, b and c represent the products of partial proteolysis of the glycoproteins of VSV Indiana, Cocal and tsY1, respectively. The bands in the lanes corresponding to VSV Cocal and tsY1 glycoproteins were scanned using the Joyce-Loebl microdensitometer and it was found that the same number of bands were obtained in both the cases and the extent of migration of the bands was similar.
Lack of Glycosylation of tsyl G Protein at Nonpermissive Temperature

The oligosaccharide moiety of G protein contains glucosamine and mannose residues present as a core with branched chains containing the terminal sugars, galactose and sialic acid residues (Etchison et al., 1977). In the initial step of the glycosylation an en bloc transfer of a high mannose residue and glucose containing oligosaccharide from the dolichol lipid carrier to asparagine residue occurs (see Fig. 1.3) (Robbins et al., 1977). In the subsequent steps the mannose residues are trimmed and terminal sugars are added in the Golgi apparatus (Robbins et al., 1977; Tabas and Kornfeld, 1979; Bretz et al., 1980).

Cells infected with VSV Cocal wild type or tsyl virus were labeled with $[^3]$H]mannose at $32^\circ$C or $39^\circ$C. Cocal G protein was labeled at both the temperatures (Figure 4.7, lanes c and e). In contrast, tsyl G protein produced at $32^\circ$C only contained mannose residues in the oligosaccharide chain attached to the protein (Figure 4.7, lane b). The absence of any mannose label in tsyl G at $39^\circ$C (Figure 4.7, lane d) suggests that at $39^\circ$C tsyl G protein is not glycosylated. This was further confirmed by labeling with $[^3]$H]glucosamine and $[^3]$H]galactose (Figure 4.8). Both of these sugar residues were again absent in tsyl G at $39^\circ$C. The presence of galactose in tsyl G at $32^\circ$C shows that at $32^\circ$C the processing of the core oligosaccharide and transfer of the terminal sugars onto the G protein occurs in tsyl infected cells. In addition, neuraminidase digestion of the G protein reduced the size of tsyl G protein synthesized at $32^\circ$C (M. Buller, unpublished results).
Fig. 4.7: Autoradiogram of [\(^3\)H]mannose-labeled proteins from cells infected with wild type VSV Cocal or tsY1 virus at 32 °C or at 39 °C, separated on a SDS-10% polyacrylamide gel. Lane a, \(^{35}\)S-methionine-labeled Cocal viral proteins; lanes b, c, d and e represent [\(^3\)H]mannose-labeled proteins synthesized by cells infected with tsY1 at 32 °C, VSV Cocal at 32 °C, tsY1 at 39 °C and VSV Cocal at 39 °C, respectively.
Fig. 4.8 Autoradiogram of $[^3\text{H}]$glucosamine and $[^3\text{H}]$galactose labeled ts$^0_Y$ and Cocal viral proteins along with Cocal $[^35\text{S}]$methionine-labeled viral proteins and separated on a SDS-5-12.5% gradient polyacrylamide gel. Lanes b-e, represent intracellularly synthesized proteins in the presence of $[^3\text{H}]$glucosamine, lane b, on infection with VSV Cocal wild type at 32 °C, lane c, on infection with VSV Cocal wild type at 39 °C, lane d, ts$^0_Y$ at 32 °C and lane e, ts$^0_Y$ at 39 °C. Lanes f-i represent proteins labeled in the presence of $[^3\text{H}]$galactose in the same order.
Removal of sialic acid in the G protein has been shown to lower the viral infectivity almost a 1,000-fold (Schlomer and Wagner, 1975) and if tsy1 G at 32°C was devoid of sialic acid it would not reach infectivity titres close to wild type as well as VSV Indiana.

It appears, therefore, that the G protein synthesized at the nonpermissive temperature by tsy1 infected cells lacks any glycosyl residues. This also explains the observation that the $^{35}$S-labeled G synthesized at 39°C has the same mobility in a gradient gel (Figure 4.3) as that synthesized in cells infected with Cocal at either 32°C or 39°C in the presence of tunicamycin.

**tsy1 G Protein Synthesized at Permissive Temperature Contains One Oligosaccharide Moiety**

In order to confirm the above suggestion about the number of glycosylation sites present on the G protein of tsy1 virus released at 32°C in comparison to the wild type VSV Cocal G protein, tryptic glycopeptide analysis was carried out. Since the number of oligosaccharide chains attached to the wild type VSV Cocal G protein was unknown, the G protein of VSV Indiana was used as a control for this study. $[^3H]$Mannose labeled viral glycoproteins of the three viruses were isolated, purified and digested with trypsin as described in the Methods. The tryptic peptide analysis was first carried out by a two dimensional separation consisting of electrophoresis followed by chromatography. As seen in the figure 4.9, the left hand side panel corresponding to the $[^3H]$mannose labeled glycopeptides of VSV Cocal shows 2 spots that form a doublet and the right hand side panel shows 4. This result was inconclusive and therefore HPLC was used.
Fig. 4.9 Autoradiogram showing the separation of [3H]mannose-labeled tryptic peptides of the glycoproteins of VSV Cocal and Indiana on a 20x20 cm polygram SIL-N-HR silica gel chromatography sheet (Brinkman). The isolated glycoproteins were treated with trypsin in a buffer containing 50 mM ammonium bicarbonate, 1 mM CaCl₂ and 50 μg trypsin per ml for 6 h and then spotted on the sheet at the origin marked 'O'. Electrophoresis was carried out in the first dimension as indicated at 130 V for 24 h using pyridine, acetic acid, water (1:10:100;v/v/v) and then dried. The dry chromatogram was cut into two halves and each half was chromatographed for 3 h in a tank containing n-butanol, pyridine, acetic acid, water (5:4:1:4;v/v/v/v) in the second dimension as shown by the arrow. After drying the chromatogram, the two halves were put together as before and sprayed evenly with NEN Enhance and exposed to XAR-5 film for about two weeks.
Fig. 4.9

Cocal Indiana

G G
Fig. 4.10 Photograph showing the separation of $[^3]$Hmannose-labeled tryptic peptides of the glycoproteins of VSV Indiana, Cocal, and tsY1. Labeled glycoproteins were isolated from unfixed wet gels and trypsinized, as described in the Methods section. The sample was then lyophilised and suspended in 5.25 mM sodium acetate, pH 5.05 and loaded on the HPLC column and eluted using solution A (5.25 mM sodium acetate, pH 5.05 with 5% THF in water) at 100% and gradually increasing the proportion of solution B (10% THF in acetonitrile) to 100%. The fractions containing the peptides eluted during a 60 min period were dried and analyzed for radioactivity and the plots are shown in the figure. The left panel shows glycopeptides obtained from VSV Indiana G protein, the middle panel shows glycopeptides from VSV Cocal wild type G protein and the right panel shows the glycopeptides obtained from tsY1. Long arrows indicate the major peaks and the arrow heads indicate the minor peaks.
There are several advantages with the use of HPLC. The separation is fast, quantitative and highly sensitive. Different solvent systems were tried but the one that gave the best separation with a 90% recovery in the major peaks was used. An analysis of VSV Indiana G protein showed the presence of two major glycopeptide peaks (Figure 4.10, left panel) and a number of minor peaks. This pattern is very similar to the earlier observation (Robertson et al., 1978) suggesting that VSV Indiana has two glycosylation sites. Cocal G protein also showed 2 glycopeptides but with a different elution pattern (Figure 4.10, middle panel). It was found that Cocal G resisted trypsin digestion, and even after increasing the concentration of Ca\(^{2+}\) to 10 mM, there was still a minor peak corresponding to the undigested G protein. The elution profile was reproducible and the shift between the different injection was less than 1 fraction. The tsY1 G protein synthesized at 32\(^\circ\)C showed the presence of a single \[^{3}H\]mannose containing peptide (Figure 4.10, right panel). Furthermore, the position of this glycopeptide was shifted from the positions of the two glycopeptides of Cocal G. The single peak suggests the presence of a single oligosaccharide chain in tsY1 G protein synthesized at 32\(^\circ\)C.

**Localization of G of tsY1 at 39\(^\circ\)C**

The G protein synthesized in cells infected with temperature sensitive mutants of VSV Indiana belonging to group(V) is defective in its pathway from ER to Golgi to plasma membrane. Mutants such as ts045, tsL513 and tsM601 show wild type characteristics at the permissive temperature. At the nonpermissive temperature the transport of the glycoprotein is blocked at the stage of transport from the ER to
Golgi as judged from the Endo H sensitivity at chase times at which the wild type shows Endo H resistance (Zilberstein et al., 1980). The G protein of these mutants is normally glycosylated in the ER with 2 Asn-linked Glc$_3$Man$_9$GlcNAc$_2$ oligosaccharides. The very first oligosaccharide processing reactions, which are localized in the rough ER involve removal of the glucose and possibly one of the mannose residues (Grinna and Robbins, 1979). All subsequent Golgi-mediated carbohydrate processing reactions are effectively blocked and the G is localized in the ER despite being glycosylated.

In the presence of tunicamycin (2 µg/ml) there is complete inhibition of glycosylation in VSV infected L cells. However, the unglycosylated G is inserted into the endoplasmic reticulum membrane efficiently and its signal sequence is cleaved but its transport out of the ER is blocked (Morrison et al., 1978; Irving et al., 1982). Thus it would seem that the unglycosylated G protein of tsY1 synthesized in infected cells at the nonpermissive temperature (39°C) should behave similarly to the G synthesized in tunicamycin treated cells. As shown earlier its insertion and efficient cleavage of its signal peptide is evident from the NH$_2$-terminal sequence of the G synthesized in infected cells at 39°C. Having reached the ER its further transport from ER should be similar to the unglycosylated G in tunicamycin treated cells i.e. it remains localized in the ER. This expectation is further reinforced by the fact that despite further processing the G of VSV Indiana mutants is also blocked in the ER.

In order to confirm the evidence suggesting that G of tsY1 synthesized at 39°C is localized in the endoplasmic reticulum membrane
at time points when normally it should have reached plasma membrane, a subcellular fractionation of cells infected with tsY1 at 39°C labeled with [35S]methionine for 20 minutes and chased for 30 minutes, 4.5 hours post-infection was carried out as outlined in Figure 4.11. The fractions were then treated with viral protein specific antibody, the virus specific proteins were analyzed by gel electrophoresis and a microdensitometric scan of the N and the G protein was obtained. The ratio of G to N was determined to correlate it to the extent of change in the presence of G relative to that of N in the intact cell suspension. As can be seen from the Table 4.3, the ER fraction has a high G to N ratio and so does the P-15 fraction. This fraction is the pellet obtained after centrifugation of S-4 in an Eppendorf centrifuge for 20 minutes at approximately 15,000xg and is thought to contain mitochondria and rough endoplasmic reticulum. Figure 4.12 shows the protein profile of the fractions. Since the P-15 fraction had the highest G to N ratio it was used to determine the extent of protection of G in the presence of trypsin. It has been demonstrated that on insertion into the ER a small portion of the G protein corresponding to the COOH-terminus stays on the cytoplasmic side of the ER such that addition of proteolytic enzymes like trypsin to S-4 extracts results in the removal of this COOH-terminal portion without affecting the remaining portion of the protein which is inaccessible to the enzyme by virtue of being inserted into the membrane or being present on the luminal side of the ER membrane (Morrison et. al., 1978; Zilberstein et. al., 1980; Irving et. al., 1982). The analysis of such a digestion by SDS-gel shows a G protein of faster mobility as
Fig. 4.11
Cell Fractionation Scheme to Determine the Localization of G Protein of tsYI in L Cells Infected at 39 °C

L cells

<table>
<thead>
<tr>
<th>tsYI</th>
<th>m.o.i. 30</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5 h</td>
</tr>
<tr>
<td></td>
<td>39°C</td>
</tr>
</tbody>
</table>

Harvest, wash and label

$[^{35}S]$methionine 20 min
and chase with nonradioactive methionine for 30 min

Harvest cells

Dounce 5x5 times
4,000xg for 10 min

S-15

15,000xg 20 min

P-15

(Mitochondrial fraction)

Layer on discontinuous sucrose density gradient

150,000xg for 3 h

1.5 M

150,000xg 3 h

0.5 M

1.5 M

2.0 M

membranous material

membrane-bound polysomes (NBP)

free polysomes

NBP
Fig. 4.12 Autoradiogram of SDS-10% (lanes a-g) and gradient 5-12.5% (lanes b-k) polyacrylamide gels showing the separation of proteins obtained from fractions of L cells infected with tsY1 at 39 °C in the presence of \[^{35}S\]methionine. The fractionation scheme is shown in Fig. 4.11. Lane a represents the \[^{35}S\]methionine labeled viral proteins of VSV Cocal. Lane b represents the virus specific products present in the L cells before fractionation. Lane c represents the proteins of the S-15 fraction. Lane d represents the proteins of the P-15 fraction (mitochondrial pellet). Lane e represents the products of the cell homogenate after cell disruption with a Dounce homogenizer. Lane f represents the MBP fraction obtained from the 1.0 M and 1.5 M interface of a sucrose step gradient, followed by centrifugation. Lane g represents the polysomal fraction from the sucrose step gradient pellet. Fractions corresponding to lanes b-g were separated on another SDS-10% polyacrylamide gel using an equal amount of low radioactivity in each lane. The autoradiogram was then scanned using a Joyce, Loebl microdensitometer and the area of peaks corresponding to the G and N bands in each case are tabulated in Table 4.3. Lanes h and j represent the analysis of the P-15 fractions in the absence of trypsin. Lanes l and k represent the P-15 fraction treated with 50 µg/ml trypsin for 6 h at 37 °C and at 25 °C for 30 min, respectively.
### Table 4.3

Amount of G found in different fractions relative to N

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>G/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspension</td>
<td>0.164</td>
</tr>
<tr>
<td>S-15 (post mitochondrial supernatent)</td>
<td>0.00016</td>
</tr>
<tr>
<td>P-15 (mitochondrial pellet)</td>
<td>1.54</td>
</tr>
<tr>
<td>Homogenate</td>
<td>&lt; 0.34</td>
</tr>
<tr>
<td>Membrane bound polysomes</td>
<td>0.6</td>
</tr>
<tr>
<td>Free polysomes</td>
<td>0.0009</td>
</tr>
</tbody>
</table>
compared to the untreated. When the P-15 fraction was treated with trypsin (50 μg/ml) for 6 hours and the trypsin inactivated by soybean trypsin inhibitor (100 μg/ml) more than 90% of the unglycosylated glycoprotein was recovered in the cleaved form (of a higher mobility) as seen in lane i, with lane h being the control lane. Under milder conditions i.e. 50 μg/ml trypsin treatment at 25°C for 30 minutes and by increasing the amount of trypsin inhibitor to 200 μg/ml, not only was the recovery of the glycoprotein in the cleaved form around 80% but the extent of protection was enhanced (lane k). Thus a combination of indirect and more direct evidence indicates that the localization of the G at 39°C in tsy1 infected cells to be predominantly in the rough ER at chase period of 30 minutes when normally it reaches the cell surface.
Section III: Study of the Fatty Acid Attachment of the Glycoproteins of VSV Serotypes and Two Other Rhabdoviruses.

Glycoproteins present in a number of enveloped viruses have been shown to contain covalently bound fatty acid (palmitic acid) residues (Schmidt, 1982). In the case of VSV Indiana glycoprotein, the fatty acid molecules attached to the glycoprotein are localized in the carboxy-terminal membrane spanning region of the G protein providing an additional stabilizing interaction with the membrane (Capone et al., 1982). The covalent nature of the linkage of 1-2 moles of fatty acid per mole of protein was deduced from the observations that the protein bound fatty acids could not be released with denaturants such as SDS, urea, or guanidine hydrochloride or by exhaustive extraction with organic solvents (Schmidt et al., 1979; Schmidt and Schlesinger, 1979). Mild alkaline hydrolysis (methanolic NaOH) quantitatively removes the fatty acids which are then recovered as the methyl ester derivatives, with complete absence of phospholipid and glycerol, indicating that the fatty acids were covalently attached by ester linkage directly to the polypeptides backbone of the glycoprotein.

The esterification of palmitate to proteins is a modification in search of a function. A temperature sensitive mutant of VSV defective in the transit of its glycoprotein from the Golgi complex to the plasma membrane at the nonpermissive temperature was reported to be due to a lack of fatty acid acylation, thus suggesting a possible role for the covalently bound fatty acid in the transport of the protein in the cell (Zilberstein et al., 1980). However, later results showed that some nonacylated G was transported to the cell surface but not
incorporated into virions suggesting that fatty acid acylation of the
glycoprotein was essential for the assembly and budding of the virus
from the host cell (Schlesinger and Malfer, 1982; Lodish and Kong,
1983).

In order to examine whether the serotypes of VSV New Jersey,
Cocal, tsy1 and other rhabdoviruses Piry and Chandipura have fatty
acids attached to their glycoprotein similar to the one on VSV Indiana
G the study of palmitic acid binding was undertaken. It has been known
for more than a decade that the lipid composition of the envelope of
the serotypes of VSV viz. Indiana and New Jersey differ in the level
of neutral lipids (McSharry and Wagner, 1971). A correlation of the
findings of the above mentioned studies reveals a possible role for
fatty acid attachment.

Labeling of G Protein with $[{}^{3}H]p$almitate
Virions labeled with either $[{}^{35}S]m$ethionine or $[{}^{3}H]p$almitate
were obtained by incubating virus infected L cells in the presence of
the radioactive precursors. The viral proteins were separated by
SDS-polyacrylamide gel electrophoresis. The $[{}^{35}S]m$ethionine labeled G
proteins (shown in Fig. 5.1) for Indiana (lane a), New Jersey (Concan)
(lane b), Cocal (lane c) and tsy1 (lane d) show their distinctive
electrophoretic mobility. The $[{}^{3}H]p$almitate labeled viruses in lanes
e, f, g, h corresponding to Indiana, New Jersey (Concan), Cocal and
tsy1 viruses, respectively, on electrophoresis show the lipid bands at
the dye front. The G protein of VSV Indiana was also labeled with
$[{}^{3}H]p$almitic acid. No $[{}^{3}H]p$almitate labeled G protein was observed in
the cases of New Jersey (Concan), Cocal and tsy1. Similarly, the
Fig. 5.1 Autoradiogram of a SDS-10% polyacrylamide gel showing the separation of viral proteins. Lanes a-d represent \[^{35}\text{S}]\text{methionine}-labeled VSV Indiana, Cocal, New Jersey and ts \(\gamma\) viral proteins, respectively and lanes e-h correspond to \[^{3}\text{H}]\text{palmitate}-labeled viral proteins in the same order.
labeling and analysis by SDS-10% polyacrylamide gel of other VSV New Jersey strains (Figure 5.2) reveals no $^3$H]palmitate labeling of the G protein in lanes corresponding to New Jersey (Concan) (lane a), New Jersey (Missouri) (lane b) and New Jersey (Hazelhurst) (lane e). Piry (lane c) and Chandipura (lane d) viruses, however, show $^3$H]palmitate labeled G. The staining of these labeled viruses shown in Figure 5.3, and Figure 5.4, show the physical presence of all viral proteins including the G proteins of the viruses not labeled with $^3$H]palmitate. Figure 5.3 shows the staining of the labeled viruses with Coomassie brilliant blue G250. Figure 5.4 shows the silver staining of labeled viruses. Thus despite the physical presence of all five proteins in roughly equal amounts in the viruses only Indiana, Piry and Chandipura are labeled with $^3$H]palmitate in the G protein. Metabolic incorporation of $^3$H]palmitate via the amino acid pool is nil as judged by lack of labeling of proteins other than G and the palmitate incorporated is by direct linkage to G.

The results of the $^3$H]palmitate labeling were similar in two other cell lines (Vero and chick embryo fibroblasts as shown in Figures 5.5 and 5.6, respectively and over a temperature range between 32°C to 39°C, to those obtained with L cells at 37°C or 39°C.

Quantitation of the Amount of Virus Released and Relative Amounts of G Protein Produced

In order to establish that the lack of $^3$H]palmitate cannot be accounted for by low production of the virus or a deficiency of G within the virus, the amount of virus released from the same number of cells in each of the three cases was determined and is shown in Table
Fig. 5.2 Autoradiogram of a SDS-10% polyacrylamide gel showing separation of viral proteins. Lane M shows $^{35}$S methionine-labeled VSV New Jersey marker and lanes a-e represent $^3$H palmitate-labeled viral proteins of VSV New Jersey (Concan), New Jersey (Missouri), Piry, Chandipura and New Jersey (Hazelhurst) respectively.
Fig. 5.3 Photograph of a Coomassie brilliant blue G250-stained SDS-10% polyacrylamide gel showing proteins in $[^3]$H]palmitate-labeled virus particles. Lanes a-d correspond to virions of VSV Indiana, New Jersey, Cocal and tsY1 respectively. The staining was carried out on gels fixed in 7% glacial acetic acid by treatment of the fixed gel with filtered 0.04% Coomassie brilliant blue G250 in 3.5% HClO$_4$ for 0.5 h as described (Reisner et. al., 1975).
Fig. 5.4 Photograph of a silver stained SDS-10% polyacrylamide gel showing the proteins present in [3H]palmitate-labeled virus particles. Lane M corresponds to VSV Indiana proteins and lanes a–e correspond to VSV New Jersey (Concan), New Jersey (Missouri), Firy, Chandipura and New Jersey (Hazelhurst), respectively.
Fig. 5.5 Autoradiogram of a SDS-8.25% polyacrylamide gel showing the separation of [3H]palmitate labeled intracellular proteins. Lane M represents the [35S]methionine labeled proteins of VSV Indiana as size marker. Lanes a–d and k represent labeling of infected L cells in the presence of [3H]palmitate. Lanes a and b, intracellular proteins of VSV Coval at 32 °C and 39 °C respectively; lanes c and d tsyl at 32 °C and 39 °C respectively; lanes e–j represent the intracellular proteins in Vero cells infected with VSV Coval at 32 °C and 39 °C (lanes e and f), tsyl at 32 °C and 39 °C (lanes g and h), Indiana at 32 °C and 39 °C lanes i and j, respectively.
Fig. 5.6 Autoradiogram of a SDS-10% polyacrylamide gel showing the products of the labeling of VSV infected CEF cells in the presence of [3H]palmitate at 35°C. Lanes b, c and d correspond to extracts of cells infected with VSV Indiana, Cocal and New Jersey, respectively. Lane a represents [35S]methionine labeled VSV Indiana viral proteins.
5.1. Also shown are G/M ratios determined by microdensitometric analysis from a Coomassie brilliant blue R250 stained gel of viral proteins (Figure 5.7) which had $^{3}$H]palmitate incorporation of $1 \times 10^6$ cpm in each case.

As seen from the Table 5.1, the amount of virus released is comparable and so is the amount of G protein synthesized in relation to M. Therefore, the lack of $^{3}$H]palmitate labeling of G proteins of New Jersey or Cocal serotypes could not be due to decreased virus production or deficient G protein present in the virions.

**Biological Activity of Virions Lacking Palmitate**

The infectivity of virions labeled with $^{3}$H]palmitate was determined by assaying the plaque forming activity of an aliquot of the labeled supernatant. The amount of virion present was determined by separating them on a 10-40% sucrose density gradient and counting the radioactivity under the corresponding peaks. Data presented in Table 5.2 show that although the yield of infectious virus in the case of Cocal and New Jersey was less than that of the Indiana serotype, the specific infectivity in each case was similar. This shows that both Cocal and New Jersey virions lacking palmitate have the same biological activity as Indiana virions containing palmitate covalently attached to G protein. Thus, fatty acid attachment has no role in infectivity of the New Jersey and Cocal viruses.

**Effect of Blocking of Fatty Acid Acylation**

The antibiotic cerulenin has been used to study the effect of blocking of fatty acid acylation on VSV replication. The antibiotic inhibited both of the fatty acid acylation of G protein and the
Fig. 5.7 Photograph of a Coomassie brilliant blue R250-stained SDS-10% polyacrylamide gel showing proteins present in \(^3\text{H}\)palmitate-labeled virus particles. Lanes a-c correspond to the proteins of VSV Indiana, New Jersey and Cocal, respectively. The amounts of the virus were similar. The gel was scanned using a Joyce, Loebl double beam recording microdensitometer. The G/N ratios determined from this gel are tabulated in Table 5.1.
Table 5.1

Quantitation of the amount of virus produced from equivalent amounts of infected cells and the amount of G present in the different serotypes of VSV in relation to M.

<table>
<thead>
<tr>
<th>Serotype of VSV</th>
<th>Amount of virus produced[^b]</th>
<th>G/M[^+ \text{ ratio}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indiana</td>
<td>43</td>
<td>0.08</td>
</tr>
<tr>
<td>New Jersey</td>
<td>41</td>
<td>0.11</td>
</tr>
<tr>
<td>Cocal</td>
<td>40</td>
<td>0.10</td>
</tr>
</tbody>
</table>

[^b]: Virus harvested from 100 mm plate containing 6 x 10^6 cells was diluted in NTE buffer and the absorbance at 260 nm and 280 nm was measured. The protein concentration was then calculated as described by Warburg and Christian (1941).

[^+]: The G/M ratio was determined from the area of the G and M peaks of the microdensitometric scans of the Coomassie blue stained gel of viral proteins of the three serotypes (Fig. 5.7).
Table 5.2

Infectivity of $[^3]$H palmitate-labeled virions

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Infectivity pfu/ml of supernatant</th>
<th>Virus particles $[^3]$H palmitate cpm/ml of supernatant</th>
<th>Specific infectivity pfu/10^6 cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indiana</td>
<td>$1 \times 10^9$</td>
<td>$1.6 \times 10^6$</td>
<td>$6 \times 10^8$</td>
</tr>
<tr>
<td>Cocal</td>
<td>$7 \times 10^8$</td>
<td>$1.0 \times 10^6$</td>
<td>$7 \times 10^8$</td>
</tr>
<tr>
<td>New Jersey</td>
<td>$4 \times 10^8$</td>
<td>$1.0 \times 10^6$</td>
<td>$4 \times 10^8$</td>
</tr>
</tbody>
</table>

The infectivity of the $[^3]$H palmitate-labeled virus present in the supernatant fraction was assayed as described (Tonoguzo and Ghosh, 1977). The amount of $[^3]$H palmitate-labeled virus particles present in the supernatant fluid was determined by purifying the virus by layering an aliquot on a 10-40% sucrose density gradient in 0.1% BSA, 150 mM NaCl, 10 mM Tris-HCl, pH 7.6 and 1 mM EDTA and centrifuging at 85,000 × g for 1 h at 4°C. The radioactivity in the virus peak was determined.
production of VSV particles by 80-90% (Schlesinger and Malfer, 1982). The nonacylated G protein, however, was transported to the cell surface. It was therefore, inferred that the fatty acid acylation plays an important role in the virus assembly and budding. Since the Cocal G protein did not contain palmitic acid, the effect of cerulenin on the replication of Cocal virus was tested. At the concentration of 40 μg/ml at which almost complete inhibition of fatty acylation of VSV Indiana G occurred there was on an average 53% inhibition of protein synthesis (Table 5.3). After correction for the inhibition of protein synthesis by cerulenin it was found that the production of virus particles (Figure 5.8) was inhibited by 85% and the infectivity was inhibited by 96% in the presence of cerulenin (Table 5.4). This was in agreement with the earlier report stating that there was a 10-fold decrease in virus particles and in infectivity (Schlesinger and Malfer, 1982). In contrast, the production of radioactive Cocal virus particles and the infectivity of the particles was blocked only to the extent of 18% and 23% respectively (Table 5.4). Since blocking of fatty acid acylation by cerulenin had no significant effect on VSV Cocal replication, its G protein is not acylated with a fatty acid other than palmitic acid. The results also show that acylation of G protein with a fatty acid has no role in the maturation and budding of the Cocal virus particles.

The effect of cerulenin on the synthesis of viral proteins is shown in Figure 5.9. The Cocal virus particles synthesized in the absence and in the presence of the antibiotic show similar viral protein profile (lanes c, d). In the case of Indiana serotype, however
Table 5.3

Effect of cerulenin on protein synthesis in infected cells

Cells infected with VSV serotypes were labeled in the presence and in the absence of cerulenin (40 μg/ml) with [35S]methionine. The cells from the monolayers were harvested at the same time as the virus particles were harvested from the medium, suspended in PBS and an aliquot was removed for determining the TCA-precipitable counts. The counts presented as cpm/5 μl of the cell suspension are shown below.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Cerulenin</th>
<th>Percent Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indiana</td>
<td>5.71 x 10^5</td>
<td>2.73 x 10^5</td>
</tr>
<tr>
<td>Cocal</td>
<td>3.66 x 10^5</td>
<td>1.77 x 10^5</td>
</tr>
</tbody>
</table>
Fig. 5.8 Effects of cerulenin on cells infected with VSV Indiana and Cocal. The antibiotic was added 3 h post-infection for 1 h at a concentration of 40 μg/ml. The media from cells labeled 1 h with \([^{35}S]\)methionine was examined by isopycnic (25-40%) sucrose gradient centrifugation in a SW27 rotor at 25,000 rpm for 4 h. The radioactivity was plotted against fraction number (---), depicts the normal infected cells and (--*) depicts the cells treated with cerulenin. The integration of the peak fraction is shown in Table 5.4.
Fig. 5.9 - Autoradiogram showing the effects of cerulenin (40 μg/ml) on virus production and on fatty acid attachment to the glycoprotein. Virus infected cells were treated with cerulenin and labeled with
$[^{35}S]$methionine or $[^3H]$palmitic acid as described in the legend of Table 5.4. The released virus was harvested 8 h post-infection, purified as described earlier (Capone et al., 1982), disrupted in SDS-containing sample buffer and the proteins were separated on a SDS-10% polyacrylamide gel. Lanes a and c, $[^{35}S]$methionine-labeled Indiana and Cocal virus, respectively; lanes b and d, $[^{35}S]$methionine-
labeled Indiana and Cocal viruses, respectively, obtained after cerulenin treatment; lanes e and f, $[^3H]$palmitate-labeled Indiana
virus obtained in the absence and in the presence of cerulenin,
respectively; lanes g and h, $[^3H]$palmitate-labeled cell extracts from
the VSV Indiana infected cells untreated and treated with cerulenin,
respectively.
Table 5.4

Effect of cerulenin treatment on production of virus particles

<table>
<thead>
<tr>
<th>Percent reduction after cerulenin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indiana</td>
</tr>
<tr>
<td>Labeled virus particles</td>
</tr>
<tr>
<td>Infectious particles</td>
</tr>
</tbody>
</table>

Confluent chick embryo fibroblasts in 100 mm plates were infected with Indiana or Cocal viruses. At 3 h post-infection, cerulenin was added to 40 μg/ml for 1 h and the cells were subsequently labeled in the presence of 20 Ci of $^{35}$Smethionine/ml for 4 h. The virus released 8 h post-infection was assayed for the $^{35}$Smethionine incorporated and for plaque forming activity. The assay for the $^{35}$Smethionine labeled virus was carried out as follows. An aliquot of the virus containing medium was layered on a 25-40% sucrose density gradient in 0.1% BSA, 150 mM NaCl, 10 mM Tris-HCl, pH 7.6 and 1 mM EDTA. The radioactivity in the peak of labeled virus obtained after centrifuging at 85,000×g for 4 h at 4°C was determined.
the inhibition of fatty acid acylation in the presence of cerulenin was clearly observed (lanes f and h) and so was the marked reduction in virus particles after cerulenin treatment (lanes b) as compared to the untreated (lane a).
Discussion

The purpose of the studies reported in this thesis has been to obtain a better understanding at the molecular level of the domains required in the biosynthesis of functional glycoproteins. Specific domains take the form of topogenic sequences (Blobel, 1980), glycosylation sites (Hubbard and Ivatt, 1981), fatty acylation sites (Magee and Schlesinger, 1982) or as oligosaccharide chains and fatty acid residues. Since glycoprotein molecules of enveloped viruses mimic the biosynthesis of the cellular proteins and are easy to study, they serve as models for the study of the biogenesis of plasma membrane proteins in general. A large body of knowledge has become available during the course of this study and an attempt is made in the discussion to relate these findings to contemporary knowledge in this field.

**NH₂-terminal Sequences of VSV Glycoproteins**

The results presented in Section I are discussed here in two parts. The first part deals with the NH₂-terminal sequences of the in vitro synthesized unglycosylated forms of the glycoproteins of the VSV serotypes and the second part deals with NH₂-terminal sequences of the mature form of the glycoprotein.

**NH₂-terminal Sequence of the Unglycosylated Form of G Synthesized In Vitro**

The amino acid sequence of the NH₂-terminus of the primary translation product of the G protein from two serotypes of VSV, New Jersey and Cocal has been determined by microsequencing of radiolabeled proteins. The amino acid sequence for the signal sequence of the
unglycosylated in vitro synthesized G proteins are presented in Figure 6.1, together with the amino acid sequence of the signal peptide of the protein of VSV Indiana (San Juan) serotype (Rose and Gallione, 1981) and VSV New Jersey Ogden strain (Gallione and Rose, 1983) as determined from the nucleotide sequences of the cDNA of G mRNA. The signal sequences of the two Indiana strains and the two New Jersey strains (Figure 6.1) are 16 amino acid residues long. The signal sequences of the two strains of each of the serotypes are identical except for differences in a few amino acids. In contrast, the signal sequences between the three serotypes (Indiana, New Jersey and Cocal) show a number of differences, for example, the length of the signal sequence for the Cocal serotype is 17 amino acids. Incidentally, the Cocal G has the largest size amongst the G proteins of the three serotypes. However, there has been no correlation found between the size of the protein and its signal sequence (Austen, 1978; Garnier et al., 1980); it would thus be unwise to attach any significance to the longer chain length of Cocal G protein. Overall, amongst the VSV serotypes, the general nature of the signal sequence (Austen, 1978; Garnier et al., 1980; and Kreil, 1981) is maintained.

The general nature of the signal sequence has become clear from signal sequence comparisons of a large number of secretory and membrane proteins (Figure 6.2) (Von Heijne, 1983). Genetic experiments have shown that the mutations affecting specific residues within the signal sequence of proteins destined for the periplasmic space of E. coli (which can be regarded as equivalent to secretory proteins as well as plasma membrane proteins) prevent passage of the polypeptide across the
Fig. 6.1

SIGNAL SEQUENCES OF VIRAL GLYCOPROTEINS

a. INDIANA SAN JUAN

b. INDIANA TORONTO
   MET-LYS-CYS-LEU-LEU-TYR-LEU - PHE-LEU-PHE-PHE- LEU-PRO-LYS

c. COCAL
   MET-LYS-PHE-LEU-LEU-LEU-LEU-PHE-ILE-LEU-PRO-LYS

d. NEW JERSEY CONCAN
   MET-LEU - PRO-LEU-PHE-LEU - LEU - VAL - PRO-LEU - LYS

e. NEW JERSEY OGDEN

* Lingappa et al. (1978) reported H's for this position.
Fig. 6-2 Collection of eucaryotic signal sequences
The sequences have been aligned from their known cleavage sites
(between positions -1 and +1). Amino acids are symbolised by one
letter code. Proteins are as follows: 1: whey phosphoprotein, rat;
2: α-1 acid glycoprotein, rat; 3: α-thyrotropin, mouse; 4: insulin,
hagfish; 5: insulin, anglerfish; 6: insulin, human; 7: insulin I, rat;
8: insulin II, rat; 9: β-casein, ovine; 10: χ-casein, ovine; 11: α-lact-
albumin, ovine; 12: β-lactoglobulin, ovine; 13: α-s1 casein, ovine;
14: α-s2 casein, ovine; 15: glycoprotein, YSV; 16: VLDL-II, cockerel;
17: melittin, bee; 18: lactin, rat; 19: placental lactogen, human;
20: β-choriogonadotropin, human; 21: α-choriogonadotropin, human;
22: uteroglobin, rabbit; 23: growth hormone, rat; 24: growth hormone,
human; 25: growth hormone, bovine; 26: parathyroid hormone, bovine;
30: liver albumin, rat; 31: tropoelastin B, chicken; 32: ovomucoid,
chicken; 33: lysozyme, chicken; 34: conalbumin, chicken; 35: α-1 anti-
trypsin, human; 36: prostatic binding protein, rat; 37: prostatic
binding protein c2, rat; 38: glycoprotein, AD virus; 39: apolipoprotein
Al, rat; 40: glycoprotein, rabies virus; 41: hemagglutinin, human influenza
Victoria; 42: hemagglutinin, human influenza Japan; 43: hemagglutinin,
avian influenza FPV; 44-50: leukocyte interferon, human; 51: immune
interferon, human; 52: fibroblast interferon, human; 53-56: λ-immuno-
globulin, mouse; 57-59: λ-immunoglobulin, mouse; 60, 61: γ-immuno-
globulin, mouse; 62: H-chain immunoglobulin, mouse; 63-66: embryonic
VH-immunoglobulin, mouse; 67: H-chain immunoglobulin, mouse;
68: trypsinogen 1, canine; 69: trypsinogen 2, canine; 70: chymo-
trypsinogen 2, canine; 71: carboxypeptidase A, canine; 72: amylase,
canine; 73, 74: amylase, mouse; 75: amylase, rat; 76: α-lactalbumin,
rabbit; 77: α-lactalbumin, porcine; 78: carboxypeptidase A, rat.
membrane (Bassford and Beckwith, 1979). DNA sequence analysis of
mutant genes of the lambda receptor protein (Emr et al., 1980) which
do not reach the outer membrane but remain within the cytoplasm, and
other studies of polypeptides of the maltose binding protein (MBP) of
E. coli (Bedouelle et al., 1980) which cannot be transferred to the
periplasmic space, have shown that all the mutations involve either
substitutions in the signal sequence from a single hydrophobic or
uncharged amino acid to a charged one, or small deletions within the
hydrophobic portion of the signals. It was also found that a change
from one hydrophobic amino acid to another did not drastically affect
the transport of the MBP to the periplasmic space (Bedouelle et al.,
1980). Thus, one of the general properties of the signal sequence can
be said to be a presence of a high proportion of hydrophobic amino
acids. The next question is; how are these hydrophobic amino acids
distributed? A sample of 34 hydrophobic core segments from known
signal sequences have been analyzed with regard to the mean
hydrophobicity of each position in the segment (Von Heijne, 1982). The
results showed that these core segments are not uniformly hydrophobic;
instead the more hydrophobic residues are concentrated around the
midpoints of the segment. A similar analysis carried out on a sample
of 12 membrane spanning segments from the transmembrane proteins
indicate that the hydrophobic residues are much more evenly distributed
along the segments (Von Heijne, 1982). An analysis of the signal
sequence of the G proteins of VSV serotypes suggests that a hydrophobic
amino acid could be present in any position except the amino acid
position just prior to the signal sequence cleavage site, but the
extent of hydrophobicity is maximum in the central region:

Indiana  H---HHHH-HHHHHH---

Cocal  H---HHHHHHHHHH---

New Jersey  HHHHHHHHHHHH-

H strands for hydrophobic amino acid i.e. amino acid with hydrophobicity index (HI) > 1

Another common pattern that has evolved from sequence analysis has been a charged amino acid (e.g., Lys) in close proximity to the NH$_2$-terminal methionine. This charged residue has not been found in the case of VSV New Jersey Concan and Odgen strains and is also not found in the acetylcholine receptor subunit, influenza hemagglutinin and Herpes gD signal sequences and it is quite doubtful whether it has any significance in recognition by the SRP. A mutation replacing the lysine at the NH$_2$-terminus of the signal sequence of E. coli MBP by a hydrophobic amino acid resulted in a block in the export of this protein into the periplasmic space (Bedouelle et al., 1980). It is quite possible that the recognition mechanism of the signal sequence is slightly different in procaryotes and eucaryotes with regard to this requirement for a basic amino acid at the NH$_2$-terminus.

Yet another feature has been the presence of proline, an amino acid which is rarely found in inner helical or sheet structures and which occurs within 6 residues of the cleavage site of several signal sequences analyzed (Austen, 1978; Von Heijne, 1983). In many cases $\beta$-turns are predicted close to the cleavage site, bringing about efficient exposure of the cleavage site to the signal peptidase. A comparison of the signal sequence of VSV serotypes does show the
presence of proline in the first 6 residues from the cleavage site in both the New Jersey strains, in Cocal and in Indiana Toronto strain but is surprisingly absent in the case of the San Juan strain of Indiana. Indiana San Juan has another secondary structure disrupting residue viz glycine. Thus the disruption of secondary structure in this region is of vital importance and it is achieved either by proline or glycine. Sequence homologies are conspicuously absent from all the signal sequences looked at so far. Even the VSV serotypes which show a considerable degree of homology within the mature glycoprotein region fail to show a consensus sequence even within the central hydrophobic core. Thus one can say that the overall hydrophobicity of the signal sequence appears to be the only factor affecting its functional effectiveness. This is very clearly demonstrated by the signal sequences of G of VSV serotypes which show an almost identical HI despite the lack of appreciable homology in this region.

Predictions of secondary structure made by locating 6 or 5 residue nucleation sites, and 4 residue boundaries (Chou and Fasman, 1974) with the use of conformation parameters (Chou and Fasman, 1978) have revealed that the central portions of all the signal sequences analyzed were strongly predicted to be involved in repeating structures, either α-helix or β-sheets of 6–12 residues in length (Austen, 1978). Thus the amino terminus of VSV G has been predicted to be involved in a β-structure followed by α-helix, and then by a lack of any repeating structure, with the region lacking repeating structure falling in the signal sequence cleavage region. The secondary structure of the signal sequences of other serotypes would be essentially similar.
A signal sequence would be expected to contain two different and largely independent 'signals'. One of which forms a hydrophobic core described above, presumably responsible for initiating transfer of protein by binding to the signal recognition particle (Walter et al., 1981) and a second one conferring cleavage specificity. It has long been known that some part of the cleavage specificity resides in the last residue of the signal sequence, which invariably is one with a small, uncharged side chain e.g., Ala, Gly, Ser, Cys (Austen, 1978; Garnier et al., 1980). This led to the suggestion that the active site of the proteinase (signal peptidase) cannot accommodate bulky side chains (Austen, 1978). A recent study attempting to predict cleavage sites found some additional patterns regarding the signal sequence protease complex. These observations are based on a sample of 78 eucaryotic signal sequences (Fig. 6.2) and it has been suggested that positions −1 and −3 seem to be strongly selected for small, neutral residues, whereas positions +1, −2 and −4 seem to accommodate almost any residue. No proline was found from position −3 to +1 and further this region is often separated from the hydrophobic core of the signal sequence by the strong helix-breakers Pro or Gly (Von Heijne, 1983).

An inspection of the signal sequences of two serotypes of VSV Cocal and New Jersey reveals the presence of a neutral small sized amino acid in the −1 position a valine in −3 position of VSV Indiana San Juan and Indiana Toronto, with an isoleucine in position −3 of New Jersey Odgen. This fulfills the proposed rule referred to as the (−3, −1) rule for acceptable cleavage site, i.e., it must have either Ala, Ser, Gly, Cys, Thr or Gln in position −1 and must not have an aromatic
(Phe, His, Tyr, Trp) charged (Asp, Glu, Lys, Arg)) or large polar (Asn, Gln) residue in position -3 (Perlman and Halverson, 1983; Von Heijne, 1984).

NH₂-terminal Sequence of the Mature VSV Glycoprotein

In this work, the amino acid sequence of the NH₂-terminus of the intracellular G protein of VSV New Jersey and Cocal was determined from the radiolabeled proteins. A comparison of these amino acid sequences with that of VSV Indiana showed considerable homology (Table 3.2). Further, comparison of the NH₂-terminal sequence of the mature glycoproteins isolated from the virion Figure 6.3 shows that the sequences for the Indiana, Cocal and New Jersey serotypes are highly conserved for the first 24 residues examined. A conserved consensus sequence for the NH₂-terminal domain of VSV glycoprotein can thus be deduced. This consensus sequence has a stretch of hydrophobic amino acids, and lies within a span of 11 amino acids with lysine residues at the ends, a region that could associate with membranes. The few differences that are evident can be accounted for by single base changes in most cases, when the San Juan strain is used as a basis for comparison. Hence, the Indiana San Juan strain may have evolved earlier than the Indiana Toronto, Cocal and New Jersey Concanc and Odgen strains. The optimum alignment of the protein sequences predicted from mRNA sequences of VSV Ind and VSV NJ showed that the identity of the amino acid sequence of the complete glycoprotein is 59% (Gallione and Rose, 1983). Thus, the considerable homology observed within the first 10 amino residues of the mature G of the three serotypes, is in contrast to both the overall homology of 59% and the 18% homology found
FIGURE 6.3  AMINO TERMINAL SEQUENCES OF MATURE GLYCOPROTEINS FROM DIFFERENT SEROTYPES OF VSV

\[
\begin{array}{c}
\text{a) INDIANA SAN JUAN} & \text{LYS PHE THR ILE VAL PHE PRO HIS ASN GLN LYS GLY ASN TRP LYS ASN VAL PRO SER ASN TYR HIS TYR CYS} \\
\text{b) INDIANA TORONTO} & \text{LYS PHE THR ILE VAL PHE PRO TYR ASN GLN LYS GLY ASN TRP LYS ASN VAL PRO SER ASN TYR HIS TYR CYS} \\
\text{c) COGAL} & \text{LYS PHE SER ILE VAL PHE PRO GLU SER GLN LYS GLY ASX TRP LYS ASN VAL PRO SER TYR TYR TYR} \\
\text{d) NEW JERSEY CONCAN} & \text{LYS ILE GLU ILE VAL PHE PRO GLN HIS - LYS GLY - TRP LYS - VAL} \\
\text{e) NEW JERSEY OGDEN} & \text{LYS ILE GLU ILE VAL PHE PRO GLN HIS THR THR GLY ASP TRP LYS ARG VAL PRO HIS GLU TYR ASN TYR CYS} \\
\text{CONSENSUS SEQUENCE} & \text{LYS - ILE VAL PHE PRO - - - GLY ASN TRP LYS - VAL PRO - - TYR - TYR CYS}
\end{array}
\]

*This figure was taken from Kotwal et. al. (1983).*

a) determined from cDNA of cloned gene (Rose and Gallione, 1981).
b) determined by amino acid sequence (Irving et. al., 1979).
c) and d) determined by amino acid sequence (Kotwal et. al., 1983).
e) determined from cDNA of cloned gene (Gallione and Rose, 1983).
in the signal sequence and further emphasizes that there is a definite role for this highly conserved NH$_2$-terminal domain of the glycoprotein. A comparison of several of the mature NH$_2$-terminal regions after signal sequence cleavage, of secretory and membrane proteins (Von Heijne, 1983) does not reveal any definite pattern for this region, suggesting that this region probably has no role in recognition by the signal peptidase enzyme. There is, however, evidence which suggests that despite a yeast glycoprotein having a similar signal sequence to a secretory protein, the signal sequence is not removed as it is in the secretory protein. The probable reason in this case is that the sequences beyond the signal sequence cleavage site is different (Julius et al., 1984). Nevertheless, the balance of evidence suggests that it is only the region in the close vicinity -3 to +3 of the cleavage site that is important for recognition by the signal peptidase (Von Heijne, 1983). This region may also play a role in its association with the lipid bilayer. Although, there is no homology between the NH$_2$-terminal regions of membrane glycoproteins from different viruses, there is conservation within the virus strain or subtypes; as observed in the case of VSV the homology in this region is in sharp contrast to the non homology in the surrounding region as revealed by weak immunological cross reactivity and sequence comparisons. It has been observed that the NH$_2$-terminal sequence of the membrane glycoproteins of HA$_2$ of different strains as well as different groups of influenza virus is highly conserved (Skehel and Waterfield, 1975; Verhoeven et al., 1980; Air et al., 1981; Winter et al., 1981). The three-dimensional structure of the haemagglutinin (HA) membrane
glycoprotein of influenza virus has been determined at a 3 Å resolution (Wilson et al., 1981), and this structure reveals that the NH₂-terminus of the HA protein is in close proximity to the external surface of the membrane. It was further found that the orientation of the protein within the viral envelope is the same as it is within the cellular ER membrane during the biosynthesis of the glycoprotein. However, in this case, the exception is that the NH₂-terminus faces the lumen of the ER and COOH-terminus is facing the cytoplasm, whereas in the viral envelope the polarity is reversed, i.e. the NH₂-terminus faces the exterior and the COOH-terminus is within. Thus, one can use the findings obtained for the orientation of the protein in the viral envelope and in this case extrapolate them to the ER membrane. The NH₂-terminus of VSV G protein was shown to be protected from aminopeptidase digestion by the viral membrane (Irving et al., 1979; Capone et al., 1982). Although it has basic amino acids and a stretch of hydrophobic amino acids it is unlikely to span the membrane, so that the protection from digestion by aminopeptidase could be a result of the interaction of basic residues with the phosphate residue in the lipid bilayer and the hydrophobic stretch with a small region inside the bilayer.

This conserved hydrophobic sequence is also present at the NH₂-terminus of the fusion glycoprotein F₁ of Sendai virus (Gething et al., 1978) and is suggested to participate in fusion of viral and host cell membranes. The biological role or function of the conserved NH₂-terminal domain of the VSV G proteins which is in close association with the membrane has been recently investigated. Based upon the
observation that the NH₂-terminus of G protein is highly conserved in many strains of VSV (Roe and Callioni, 1981; Kotwal et al., 1983) a synthetic peptide corresponding to the NH₂-terminus of VSV glycoprotein was synthesized. The synthetic peptide has been shown to possess hemolytic activity and may reflect analogous function of the in situ G protein to the NH₂-terminus of G protein (Schlegel and Wade, 1984). It is also possible that the interaction of this region in VSV G and in influenza HA during the cotranslational transport across the ER with the inner side of the membrane serves to orient the protein in such a way as to expose its glycosylation sites to the glycosylating enzymes preventing its premature folding into a 'compact' structure before being glycosylated. The orientation seen in the mature virus is a retention of the orientation in the ER acquired during its biosynthesis and the membrane associating regions of the protein contribute to the final orientation.

Phenotypic Characterization of a Temperature Sensitive Mutant

Temperature sensitive mutants are a type of conditional lethal mutants. The term "conditional lethal" was suggested by Hadorn to describe mutants whose growth could be prevented by altering an environmental parameter (Hadorn, 1951). In mammalian viral genetics, the bulk of the "conditional lethal" mutants are of the temperature sensitive (ts) variety. In the case of VSV ts mutants isolated by Pringle, the permissive temperature is 32°C whereas the nonpermissive or restrictive temperature is 39°C (Pringle, 1970). Temperature sensitivity is the result of an amino acid
substitution which causes a change in the conformation of the polypeptide and this renders it inactive at the restrictive temperature.

Complementation analysis examines the infection of cells with two "conditional lethal" mutants under nonpermissive conditions. The cooperation of two mutants to produce an increase in progeny yield of both genotypes is expected if the genetic lesion resides in different polypeptides. Mutants, which do not produce an increase in progeny yield when coinfeated at the nonpermissive temperature are said to belong to the same "complementation group". Temperature sensitive (ts) mutants of Indiana serotype of VSV defective in the structural gene of G correspond to the complementation group (V). They have been isolated on the basis of their ability to grow at the two temperatures of 32°C (permissive) and 39°C (nonpermissive) (Lafay, 1974; Lodish and Weiss, 1979; Pringle, 1982). Studies on VSV Indiana mutants which belong to the group (V) have demonstrated the presence of two subclasses. At the nonpermissive temperature mutants such as ts045, tsM501 and tsL513 synthesize G protein blocked in the transport from rough ER to the Golgi complex (also described as the preGolgi stage). The defective G protein is glycosylated at both sites with high mannose core oligosaccharide which is endo H sensitive (Knipe et al., 1977; Lodish and Weiss, 1979; Zilberstein et al., 1980). Processing of the core oligosaccharide (Robbins et al., 1977; Tabas and Kornfeld, 1979) addition of terminal sugar residues (Bretz et al., 1980), and covalent linkage of fatty acids (Schmidt and Schlesinger, 1980) occurring in the Golgi complex is also blocked at the nonpermissive temperature. The
the only ts mutant which belongs to the other class of G mutants, after reaching the Golgi from the ER does not proceed to the cell surface. The glycoprotein is fully processed including the addition of terminal sugars except fucose. The attachment of fatty acid is, however, blocked and only a part of the nonacylated glycoprotein moves to the cell surface (Zilberstein et al., 1980; Lodish and Kong, 1983).

The mutant tsγ1 described in this thesis is distinct from both of these classes. Earlier studies on this mutant have suggested that this mutant is defective in glycosylation at both the temperatures (Buller M., unpublished observation). Studies described here clearly demonstrate that at the permissive temperature (like the two classes of G mutants described above) tsγ1 is glycosylated at only one of the two sites in the wild type. At the permissive temperature it has a complete oligosaccharide chain as in the wild type G protein and pulse labeled tsγ1 G synthesized at 32°C becomes Endo H resistant after a chase period of about 30 minutes. The susceptibility to neuraminidase (Buller M., unpublished observation) and the incorporation of galactose reported in this thesis further shows that the terminal sugars are added normally and the mutant glycoprotein is transported via the Golgi apparatus.

At the nonpermissive temperature the production of infectious particles is drastically reduced to about 0.001%. As in the case of the other G mutants (Lodish and Weiss, 1979), noninfectious particles lacking only the G protein comprise about 35% of the particles obtained at the permissive temperature, thus confirming that assembly and
budding of the virus can occur in the absence of the G protein. In contrast to the other mutant glycoproteins, ts \( \gamma \) G protein synthesized at 39\(^\circ\)C is completely unglycosylated. The majority of the G protein is localized in the rough ER. This is supported by NH\(_2\)-terminal sequence determination showing that the signal peptide is removed from the unglycosylated protein as a consequence of being inserted into the rough ER. This is in agreement with an earlier observation that the inhibition of glycosylation does not prevent the glycoprotein from being inserted into rough ER or from its signal sequence getting cleaved (Irving and Ghosh, 1982). The unglycosylated G does not reach the Golgi or the plasma membrane in appreciable amounts (\(<10\%\)), thus suggesting that the glycosylation is essential for efficient transport of the protein from the ER to Golgi and finally to the plasma membrane.

The synthesis of the G polypeptide in Cocal and ts \( \gamma \) infected cells at 39\(^\circ\)C was not observed to affect either the subsequent glycosylation, or incorporation into virions on downshift to 32\(^\circ\)C. Furthermore, G polypeptide synthesized and glycosylated at 32\(^\circ\)C was assembled into infectious virus at 39\(^\circ\)C (M. Buller, unpublished observation). This suggests that conformational constraints at 39\(^\circ\)C are responsible for the lack of glycosylation. In other words, the conformation of the protein at 39\(^\circ\)C is such that the glycosylation site to which oligosaccharide chain is attached at 32\(^\circ\)C is inaccessible to the glycosylating enzyme which transfers the preassembled high mannose oligosaccharide chain to the asparagine residue in the glycosylation site of the protein. This also suggests that the amino acid substitution which is responsible for the conformational change at 39\(^\circ\)C
is in the vicinity of the consensus glycosylation site, but not in it. This is further supported by the finding that the elution profiles of the tryptic glycopeptides from the G proteins of Cocal and tsγ1 synthesized at the permissive temperature showed that retention times on the HPLC column were different. The single glycopeptide peak of tsγ1 did not coincide with either of the two peaks of Cocal. This shift can be attributed to an amino acid change within the tryptic glycopeptide and since the glycosylation at 32°C within this peptide is normal it is probable that the change in the amino acid composition also results in change in conformation of the protein at 39°C. One obvious question then is whether there is a double mutation or a single one in the glycoprotein gene? There can be no definite answer unless a complete nucleotide sequence of the G gene of both the wild type and the mutant is determined. But one could speculate that the results can explain two possibilities. One is that the lesion, most likely a single base change causes an amino acid substitution in the vicinity of one of the glycosylation site which brings about a conformational change at 39°C and prevents glycosylation at that site and that there is another lesion which affects the second glycosylation site at both temperatures. The other possibility is that there is only one lesion most likely close but not within the first glycosylation site (since glycosylation is a cotranslational event the site to appear first in a B-turn would be the first to be glycosylated) that affects the exposure of the second site at 32°C and at 39°C affects the conformation in such a way as to bury the glycosylation sites within the polypeptide backbone making it inaccessible to glycosylating enzymes.
Role of Oligosaccharide Chains in Transport

Oligosaccharide moieties are ubiquitous components of surface membrane proteins, secreted proteins and viral envelope proteins. The precise function of the oligosaccharide has been a subject of speculation and research for a number of years. It has been suggested that they may serve to direct the migration of the glycoprotein to its ultimate location in the cell (Eylar, 1965). Therefore, a failure to glycosylate a membrane or secreted protein would result in the inability of the glycoprotein to reach the cell surface or be secreted from the cell. Studies on immunoglobulin secretion have supported this theory (Hickman et al., 1977). Other studies have demonstrated that the failure to glycosylate the major cell surface protein of CEF did not alter the ability of the protein to reach the cell surface. However, the intracellular degradation of the major cell surface protein increased 2 to 3 fold in the absence of glycosylation. Thus, in this case carbohydrate stabilizes this protein against proteolytic degradation.

Studies dealing with the role of carbohydrate in the life cycle of enveloped RNA viruses have also produced variable results. Leavitt et al. (1977) have demonstrated that a failure to glycosylate the viral glycoproteins of both Sindbis virus and the San Juan strain of VSV results in the inability of the glycoproteins to migrate to the cell surface. In addition, in the absence of glycosylation the release of virus particles is inhibited by 99%. A study of the localization of the glycoprotein in tunicamycin treated VSV infected cells, revealed that the glycoprotein attached to the ER membranes normally but did not
move to low density membranes forming the Golgi complex (Morrison et al., 1978).

In contrast to the above results there is a report suggesting that the absence of carbohydrate does not prevent the efficient insertion of the influenza glycoproteins into the plasma membrane (Nakamura and Compans, 1978). Thus, carbohydrate may be required for transport of some viral glycoproteins to the cell surface but not for others.

This observation has been supported by studies on the requirement for carbohydrate in morphogenesis of two different strains of VSV of the Indiana serotype the San Juan and the prototype strain (Orsay) (Gibson et al., 1979). At 30°C, in the presence of glycosylation inhibitor tunicamycin, cells infected with the San Juan strain release only 5 to 10% of the normal yield of virus particles, whereas the prototype strain releases between 45 and 70%. This marked difference in glycosylation requirement suggests that, carbohydrate is not critical for efficient morphogenesis of the prototype strain of VSV at 30°C, whereas it is essential for a similar function in the San Juan strain. Furthermore, whenever unglycosylated G does move to the surface, it is incorporated normally into virus particles, and these have normal specific infectivity (Gibson et al., 1978). At 39.5°C, prototype infected cells treated with tunicamycin release only 4% of the normal yield of virus (Gibson et al., 1979).

The glycoproteins of the two strains are quite different. The peptide patterns of the San Juan G protein and the prototype G protein were different when compared by partial peptide analysis (Burge and
Huang, 1979). This difference in primary sequence could be a cause of the difference in glycosylation requirement for the maturation to the cell surface. There are also minor differences in the NS protein peptide maps in these strains and the RNA fingerprints of the different isolates of the Indiana serotype differ in their oligonucleotide pattern by 50-75% for the G gene, 20% for the NS gene and 15% for the L gene (Freeman et al., 1979). The observations raise concerns about whether the difference in carbohydrate requirement between the two strains are a result of differences in G proteins or to some other undefined differences between the two strains.

A recent study reveals that mutational changes within the G protein of the same strain of virus (prototype or Orsay) alters the requirement for carbohydrate at 30°C. Group(V) or G protein mutants of VSV Indiana ts045 and ts044 like their prototype parent did not require carbohydrate for efficient morphogenesis; this was shown by the study of the virus released in the presence of tunicamycin (Chatis and Morrison, 1981). In contrast, the G protein of another group(V) mutant, ts0110 was totally dependent upon carbohydrate addition for migration to the cell surface. Furthermore, no ts0110 particles were released in the absence of glycosylation. The wild type prototype strain and the two mutants derived from the strain ts045 and ts044 did require carbohydrate at 39°C for insertion of the G protein into the plasma membrane and virion formation as revealed by tunicamycin inhibition of glycosylation (Chatis and Morrison, 1981).

The studies on tsyl reported in this thesis have revealed that at the nonpermissive temperature the G protein is not glycosylated and
its transport to the plasma membrane is affected. The G protein at 39°C in this case remains inserted in the ER membrane. Inhibition of glycosylation with tunicamycin in cells infected with wild type VSV Cocal at 39°C revealed a pattern similar to the mutant at 39°C.

The presence of one oligosaccharide chain in the mutant tsY1 in comparison to two in the case of wild type VSV Cocal at the permissive temperature (32°C) suggests that the transport from the ER to the Golgi and finally to the plasma membrane is as efficient with one chain as it is with two chains.

This taken together with the studies of the other mutants provide strong evidence that the amino acid sequence of the G protein determines the importance of carbohydrate for insertion of the G protein into plasma membranes. Mutants isolated from the same strain of VSV and which fall into the same complementation group have different requirements for carbohydrate addition for insertion of the G protein into the plasma membrane. Furthermore, the study on tsY1 and other mutants reinstates an earlier suggestion by Gibson et al. (1979) that the conformation of the polypeptide is of ultimate importance in efficient migration through the cell. Molecules of some strains/mutants may obtain this conformation in the absence of glycosylation whereas others may require at least a partial glycosylation and still others may require complete glycosylation in order to assume the proper three-dimensional structure.

**Fatty Acid Acylation of Proteins**

The first report for possible covalent attachment of lipid to proteins in higher organisms was the analysis of an organic solvent
extractable protein from brain myelin (Folch-Pi and Lees, 1951; Sherman and Folch-Pi, 1971). The discovery of a lipoprotein in the E. coli cell wall was the first example of their presence in procaryotic organisms (Braun and Rehn, 1969). A structural study of the lipoprotein showed that fatty acids were bound in both ester and amide linkages to the protein (Hantke and Braun, 1973). The evidence for the covalent binding of fatty acids to membrane associated proteins of enveloped viruses came from the studies of two glycoproteins, E1 and E2 of Sindbis Virus (Schmidt et al., 1979; Schlesinger, 1981). The first demonstration that the VSV G protein contains tightly bound fatty acid residues was based on the observation that labeling VSV with [3H]palmitate resulted in incorporation of the label not only into the viral phospholipids but also into G proteins (Schmidt and Schlesinger, 1979). The [3H]palmitate bound to the G protein was retained even after denaturation, extraction with organic solvents, and SDS polyacrylamide gel electrophoresis. However, the label could be released by mild alkaline treatment (methanol/NaOH) and recovered as the palmitate methyl ester. This led to the suggestion that the fatty acid molecules were covalently bound to the polypeptide backbone of the G protein by ester linkage. Using this approach it has been shown that proteins of a wide range of organisms have lipid moieties covalently bound to them (Table 6.2).

The examination of the glycoproteins of various vesiculo viruses for the presence of fatty acid (palmitic acid) discussed in section III, showed that while the mature G protein of VSV Indiana serotype and Piry and Chandipura viruses contain palmitic acid
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<th>VIRUS</th>
<th>ACYLATED PROTEIN</th>
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<tr>
<td>VESICULAR STOMATITIS VIRUS</td>
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<tr>
<td>(Indiana Serotype)</td>
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<tr>
<td>Chandipura 2</td>
<td></td>
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<tr>
<td>FVY 2</td>
<td>G protein</td>
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<td>SINDbis VIRUS 3</td>
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<tr>
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<td>C protein</td>
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<tr>
<td>FOAL PLAGUE VIRUS 4</td>
<td>E1, E2</td>
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<tr>
<td>NEW CASTLE DISEASE VIRUS 4</td>
<td>HA (Hemagglutinin)</td>
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<tr>
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<td>E2 Glycoprotein</td>
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<td>HERPES SIMPLEX VIRUS 8</td>
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**II. PROCARYOTIC ORIGIN**

| BACILLUS SUBTILIS 9          | Penicillinase                         |
| E. COLI                     |                                       |
| PROTEUS CAMBRIUM 11         | Marin lipoprotein                     |

**III. MAMMALIAN ORIGIN**

| Brain myelin proteolipoprotein 2, 12 | 25-30 membrane proteins |
| Human transferrin receptor 13      |                         |
| Membrane-associated tissue culture cell proteins 14 | |
| Butyrophilin and Xanthine Oxidase of milk fat globule membrane 15 | |
| Bovine Rhodopsin 16                |                         |

**MEMBRANE PROTEINS NOT FOUND TO BE ACYLATED WITH FATTY ACID**

| VESICULAR STOMATITIS VIRUS 2, 17 | C protein |
| (NEW JERSEY AND CACH SEROTYPE)  | NA (Neuraminidase) |
| INFLUENZA VIRUS 4                | NA (Neuraminidase) |
| NEW CASTLE DISEASE VIRUS 4       | NA (Neuraminidase) |
| CORONA VIRUS 4                   | HA (Hemagglutinin) |
| HERPES SIMPLEX VIRUS 8           | E1 Glycoprotein |
| Human homologue of murine T200 glycoprotein 17 | GC and RAB |

**REFERENCES**

residues, the glycoproteins of Cocal and New Jersey serotypes do not contain palmitic acid, showing that fatty acylation may be a common but not general occurrence.

**Site of Fatty Acid Acylation**

The only direct evidence for the attachment of fatty acid to a precise site in a membrane protein is the fatty acid (myristic) acylation of the amino terminal glycine in the murine leukemia virus gag protein precursor (Henderson et al., 1983). Other lines of indirect evidence have led to the postulate that the fatty acids are linked covalently to the protein by simple ester bonds, probably through the side chains of hydroxyl group containing amino acids (Ser, Thr, Tyr). The fatty acid attached to the protein is labile to transesterification (methanol/KOH treatment) and hydroxylaminolysis, e.g. Sindbis E1, E2 and VSV G proteins (Schmidt et al., 1979; Schmidt and Schlesinger, 1979).

Transesterification and hydroxylaminolysis are reactions characteristic of ester linkages. A study of the kinetics of release of fatty acid bound to E2 and VSV G revealed that the release of esterified fatty acid is rapid ($t_{1/2} < 10$ min at $23^\circ\text{C}$) in these proteins in comparison to E1 (Magee and Schlesinger, 1982). A study of the lability of serine esters compared to threonine esters showed that the former are much more labile than the latter (Lenard, 1964).

Characterization of a thermolysin protected peptide of the VSV G protein containing the fatty acid residue attached to the protein by peptide mapping and sequencing have shown that the fatty acid is
present in the membrane spanning hydrophobic sequence of the VSV G protein (Capone et al., 1982). It has also been demonstrated that a few other viral membrane glycoproteins possess covalently attached fatty acid residues within their membrane spanning regions e.g. influenza haemagglutinin (Schmidt, 1982a), Sindbis E1 and E2 glycoproteins (Rice et al., 1982), transforming protein pp60src of RSV (Sefton et al., 1982) and the transferrin receptor from cultured cells (Omary and Troubridge, 1981).

Despite the efforts to find the actual linkage sites of the fatty acid residues there has been no investigation thus far that has definitely located the fatty acid containing amino acid. This has largely been due to the difficulties of working with these extremely hydrophobic peptides during sequence analysis (Capone et al., 1982; Magee and Schlesinger, 1982; Schmidt, 1982b).

There is a suggestion that in the case of VSV G protein, the fatty acid may be esterified directly to serine residues (Schmidt and Schlesinger, 1979; Magee and Schlesinger, 1982). This suggestion is based on the finding that the kinetics of release of fatty acid from G protein by hydroxylamine at neutral pH is similar to the lability of serine esters than to the the other possible esters of hydroxyl amino acids. It has also been shown that a fatty acid containing peptide recovered from a proteolytic digestion of [3H]palmitate-labeled G protein of VSV Indiana contained a high proportion of serine residues. Despite these findings, the esterification to the side chains of the other hydroxy amino acids threonine and tyrosine or to the SH-group of cysteine is quite likely.
The sequence of the cloned G gene of the Odgen strain of the New Jersey serotype (Rose and Gallione, 1983) has revealed an interesting possibility for the fatty acylation site. In agreement with the results reported in the section III it was observed that in the case of New Jersey odgen strain there is absence of palmitic acid in the G protein. A comparison of the COOH-terminal sequence of G protein of Indiana serotype (Rose and Gallione, 1981) and the New Jersey Odgen strain (Rose and Gallione, 1983) indicates that in positions 467 and 474 of Indiana G protein have serine residues whereas glycine residues are present in the same two positions in the New Jersey G protein. Also, in the case of New Jersey G protein there is a serine in position 497 whereas in the same position there is a cysteine in the case of VSV Indiana. Thus the lack of fatty acylation in the New Jersey G may be a direct result of the lack of any one or all of these esterification sites. Therefore both of these serines and/or cysteine could serve as acceptor sites for fatty acid attachment. Whether cysteine is the site for fatty acylation, or its presence is required for fatty acylation at other sites is yet to be established. But a recent report indicates that presence of cysteine in the cytoplasmic domain of VSV Indiana G is required for palmitate addition. In order to identify the probable site of acylation, oligonucleotide-directed mutagenesis was used to change the sequence of cDNA corresponding to the cytoplasmic domain of VSV G protein. The mutagenized cDNA clones were expressed in eucaryotic cells. It was found that when a single codon specifying cysteine in this domain was changed to a codon specifying serine expression of the mutant gene
resulted in the synthesis of a G protein lacking palmitate (Rose et al., 1984).

Role of Fatty Acid Acylation of Membrane Proteins

Fatty acid attachment to proteins is a post-translational modification in search of a function. To date there have been no definite role assigned to fatty acylation to the protein although there are indications that they play an important role.

A study of a temperature sensitive mutant of VSV Indiana serotype has shown that at the nonpermissive temperature the G protein fails to become acylated with fatty acid and did not reach the cell surface. Based on this observation a possible role of the fatty acid in the intracellular transport of the protein was suggested (Zilberstein et al., 1980). However, this mutant glycoprotein has another temperature sensitive defect viz. aberrant glycosylation which may also contribute to the observed effect. Also, further studies of this mutant have shown that some nonacylated G does move to the cell surface though not into virions (Lodish and Kong, 1983). The studies reported in this thesis have shown that of the three different serotypes of VSV tested, two did not contain any fatty acylated G protein, suggesting that nonacylated G protein can migrate to the cell surface.

Since fatty acids are linked to the membrane spanning regions of acylated glycoproteins, it has been proposed that the fatty acid may function to anchor the protein in the lipid bilayer of the virus envelope or the cell membrane (Schmidt et al., 1979; Omary and Trowbridge, 1981; Capone et al., 1982; Schmidt, 1982). This view is
supported by amino acid sequence data which reveals a high proportion of hydrophobic and hydroxy amino acid (mainly Serines) within the membrane associated portion of a number of glycoproteins. Fatty acid moieties in this region may function to anchor the protein by providing additional hydrophobic moieties. This suggestion has been reiterated by a recent report by Omary and Trowbridge (1981) which stated that the process of acylation and deacylation could modulate the affinity of a glycoprotein for the membrane. This would offer a means of regulating certain biological activities expressed on the cell surface. It has also been shown that the membrane form of immunoglobulin IgM when chemically acylated with fatty acid acquires the ability to bind to liposomes whereas the naturally occurring IgM does not (Huang et al., 1980). The transforming protein of RSV, which is initially synthesized as a cytoplasmic protein associates with plasma membranes after its amino terminus is fatty acylated (Sefton et al., 1982).

The 'anchor hypothesis' would be acceptable if only it could explain why many membrane associated proteins of viral and cellular origin do not become acylated during their biosynthesis (Omary and Trowbridge, 1981; Schmidt, 1982; Kotwal and Ghosh, 1984). One of the best characterized of the non acylated species is the μ-chain of the membrane form of IgM. This is known to be tightly bound to the plasma membrane and contains 10 hydroxyamino acids out of a total of 26 amino acids within the membrane spanning sequence and still does not contain fatty acid (Rogers et al., 1980; Schmidt, 1983).

The most attractive common feature of acylated glycoproteins of viral origin is their fusion activity. The best studied examples of
this property are F protein of fowl plaque and Sendai virus, the hemagglutinin (HA₂) of influenza viruses, the E1 and E2 of Sindbis virus and the E2-protein of Corona viruses (Scheid and Choppin, 1974; Huang et al., 1980; Klenk and Rott, 1980; Schmidt, 1982). The potential participation of fatty acids in the induction of fusion is further supported by the presence of oleic acid, a fatty acid long known for its fusogenic properties (Akkong et al., 1973) in the fatty acid fraction released from Sindbis virus glycoprotein E1 (Schmidt et al., 1979). If fatty acids are involved in fusogenic activities how does one explain the function of fatty acids within the plasma membrane for different membrane proteins. It would thus seem that more knowledge regarding the position of the fatty acid is necessary before any conclusions can be drawn e.g. presumably if a fatty acid is attached to the protein embedded in the lipid bilayer then it would be unable to reach the external space for interaction with other membranes in order to initiate fusion.

The other possible role for the fatty acid could be in the final stages of the viral life cycle viz. in virus assembly and budding. This possibility is based on the examination of VSV Indiana and Sindbis virus particle formation in the presence of cerulenin, an antibiotic shown to inhibit fatty acylation of G protein (Schlesinger and Malfer, 1982). In the presence of cerulenin VSV particle production was blocked by 80-90% and incorporation of [³H]palmitic acid into the VSV glycoprotein by an equivalent amount. Nonacylated G protein was found to accumulate on the cell surface but was not incorporated into the budding virus. This suggested that while fatty acylation was not
essential for G protein migration. It is possible that the fatty acid containing G protein acquires a conformation that establishes sites for protein-protein or protein-lipid interactions and that G proteins of VSV Indiana which are devoid of fatty acid are unable to reach a conformation which can establish such interactions. On the other hand, the glycoproteins of the other two serotypes of VSV, Cocal and New Jersey may acquire a conformation suitable for interactions necessary in assembly and budding without any fatty acid. The results with cerulenin need to be interpreted carefully since cerulenin in agreement with published results (Goldfine et al., 1978) was found to inhibit protein synthesis at concentrations at which it blocked fatty acid acylation. It also inhibits cholesterol and de novo fatty acid biosynthesis (Omura, 1976) and this could well explain the effect of cerulenin on VSV New Jersey which was quite similar to that observed in the case of VSV Indiana (data not shown). A recent study of Mycoplasma capricolum has shown that cerulenin had no effect on the labeling of mycoplasma membrane proteins by [3H]palmitate (Dahl et al., 1983). It is quite likely, however, that the procaryotic fatty acyl transferases are affected differently from the eucaryotic ones. Nevertheless, it shows a lack of complete knowledge of the fatty acylating process and the mode of action of cerulenin.

An interesting possible function for fatty acid bound to the viral protein could be in the selection of certain cellular lipids to be incorporated into the maturing VSV virions. The limited genetic information of VSV (Huang and Wagner, 1966) precludes de novo synthesis of lipids coded by the viral genome. This concept has evolved from the
examination of the lipid composition of VSV Indiana and VSV New Jersey which shows that the Indiana and New Jersey serotypes of VSV have a composition in their lipid bilayer which is distinct from each other and from the cells from which the particles were released (McSharry and Wagner, 1971). According to this study, the concentration of neutral lipids and the molar ratios of cholesterol to phospholipid are higher for plasma membranes and virions than for uninfected cells. VSV New Jersey has a high concentration of cholesterol ester while VSV Indiana has a high cholesterol concentration. Cholesterol represents a greater proportion of the total neutral lipid content of VSV Indiana virus than it does in the New Jersey virus. Inspite of these differences in composition the overall structure of the viral envelope of the two serotypes in terms of microviscosity as measured by fluorescence depolarization has been found to be the same (Patzer et al., 1978). Thus, the fact that the presence or absence of G bound lipid does not affect virus envelope structure of VSV Indiana and VSV New Jersey suggests that the selection of the pre-existing cellular lipids to form new lipoprotein complexes for incorporation into virions budding from the plasma membrane makes for rigidity of the viral envelope which may be otherwise more fluid in cases where non-acylated glycoproteins such as that of VSV New Jersey are present in the viral envelope. This concept could be extended to explain the presence of fatty acid in non-viral membrane proteins.
CONCLUDING REMARKS

The NH₂-terminal sequence analysis of the in vitro synthesized unglycosylated product and the glycosylated form obtained in cells infected with VSV Cocal and VSV New Jersey were determined. The comparison of these sequences with those corresponding to the ones of VSV Indiana G protein reveal a striking unrelatedness within the signal region in contrast to the high degree of conservation of sequences close to the NH₂-terminus of the mature glycoprotein. The signal sequences of the three glycoproteins are similar in the extent of hydrophobicity and are comparable to the known signal sequences of secretory and membrane proteins in terms of the distribution of hydrophobic amino acids, presence of secondary structure disrupting amino-acid residues within the last five residues of the cleavage site and the (-3, -1), rule. This suggests that the recognition of the signal sequence by VSV G is mediated by the same SRP as that for the secretory and other membrane proteins. Also the signal peptidase which brings about the removal of the signal peptide after the transfer of the protein across the ER would be expected to be the same for the protein destined to be secreted outside or inserted into the plasma membrane.

The three dimensional conformation of the VSV glycoprotein is of vital importance during biosynthesis. Attachment of oligosaccharide chains occurs only at sites (Asn-X-Ser/Thr) located regions (possibly β-turns) which are accessible to glycosylating enzymes involved in transferring the oligosaccharide chain from the lipid carrier to the protein. Conformation also depends on the interaction of regions
within the VSV G with the ER membrane. The attachment of oligosaccharide chains also influences the conformation of the VSV protein and in turn its transport to the cell surface. In the case of tsV1 a glycosylation defective temperature sensitive mutant, the absence of one complete sugar chain in comparison to the wild type VSV G as a glycoprotein at the permissive temperature does not alter the ability to follow the normal biosynthetic pathway nor is there a significant decrease in infectivity that can be attributed to the deficiency in a sugar chain suggesting that one complete oligosaccharide is sufficient for the biosynthesis of a functional glycoprotein.

The recently discovered post-translational modification viz. the fatty acid attachment to the glycoproteins is of importance to some vesiculoviruses while it is not present in others. The lack of fatty acylation does not alter the function of the glycoprotein in the case of viruses which possess such glycoproteins. The possible role of the fatty acid could be in the selection of the preexisting cellular lipids, to be incorporated into the viral envelope. The fatty acylation site seems to be the Cys or at least Cys is an important amino acid in fatty acylation whether it is Cys or some other amino acid one would expect it to be present in exposed regions of the protein so as to be accessible to the fatty acylation enzymes.

Present Trends and Future Prospects

A question of central importance and the focus of considerable study in recent years has been the process by which proteins are transported from their site of synthesis to their ultimate subcellular
destination. Recombinant DNA technology has provided a very powerful means of examining the ability of some of the domains in the protein to direct a protein to a particular site in the membrane. Quick and easy means of assessing gene expression such as immunofluorescence and SDS-polyacrylamide gel electrophoresis have made it possible to study the effect of specific structural changes in proteins brought about by genetic manipulations. The rapid advances in the construction of hybrid genes and genes with site specific changes has been preceded by the successful cloning of the cDNA to some of the (−) strand RNA virus genes (Gething et al., 1980; Gallione et al., 1981; Rose and Gallione, 1981; Hartman et al., 1982). The expression of the VSV G protein from cloned cDNA in eucaryotic cells and its normal transport to the plasma membrane suggests the presence of all the necessary signals, and this has paved the way for studying the signals that direct membrane proteins to their appropriate locations in eucaryotic cells (Rose and Bergman, 1982). Since then, a large number of alterations have been analyzed. A deletion mutant in which DNA sequences encoding the cytoplasmic domain of the G protein was removed along with the transmembrane domain was found to be secreted slowly from the cells. It was also found that alterations in the cytoplasmic domain in the G protein of VSV by in vitro mutagenesis of the DNA encoding this domain had profound effects on the rate at which the protein was transported (Rose and Bergmann, 1983). These results stressed the importance of these domains not only in insertion of the glycoproteins in the plasma membrane but also in the efficient transport of the protein. Construction of chimeric genes with
influenza hemagglutinin and VSV G carboxy or amino terminus (McQueen et al., 1984) or polyoma middle size tumor antigen with the COOH-terminus of VSV G (Templeton et al., 1984) has revealed that the NH2-terminal sequences of VSV G protein can serve as signal sequences and the COOH-terminal sequences can provide the anchor function when substituted for similar sequences. Construction of the chimera, however, altered the conformation rendering them defective in the transport of the influenza haemagglutinin and incapable of transforming susceptible cells in the case of the polyoma middle size antigen. These studies demonstrate the inadequacy of the substitution of domains of homologous function suggestive of additional domains that become obscured or deleted by the manipulations.

Bacterial systems in which genetic manipulations have been used showed that the signal peptide was, indeed, necessary for translocation (Bassford et al., 1979). Fusion of the amino-terminal region of a cytoplasmic protein gene resulted in a hybrid protein that was exported from the cytoplasm (Silhavy et al., 1977). Mutations in this hybrid gene that blocked the export process all showed altered signal sequences (Bedouelle et al., 1980; Emr et al., 1980). However, one fusion protein containing all the amino acids of a signal peptide and some amino acids of the mature protein remained as a cytoplasmic protein (Moreno et al., 1980). The conclusions drawn from the bacterial systems supported the signal hypothesis but suggested that information within the secretory protein in addition to the signal peptide was required for secretion.

A recent study to identify the region of the protein necessary
for translocation has used genes cloned in bacterial plasmids as targets for in vitro mutagenesis. They then used the mutated genes in a eukaryotic translation-translocation system. A hybrid gene containing 23 codons of the pre β-lactamase signal sequence and 5 codons of the mature β-lactamase were fused to the α-globin such that this cytoplasmic protein became a secretory protein (Lingappa et al., 1984).

Another example of the use of recombinant DNA technology is the investigation of functional aspects of hydrophobic domains in neuraminidase of the influenza virus. It has been known that NA possesses two stretches of hydrophobic sequence one at the NH₂-terminus and the other at the COOH-terminus. However, unlike most of the known membrane proteins, the hydrophobic sequence at NH₂-terminus is not cleaved from the mature protein and remains embedded in the membrane and serves as an anchor (Fields et al., 1981; Blok et al., 1982; Hitch and Naya, 1982). Thus in order to determine which of the hydrophobic sequence of NA provides the signal function in translocation across the RER two groups using different approaches involving recombinant DNA technology arrived at the same conclusion. The group of Markoff et al. (1984), constructed, two different recombinants one lacking the region encoding the NH₂-terminal region of the NA and other lacking the region encoding the COOH-terminal region of NA and found that the NH₂-terminal hydrophobic region was required for glycosylation and surface expression of the influenza virus NA. The second group (Bos et al., 1984), constructed a hybrid in which the DNA coding for the NH₂-terminal hydrophobic sequence of NA was joined
to the DNA coding for the signal minus HA as well as to the DNA coding for the signal minus and anchorless HA. The results of expression in CV-1 cells, showed that the NH₂-terminal hydrophobic sequence of NA, in addition to its anchor function, also provides the signal function in translocation across the RER.

A further extension of the expression of a cDNA clone encoding the G protein of VSV Indiana of truncated genes encoding G proteins lacking portions of the carboxy-terminal cytoplasmic domain was the use of oligonucleotide directed mutagenesis to change the protein sequence in the cytoplasmic domain in order to identify the probable site of acylation (Rose et. al., 1984). Labeling of these truncated protein with [³H]palmitate indicated that palmitate might be linked to cysteine. This conclusion was drawn from the lack of palmitate labeling found when cysteine was deleted along with other amino acid and also when Cys was changed to Ser. This type of expression in which the fatty acylation does not occur because of lack of amino acid residue such as Cys can be useful in the study of the fate of unacylated glycoprotein. The main advantage from such studies is the absence of side effects, e.g. inhibition of protein synthesis, inhibition of cholesterol biosynthesis etc., which are prevalent on using antibiotics such as cerulenin to block fatty acylation. The nonacylated glycoprotein can be shown to reach the cell surface and can be rescued by infecting the cells with a temperature sensitive mutant like tsYM at the permissive temperature and harvesting the virus released at the nonpermissive temperature. The chemical analysis of the envelope containing the nonacylated glycoprotein in the released virus and its
comparison with the one containing acylated glycoprotein would give a clear indication of the suggested role of fatty acylation viz. the selection of pre-existing lipids for the incorporation into virus. Besides this recombinant DNA and pseudotype analysis approach, it should also be possible to obtain a reasonably good correlation between the presence and absence of fatty acid and the composition of the lipids incorporated into the envelope, by lipid analysis of the envelopes of Cocal serotype of VSV and of the Piry and Chandipura viruses.

The expression of cDNA encoding the truncated VSV G which lacks the region corresponding to the COOH-terminal membrane spanning region of the protein provided useful information regarding the role of this region not only in anchoring the protein to the plasma membrane but also its role during its intracellular transport. Similar studies could be done for the NH₂-terminal region which has been shown to be conserved in three serotypes of VSV (Kotwal et al., 1983) and also shown to possess hemolysin activity (Schlegel and Wade, 1984). The expression of cDNA clones lacking the 5' end of the gene encoding the NH₂-terminus of the mature glycoprotein could provide crucial information regarding the involvement if any, of membrane fusion in the transport of the glycoprotein.

A logical extension of the phenotypic characterization of the tsY1 is the determination of the genotypic lesion caused by the chemical mutagen. This can be achieved by annealing of the dG tailed cDNA corresponding to the G mRNA into a dG tailed Pst 1 cut pBR322 and then cloning into bacterial cells such as C-600. Further, in order to
obtain the nucleotide sequence, the bacterial cells are grown and the plasmid containing the cDNA isolated and sequenced by any of the two sequencing methods (Maxam and Gilbert, 1977; Sanger et al., 1977). This has been successfully carried out for the VSV Indiana mRNA encoding the G, N, NS and M proteins (Gallione et al., 1981; Rose and Gallione, 1981). A comparison at the nucleotide sequences encoding the wild type VSV Cocal G with that of ts Μ G would give an exact indication of what is the base change that has occurred and more importantly where in the G gene is it located. An attempt at trying to accomplish this goal was made. The poly(A)⁺mRNA was isolated from cells infected with Cocal or ts Μ and the first strand synthesis using the total poly(A)⁺mRNA of VSV Cocal as template gave the synthesis of three discrete bands possibly corresponding to G, N and NS + M. The second strand synthesis gave products in the range of 0.5 kb to 2 kb and the dC tailed double stranded DNA was annealed to the dC tailed Pst I cut pBR322 and using the RdCl₂ method (Hanahan, 1983). C-600 cells were transformed. Due to constraints of time it was only possible to analyze a few clones and 5 of these clones had a plasmid which had an insert in the PstI site whose size was around 2 kb but since during the manipulations one of the PstI sites was lost, it was decided to discontinue.

The nucleotide sequence that would become available could also be useful in comparing the 3' end of the Cocal mRNA encoding the membrane spanning regions to determine the changes that would account for the lack of a fatty acylation site.

The synthesis of peptides corresponding to conserved regions of the glycoprotein molecule may help to elucidate the function of these
conserved regions. This approach has been successfully adopted in a recent study in which a peptide corresponding to the NH$_2$-terminal 25 amino acid of VSV (Indiana, San Juan strain) G shown to be conserved in other VSV serotypes (Kotwal et al., 1983) possessed pH dependent hemolysin activity (Schlegel and Wade, 1984).

Additional peptides (with deletions and modifications) could be generated in order to establish the similarities between the hemolytic activities of the synthetic peptide and the G protein. Similar approaches can be used to define functional domains in the glycoprotein molecule such as regions interacting with the M protein or the ones involved in attachment to cells.

The lack of glycosylation of G protein of ts Y at 39°C as discussed previously could be due to a lesion in the glycosylation site or in adjoining region. This can be investigated using an approach used by Lennarz et al. (1981). This approach involves the use of membrane preparations from hen oviduct as an enzyme source and exogenous oligosaccharide-lipid as donor and the denatured or native protein synthesized at 39°C as a possible acceptor. If there were glycosylation then it would be indicative of the presence of glycosylation sites and their possible inaccessibility in the native form.
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