VESICULOVIRUS:

COMPARISONS OF PROTEIN STRUCTURE

AND

STUDIES OF THE ABNORMAL N PROTEIN OF

THE VESICULAR STOMATITIS NEW JERSEY D_1 TEMPERATURE-SENSITIVE MUTANT

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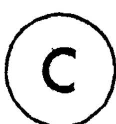

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ABSTRACT

Whereas the *Vesiculovirus* genus of the Rhabdoviridae is comprised of several more or less biochemically distinguishable serotypes, a study was undertaken to assess conservation of protein structure in *Vesiculovirus* members. It was of particular interest to assess protein structural conservation for Piry and Chandipura viruses which show only slight serological cross-reactivity with VS Indiana, the prototype of the genus. Three methods of peptide mapping were employed: tryptic peptide mapping by 2-dimensional thin-layer electrophoresis-chromatography, a novel mapping procedure which utilized limited cleavage of tryptophan peptide bonds with the chemical agent N-chloro-succinimide (NCS) followed by fractionation using SDS-polyacrylamide gel electrophoresis, as well as an adjunct to the second method where proteins labeled at their amino-termini with f[^{35}S]Met were used to derive linear oriented maps of tryptophan positions. The methionine-containing tryptic peptides of the 5 constituent proteins of members of the *Vesiculovirus*: VS Cocal, VS New Jersey (Missouri and Concan strains), ~~and Piry~~ viruses were compared to the prototype member, VS Indiana (T) standard strain, by two-dimensional thin-layer chromatography-electrophoresis. While the corresponding proteins of each virus isolate could be identified by their characteristic peptide map there were common peptides shared among the members tested. On comparison with the prototype VS Indiana(T), VS Cocal possessed common peptides from its N, M, and G proteins whereas VS New Jersey possessed common

peptides from N and M proteins. Piry virus possessed one N protein peptide in common with the prototype. Comparison of the tryptic peptides of 2 members of the VS New Jersey serotype but from separate subtypes revealed characteristic differences between these closely related viruses. The partial cleavage products resulting from NCS treatment of [^{35}S] methionine-labeled Vesiculovirus proteins were analyzed. The resulting cleavage patterns of the N and M proteins of VS Indiana(T), VS Cocal, VS New Jersey Missouri and Concan strains, Chandipura and Piry, while unique, showed a number of similarities in peptide fragment position and pattern. Pattern similarity was detectable among all the NCS patterns of the N and M proteins, but less so in the case of the Chandipura M protein. The most pronounced similarity was observed among the N protein NCS patterns of the VS viruses or between Piry and Chandipura. The M protein NCS patterns did not demonstrate as unified a relationship among Vesiculovirus members as the N protein NCS patterns, but the same two groupings of members could be made.

Cleavage of f[^{35}S]Met-labeled N and M proteins of Vesiculovirus members with NCS and subsequent SDS-PAGE separation allowed the derivation of linear oriented maps of tryptophan positions. Visual comparison of linear peptide maps showed extensive conservation of tryptophan residues among N and M proteins of the VS viruses Indiana(T), Cocal, and New Jersey (M) as well as Piry virus. The VS New Jersey (M) and Piry tryptophan map of N protein were very homologous, both containing 6 sites in similar positions. These 6 tryptophan residues

appeared to be conserved in VS Indiana(T) and VS Cocal, although these two viruses contained additional tryptophan sites. The M proteins of VS Indiana(T), VS Cocal, VS New Jersey(M) and Piry possessed 3 common tryptophan positions, although there seemed to be some form of molecular rearrangement in the case of Piry virus.

Considered in isolation, the tryptic peptide mapping analysis supports the existing serological interrelationships by demonstrating VS Indiana(T) to be closely related to VS Cocal and less related to VS New Jersey but equivocally related to Piry virus. By employing the NCS peptide mapping technique, particularly when used to prepare linear oriented maps, an ancestral relationship was demonstrated among all of the Vesiculoviruses tested. However, Chandipura and Piry viruses could be distinguished from the VS viruses suggesting a closer ancestral relationship among members within rather than between these groups.

NCS peptide mapping proved to be a valuable method of structural comparison which complemented tryptic peptide analysis under the conditions employed. Tryptic peptide mapping was much more discriminatory than NCS peptide mapping and as a consequence the former was ideal for comparison of closely related members whereas the latter method was a powerful means of detecting homology among more distantly related organisms. Given the fact that genus classification within the Rhabdoviridae is primarily serologic, relying on conservation of protein structure, this study supports the classification of the International Committee on Taxonomy of Viruses since it confirms the existence of conserved protein structures among members of the Vesiculovirus.

The D_1 N protein abnormality is manifest by an increase in electrophoretic mobility in SDS polyacrylamide gels, suggesting a decrease in apparent molecular weight of 1,000. Some of the previously described methods were employed for the elucidation of the structural basis of the N protein abnormality in the VS New Jersey(M) D_1 temperature-sensitive mutant. It was hypothesized that the D_1 protein defect resulted from premature termination due to a nonsense mutation. The N protein alteration was located on the linear map of methionine and tryptophan residues as determined by NCS and CNBr cleavage of protein labeled at its amino-terminus. This approach allowed identification of a region of ca. 2-3K molecular weight at the C-terminal end of the molecule which was required to detect the alteration. This was confirmed by removal of a similar sized portion of the C-terminal region with carboxypeptidases A and B. The tryptic peptides from the C-terminal region were identified by carboxypeptidase A plus B sensitivity and the C-terminal amino acid and tryptic peptide were identified after carboxypeptidase B treatment. The C-terminal amino acid of mutant, revertant and wild type was identified as lysine and the C-terminal tryptic peptide was indistinguishable among them. The carboxypeptidase A plus B susceptible tryptic peptides were indistinguishable as well, but a carboxypeptidase A plus B resistant tryptic peptide was altered in the D_1 N protein and was restored to wild type on reversion. Mutant N protein also differed from wild type in isoelectric point, but reversion resulted in restoration of wild type isoelectric point. From these data it was concluded that the D_1 N protein alteration was not a premature

termination, but was probably a missense mutation which results in an apparent decrease in molecular weight as detected on SDS-PAGE. It was concluded that the protein defect was not contained in the C-terminal region (ca. 2K MW), but that this portion of the molecule was required to detect the abnormality on SDS-PAGE. The region of the molecule containing the alteration was located on the linear physical map and models are presented to explain the increased mobility on SDS-polyacrylamide gel electrophoresis.

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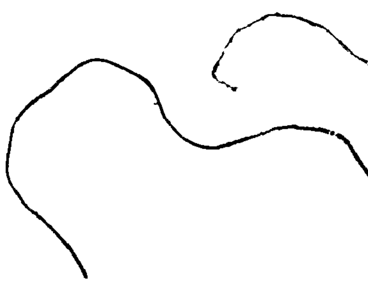
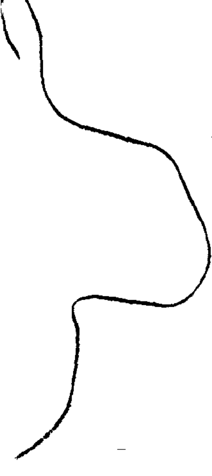


ABBREVIATIONS USED

AP	ammonium peroxydisulphate
BNPS-skatole	bromonitrophenylsulphenyl-skatole
BPB	bromphenol blue
BSA	bovine serum albumin
CPA	carboxypeptidase A
CPB	carboxypeptidase B
DFP	diisopropyl phosphorofluoridate
DOC	deoxycholate
DMF	dimethylformamide
fMet	N-formyl-methionine
Ind(T)	standard strain of Indiana serotype from Toronto
LSC	liquid scintillation counting
MEM	minimum essential medium
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
MW	molecular weight
NBCS	newborn calf serum
NCS	N-chlorosuccinimide
NJ(C)	Concan strain of New Jersey serotype
NJ(M)	Missouri strain of New Jersey serotype
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming unit
pi	post-infection
POPOP	1,4-di-2-(5-phenyloxazolyl) benzene
PPO	2,5-diphenyloxazole
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N', N'-tetramethylethylene diamine
ts	temperature-sensitive
TLC	thin layer chromatography
TLE	thin layer electrophoresis
TPCK	L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone
tRNA	transfer ribonucleic acid
V	volt
VS	vesicular stomatitis



TO MY FATHER

INTRODUCTION

Common structures are identifiable in the genes and gene products of related organisms. Living matter is more- to apply Hegel's expression- "aufgehoben" (constantly abolished and simultaneously preserved) than any other system (Zuckerkandl and Pauling, 1965). Proteins of related organisms will possess conserved structures even if they are biochemically distinct by other criteria. Evolution acts within the limits set by functional requirements and thus certain structures are conserved, since they serve specific functions of the organism.

Two areas of consideration are dealt with in this thesis: one being comparative taxonomy among members of the Vesiculovirus genus, and the other being characterization of the structural basis for a specific protein abnormality in the VS NJ(M) D_1 temperature-sensitive mutant.

Viruses, like other organisms, can be classified by identification of evolutionarily conserved protein structures. This approach to classification was used to clarify the relationships of members of the Vesiculovirus genus.

It has been observed that proteins accept mutations at rates (Wilson et al, 1977) that depend on the functions the proteins serve (Zuckerkandl, 1976). In comparing structural features of viral proteins, it will be important to know the functions fulfilled by these proteins. Information regarding the functions played by viral proteins during replication will be presented. Vesiculovirus genetics will also be discussed since much of the present understanding of the functional

relationships of viral proteins came from analysis of mutants. This will be followed by a presentation of information bearing on the relatedness of Vesiculovirus members. The final portion of this section will deal with the nature of the VS NJ(M) D_1 variant nucleocapsid (N) protein and will include an explanation of why it was chosen as a subject of study.

1. GENERAL CLASSIFICATION

Viruses with a bacilliform or bullet shaped morphology are classed in the family Rhabdoviridae, 75 members of which have been isolated from vertebrates, invertebrates and plants (Brown et al, 1979). Some members have been classified solely on the basis of morphology whereas for others, detailed physico-chemical, biochemical and biological criteria have been employed to show similarity to the prototype: vesicular stomatitis Indiana standard strain (Clewley et al, 1977). Plant virus candidates are accepted on the basis of comparison with lettuce necrotic yellows virus.

The Rhabdoviridae is made up of two defined genera: Vesiculovirus and Lyssavirus, and 61 other members with no genera established. The Vesiculoviruses are: VS Indiana, VS Cocal, VS Argentina, VS Brazil (Alagoas), VS New Jersey, Chandipura virus, Isfahan virus and Piry virus. Members of the Lyssavirus genus, previously termed the rabies subgroup, are: Rabies, Duvenhague, Kotonkan, Lagos bat, Mokola and Obodhiang. The most current reviews on the topic of Rhabdoviruses are Wagner, 1975; Knudson, 1973 and Bishop and Smith, 1977.

2. GROWTH CYCLE OF VS INDIANA VIRUS

Vesicular stomatitis Indiana serotype is the most extensively

studied Vesiculovirus and is also the prototype of the genus. Any specific values unless otherwise indicated refer to VS Indiana. All Vesiculovirus members replicate by the same strategy but do so with more or less biochemically distinct macromolecules (Brown et al, 1979).

The first step in viral replication is cellular attachment. On collision with a susceptible cell, the virus attaches to the surface by an energy independent step and subsequently enters the cell by an energy dependent event (Kohn, 1979). Internalization of virus has been reported to be by fusion with the plasma membrane and by endocytosis. Quantitative electron microscopic evidence supports both fusion and engulfment (Dahlberg, 1974). Support for both models was also obtained using serologic means. Heine and Schnaitman (1971) demonstrated viral antigen on cell surfaces after absorption using ferritin conjugated antibody, but treatment of cells with complement plus antibody after adsorption failed to lyse the cells (Fan and Sefton, 1978).

The first intracellular viral event is primary transcription of mRNA from the negative sense genome by means of the virion associated transcriptase (Flamand and Bishop, 1973). Primary transcription is independent of protein synthesis and occurs in cycloheximide treated infected cells. The mRNA products of primary transcription are immediately translated and can be isolated from polyribosomes.

The next step, namely, the process of replication in which the anti-genome positive strand template is synthesized and then copied to generate new genomes, is less well understood. Replication requires protein synthesis and is sensitive to cycloheximide. Recently replicative complexes have been isolated from infected cells in

renografin gradients (Hill et al, 1979) and genome length RNA was transcribed in vitro in the presence of an ATP analogue (Testa and Banérjee, 1979). After RNA replication commences, cells contain ribonucleocapsid (RNC) cores which contain the genome in complex with proteins L,N, and NS (Hsu et al, 1979). Maturation involves association of the core with regions of modified plasma membrane which contain virus specific G and M proteins (Cohen et al, 1971). After or concomitant with envelopment is release from the cell by a budding process. The growth cycle is complete in eight hours with virus release commencing after two hours and providing a yield of approximately 1000 pfu/cell (Wagner et al, 1963). Host macromolecular synthesis is suppressed during the infection process which culminates in cell death (Baxt and Bablanian, 1976).

The host range of VSV is extremely broad encompassing vertebrate and invertebrate cells, including moth and mosquito cell lines (Yang et al, 1969; Buckley, 1969). Enucleate cells have also been reported to support replication of VSV (Follett et al, 1974).

Serial passage of undiluted virus in cultured cells results in decreasing yields of infectious virus (Cooper and Bellet, 1959). Analysis of serially passaged virus demonstrated the existence of a smaller virus-like particle in addition to full size particles. These truncated, (T) particles, also referred to as DI (defective interfering particles), are noninfectious and interfere with the replication of infectious (B) particles (Huang and Wagner, 1966). Evidence of Sekellick and Marcus (1980) shows that coinfection of cells with T and B particles can result in a complete lack of infectious progeny. Of some

experimental use is the Madin-Darby bovine kidney (MDBK) cell line which results in progeny devoid of defective T particles (Huang and Baltimore, 1970).

3. MORPHOLOGY

The typical infectious vesicular stomatitis virion is a bullet shaped cylinder (B particle) 180±10 nm in length and 65±10 nm in diameter (Nakai and Howatson, 1968). The virion is composed of a coiled ribonucleocapsid enclosed in a unit membrane envelope. The helically coiled ribonucleocapsid has one rounded and one planar end and gives the characteristic shape to the virion. The envelope is a modified membrane of host origin with glycoprotein peplomers protruding from it.

4. VIRAL PROTEINS

Vesical stomatitis virions are composed of five structural proteins: L (large 190K MW), G (glycoprotein, 69K MW), NS (phosphoprotein 40-45K MW), N (nucleocapsid 50K MW), and M (matrix 29K MW) (Wagner, 1975). No additional proteins have been detected in infected cells (Kang and Prevec, 1970) but post-translational modifications have been detected in G, M, and NS proteins. Two host specific proteins have been observed in mature virions by examining proteins of virus produced in prelabeled cells (Lodish and Porter, 1980).

4.1 Glycoprotein. The glycoprotein (G) which exists as spikes protruding from the envelope, can be removed by proteolysis, except for a 4K MW fragment embedded in the membrane (Burge and Huang, 1970). G protein can be removed completely along with most of the membrane lipid by treatment with nonionic detergents (Cartwright et al, 1970a,b).

The G protein is subject to extensive post-translational modification and its biosynthesis has been studied in detail (Toneguzzo and Ghosh, 1978; Lodish and Rothman, 1979). Correct processing requires translation to occur in association with membranes where proteolytic cleavage and the initial steps in glycosylation take place. Additional carbohydrate groups are attached elsewhere, probably in the Golgi apparatus.

Very recently Schmidt and Schlesinger (1979) have identified a previously undescribed post-translational modification involving G protein; that of covalent attachment of fatty acid residues. In VSV, 1-2 molecules of palmitate become associated with G at some point in maturation. The completed glycoprotein becomes associated with the plasma membrane.

4.2 Matrix Protein. The matrix (M) protein underlies the viral envelope and is associated with G protein and N protein *in situ* as detected by chemical crosslinking (Dubovi and Wagner, 1977). The stoichiometric relationship of M to N is fixed whereas the stoichiometry of the M to G varies over a six fold range (Lodish and Porter, 1980b). This variation has been found to correlate with time of release from infected cells, with the early virus having less G protein. The M protein is synthesized on free ribosomes but becomes rapidly associated with sedimentable membranes. (Atkinson *et al*, 1976). Treatment of virions with the non-ionic detergent NP 40 removes G proteins and some lipids, whereas, the anionic detergent DOC removes all of the lipid, resulting in the removal of M protein as well. This suggests a lipid stabilized association of M protein with the virion (Cartwright *et al*,

1970a,b). Recently Clinton et al (1979) have detected phosphorylation of VSV M protein in vivo and determined that the phosphate is present as phosphoserine, phosphothreonine, and phosphotyrosine.

4.3 L and NS Protein. The L and NS proteins are minor constituents of the virus which remain associated with the RNP to form the ribonucleocapsid (RNC) when the envelope is removed by Triton X100 and 0.3 molar NaCl (Emerson and Wagner, 1972). The same authors could reversibly dissociate L and NS from RNC by increasing the salt concentration. Both L and NS are required for in vitro transcription of mRNA from the RNP template (Emerson and YU, 1975). NS protein has been found to exist in more than one form, whereas no alternate forms of L have been observed.

Differents forms of NS have been shown to vary in their extent of phosphorylation and in their functions. One study characterizing NS by its electrophoretic mobility on SDS-urea-acetic acid-PAGE found a preferential association with ribonucleocapsid of the less phosphorylated form (Clinton et al, 1978). Another study employing DEAE cellulose column chromatography characterized two NS forms isolated from virions and another from infected cells (Kingsford and Emerson, 1980). These forms differed in their extent of phosphorylation and only the most highly phosphorylated form was found to function in vitro to synthesize RNA, although the other forms had a stimulatory effect when added to the active form. The different forms may serve different functions in transcription, such as methylation, capping, polyadenylation and processing (Banerjee et al, 1977). To further complicate the situation, each of the two NS forms which were derived from virions,

were resolved into two components when electrophoresed in SDS-urea-PAGE.

4.4 Nucleocapsid Protein. The nucleocapsid (N) protein is abundant, comprising 30% of the total virion protein (Bishop and Smith, 1977). The N protein is firmly bound to the viral genome and can be isolated in complex with RNA as a sedimentable RNP after virion disruption with DOC (Cartwright et al, 1970b) or Triton X100 plus sodium chloride (Emerson and Wagner, 1972). The N protein confers ribonuclease resistance to the genome when complexed to form RNP (Cartwright et al, 1970a). This latter structure is required for transcription in vitro, since RNA devoid of N protein does not serve as a template (Emerson and Wagner, 1972). Transcription takes place without removing the N protein (Bishop and Roy, 1972). Removal of the N protein from the RNP requires SDS treatment or guanidinium HCl and low pH which denature the protein in the process (Cartwright et al, 1970b; Bishop and Smith, 1977).

5. RIBONUCLEIC ACID

The genome is a single strand of RNA of negative sense with an estimated molecular weight of 3.8×10^6 (Repik and Bishop, 1973). There are no significant complementary sequences as evidenced by ribonuclease resistance (Schincariol and Howatson, 1972). The genome is of negative sense since it is complementary to polysomal RNA from infected cells and RNA transcribed in vitro (Moyer et al, 1975). The complementary RNAs synthesized in vivo and in vitro are indistinguishable by size and function, both consisting of size classes of RNA having sedimentation coefficients 31S, 17S, 14.5S and 12S. The 17S and 14.5S mRNAs can be translated in vitro to produce authentic G and N protein respectively,

whereas, the 12S class contains two mRNA species and codes for M and NS proteins (Knipe _et al_, 1975). The 31S RNA has not been translated _in vitro_ but its MW is compatible with its serving as the template for L protein. The viral proteins encompass essentially all the genetic potential of the VSV genome, as far as can be predicted by the corresponding molecular weights (Banerjee _et al_, 1977), and with no evidence of gene overlap or mRNA splicing (Herman _et al_, 1978).

Evidence that transcription of the genome is sequential from the 3' end and that the gene order is 3'-N-NS-M-G-L-5' was first arrived at by determining the UV irradiation target size for expression of gene products in a cell free system which coupled transcription and translation (Ball and White, 1976). The sequential order was supported by similar studies of ultra violet light inhibition of individual mRNAs _in vitro_ (Abraham and Banerjee, 1976), as well as protein _in vivo_ (Ball, 1977). More recently direct visualization of mRNA-genome hybrids has confirmed the physical gene sequence (Herman _et al_, 1978).

Recently, Testa _et al_ (1980) showed that during VS Ind transcription _in vitro_ mRNA species are initiated independently at different promoter sites on the genome. The subsequent elongation is however, sequential, resulting in a polar effect on transcription.

5.1 _mRNA Structure_. All of the mRNAs are capped at their 5' ends and polyadenylated at their 3' ends. The sequence of the first six bases is common among the mRNA molecules, G(5')ppp(5')ApApCpApGp... (Rhodes and Banerjee, 1976). Methylation of the cap structure takes place _in vivo_ and _in vitro_ in the presence of S-adenosyl-methionine to give 7mG(5')ppp(5')Amp... .

In addition to mRNA products, a leader RNA molecule is transcribed from viral RNA in vivo and in vitro (Colonno and Banerjee, 1976). It is 48 nucleotides long, is not capped or polyadenylated and is complementary to the 3' end of viral RNA (Colonno and Banerjee, 1978). Some portions of the genome have been sequenced (Rose, 1980; McGeoch et al, 1980; Keene et al, 1980).

5.2 T Particle RNA. The genomes of T particles are deletion mutants of B particles which have the further distinction of possessing complementary termini (Schubert et al, 1979). The RNA length is proportionate to the size of the T particle (Reichmann et al, 1980). The only in vitro transcription product is a 46 nucleotide RNA (Schubert et al, 1978), which is similar to a leader molecule complementary to the anti-genome RNA found in infected cells (Leppert et al, 1979). The exception to this common type of DI is the long T particle, HRLT, derived from the Indiana HR variant which, unlike the others, does not have complementary termini (Perrault and Semler, 1979). By T1 oligonucleotide mapping and sequencing of HRLT (Clerx-von Haaster et al, 1980), and EM analysis of RNA hybrids (Epstein et al, 1980), it was concluded that it has an internal deletion in the L gene.

6. GENETICS

A variety of mutant types have been derived from Vesiculoviruses, including temperature-sensitive (ts), host-range (hr), temperature dependent host-range (tdCE) and others (see review by Pringle, 1977). Most Vesiculovirus genetics has employed temperature-sensitive mutants, a large number of which have been isolated from VS Indiana: Orsay ts mutants (Flamand, 1969), Winnipeg ts mutants

(Holloway *et al*, 1970), Glascow ts mutants (Pringle, 1970a;b), and Massachusets ts mutants (Rettenmier *et al*, 1975).

The temperature sensitive mutants are divisable into six groups (I-VI) on the basis of complementation. There is a correspondence of complementation groups among the serotypes which can be determined by comparing phenotypes (Pringle, 1977). Temperature-sensitive mutants have been classified according to their ability to synthesize RNA at nonpermissive temperatures (Pringle and Duncan, 1971), and in general mutants within a complementation group have the same phenotype.

Genetic studies show that L and N proteins serve indispensable functions in transcription. Transcription is thermolabile in some group I ts mutants and it was shown by reconstitution of transcription *in vitro* with combinations of wild type and mutant components that the defect is contained in the L protein (Hunt *et al*, 1976). Similar reconstitution experiments showed that the RNP template of a group IV mutant was defective, indicating an N protein defect (Ngan *et al*, 1974). Experiments employing RNA$^-$VS NJ group E ts mutants support the idea that NS is required in replication of RNA since some NJ group E mutants have defective NS proteins which are detectably altered on SDS-PAGE but become indistinguishable from wild type on reversion (Lesnaw *et al*, 1979; Evans *et al*, 1979). Treatment of cells with purified G protein results in an inhibition of incorporation of radioactive thymidine and uridine into DNA and RNA (McSharry and Choppin, 1978), suggesting that G protein is responsible in part for inhibition of host macromolecular synthesis during infection.

Group III ts mutants are represented by defective M proteins as evidenced by the fact that the M protein synthesized by ts O89 (group III) at nonpermissive temperature does not enter virions produced after a shift to permissive temperature (Lafay, 1974). A prepublication report by Pringle (1977) of work done by Buller and Wunner noted electrophoretic mobility shifts in M proteins of group III ts mutants O23, G32, and G33. Normal M protein was found to inhibit <u>in vitro</u> transcription while the M proteins of ts O23 and G31 from group III did not (Carrol and Wagner, 1979). Another finding which suggests a regulatory role for M protein in transcription was that cells infected with group III ts mutants had increased synthesis of viral mRNA (Martinet <u>et al</u>, 1979; Clinton <u>et al</u>, 1978). Infection with specific group III mutants under nonpermissive conditions results in the production of noninfectious particles which lack a nucleocapsid, suggesting the lack of proper interaction with N protein in assembly (Schnitzer and Lodish, 1979). A further function attributable to the M protein is inhibition of uridine uptake in infected cells, since a mutant of group III did not affect uridine uptake to the same extent as wild type or ts mutants of other groups (Genty, 1975).

Studies of the increased rate of intracellular degradation of mutationally altered polypeptides has supported the correlation of ts complementation groups with specific genes. Knipe <u>et al</u> (1977) showed that the M proteins of group III mutants (G33 and M301) were degraded 3-4 fold faster than wild type M protein at nonpermissive temperature. Similar enhanced rates of degradation were observed for N proteins of

group IV mutants (M601 and G41) and L proteins of group I mutants (G13, G11).

True recombination has not been observed in negative stranded RNA viruses. Initial reports of recombination in VSV were later found to represent complementation probably mediated by physically associated particles of each parental type (Wong et al, 1971).

Though genetic recombination has not been observed, phenotypic mixing has been shown to occur with VSV and a variety of unrelated viruses in the production of pseudotype particles (Choppin and Compans, 1970; Zavada and Rosenbergova, 1972; Zavada, 1972). Pseudotypes containing the VSV genome possess core proteins of VSV and the external membrane proteins of the coinfecting virus (McSharry et al, 1971).

The existence of pseudotype particles, the fact that ts 045 can produce virus which lacks G, and the finding that there is no stoichiometric relationship of G with virions, all suggest that few specific functions are served by G protein in virus replication.

7. IMMUNOREACTIVE SITES

Infectivity of intact virus is neutralized by reaction with hyperimmune antiserum. The neutralizing antibody recognizes G protein, since antiserum produced against virus lacking this protein due to trypsinization does not neutralize virus (Cartwright et al, 1970b). Although antiserum against trypsinized virus does not inactivate intact virus, it does inactivate tween-ether or NP 40 treated virus (Cartwright and Brown, 1972b). The latter preparations consist of structures termed "skeletons", which lack G protein and much of the membrane leaving only M, N, L, and NS (Cartwright et al, 1970a). Neutralization of skeleton

preparations occurs via anti-N antibody since anti-RNP serum inactivated skeletons, and RNP preparations were able to adsorb out the skeleton neutralizing activity in hyperimmune serum. Cartwright and Brown (1972b) were unable to detect any significant reaction with M protein by complement fixation or infectivity neutralization of skeletons. In complement fixation tests, G protein was found to fix 70% of the complement with the remainder of the complement fixed by N.

8. RELATEDNESS

The relatedness of organisms is analyzed in terms of homology or heterology of morphology and function. Comparisons can be made at both a macro and a micro level, although morphological comparisons of viruses allow only the crudest groupings of members. A more detailed examination of the structure and function of molecular constituents of the virion, specifically the viral genome and virus coded proteins, is required for fuller characterization of viruses.

Detailed comparisons of viruses must be made at the level of the gene or its product. In both cases sequence similarity is indicative of homology, but functional similarity can indicate either homology or analogy (Fitch, 1973). Molecules which serve similar functions but do so with unique structures are analagous and have arisen from convergent evolution. Homologous molecules are the result of divergent evolution from a common ancestor where structure and function have been conserved.

Several experimental procedures have been used to study the relatedness of *Vesiculovirus* members. RNA homology has been determined by hybridization, sequencing, and T1 oligonucleotide mapping. Protein structure has been compared serologically and by peptide mapping.

Several biological tests have been employed which assess the interconvertibility of viral components, such as heterotypic interference, interserotypic complementation of ts mutants, and *in vitro* transcription employing interserotypic mixtures of viral constituents.

It is appropriate at this point to catalogue the members of the vesiculovirus genus before outlining the extensive literature pertinent to their classification. Table 1 contains the serotypes recognized by the International Committee on Taxonomy of Viruses which in its listing did not note subtype relationships nor strain variants isolated from the wild (Brown *et al*, 1979).

Serotypes are distinguished by uniqueness of glycoprotein antigens but in several instances marked cross reactivity has shown specific but less than identical relationships. There are other biological and chemical features which distinguish such members and in these instances subtype status has been proposed. Federer *et al* (1967) have proposed subtyping of VS Indiana, VS Brazil, VS Cocal and VS Argentian in the Indiana serotype and Reichman *et al* (1978) have proposed classification of VS New Jersey strains into two subtypes.

Vesiculovirus members were isolated from the continents of Asia, North and South America, and Africa. The isolates are mainly from livestock but other vertebrates and invertebrates have yielded members of this group. Although only one of these viruses, Chandipura, is a human isolate, serological studies have shown widespread human infection by Isfahan and Brazil viruses in areas to which they are indigenous (Karabatsos, 1978). The VS Brazil, VS Argentina, and Isfahan viruses are not available in Canada and their importation is restricted thus

TABLE 1
MEMBERS OF THE *VESICULOVIRUS* GENUS

Serotype	Further Subdivision		Source[a]	Location
VS Indiana	Strains (4)			
	Standard (ATCC)		V	Ind. U.S.A.
	Colorado		V	Col. U.S.A.
	San Juan		V	N.M. U.S.A.
	New Mexico		I	N.M. U.S.A.
VS Cocal	-		I	Trinidad
VS Argentina	-		V	Argentina
VS Brazil	-		V	Brazil
VS New Jersey	Subtypes (2)			
	Hazelhurst:(2) Strains			
		Hazelhurst	V	Ga. U.S.A.
		Missouri	V	Miss. U.S.A.
	Concan: (3) Strains			
		Concan	V	Tex. U.S.A.
		Ogden	V	Utah U.S.A.
		Guatemala	I	Guatemala
Piry	-		V	Brazil
Chandipura	Strains (2)			
	Nagpur		V	India
	Ibaden		V	Africa
Isfahan	-		I	Iran

(a) Refers to the organism from which it was isolated, (V) vertebrate (I) invertebrate.

preventing or inhibiting their study in this country. The origins and passage history of these viruses can be found in Knudson (1973), Clewley et al (1977), Federer et al (1967), and Reichman et al (1978). The Vesiculoviruses employed for protein structural comparison in carrying out this thesis included the prototype member (VS Indiana (T) standard strain), as well as 4 other viruses (VS Cocal, VS New Jersey, Piry, and Chandipura) representing the range of relationships in the genus.

The most widely and frequently used means of classification has been serological. Relatedness is indicated by the cross-reactivity of antisera and viral antigen as detected by complement fixation or infectivity neutralization. The most extensive study of antigenic similarity involved seven members of the vesiculovirus genus: VS Indiana(C), VS New Jersey(M), VS Cocal, VS Argentina, VS Brazil, Piry, and Chandipura (Nagpur) and was carried out by Cartwright and Brown (1972b). In this study, these authors showed that the antibody directed against glycoprotein was involved in neutralizing infectivity of intact virions and that neutralization of tween-ether treated virus (so-called skeletons) was mediated by antibody directed against N protein. Complement fixing activity (CFA) was determined using intact and DOC treated virus. Reaction with intact virus involves the G protein, whereas reaction with DOC treated virus involves all of the viral proteins. CFA of reciprocal mixtures of antiserum and antigen after 30 minutes reaction showed extensive cross-reaction between VS Argentina and VS Cocal both before and after DOC treatment, but all other virus-antiserum preparations showed negligible CFA. When the experiment was repeated with incubation for 18 hr and employing intact viruses, only

trace CFA was observed among the other virus types. However, significant cros- reactivity was achieved with DOC treated viruses, VS Indiana, VS Brazil, VS Cocal, VS Argentina, and VS NJ(M). This indicated structural relatedness of their N proteins.

Similar reactions were carried out using intact virus and skeletons assayed by infectivity neutralization. Cross-neutralization of intact virus and skeletons demonstrated that only VS Brazil, VS Argentina, VS Cocal and VS Indiana shared external antigens, whereas these viruses plus VS NJ(M) shared internal antigens probably associated with N protein. The finding that VS Cocal and VS Argentina are the most closely related with less type specific cross reactivity among these viruses and VS Indiana and VS Brazil is in complete agreement with the findings of Federer et al (1967) who proposed 3 subtypes consisting of subtype 1: Indiana, subtype 2: Cocal and Argentina, and subtype 3: Brazil. The neutralization studies were more sensitive than CFA but no cross-reactivity was detected for the type specific antigen G nor for the group specific antigen N of Piry and Chandipura viruses with each other or with the other five viruses. This finding is in contradistinction to the finding of Murphy and Shope (1971) who found low but distinct levels of cross-neutralization of intact viruses. The different systems may in part explain this discrepancy since the latter authors employed mouse hyperimmune ascitic fluid, whereas Cartwright and Brown (1972b) employed guinea pig serum.

More recently Dragunova and Zavada (1979) detected a low but distinct level of cross-neutralization between VS Indiana and Chandipura virus. Highly potent immune sheep sera were used to neutralize

infectivity as detected by the plaque assay. The authors detected three components of neutralization between VSV and Chandipura virus; a complement-independent component, and a complement-dependent one which are not cell specific, as well as a neutralizing component which is complement dependent and cell specific. The cell specific neutralizing component is probably directed against the glycolipid membrane component described by Cartwright and Brown (1972a).

Initial serotyping of Isfahan virus by Tesh (1976) as reported by Karabatsos (1978) demonstrated marked complement fixing cross-reaction between this virus and Chandipura and to a lesser extent, Piry virus, in studies employing mouse hyperimmune ascitic fluid and hyperimmune mouse serum. The same data demonstrated positive cross-reaction between Piry and Chandipura viruses. However, no cross-neutralization was demonstrated among any of VS New Jersey, VS Indiana, VS Cocal, VS Brazil, Piry, Chandipura, and Isfahan, except for VS Indiana and VS Cocal when hyperimmune guinea pig sera were used. An antiserum produced by sequential inoculation of Piry, Chandipura, VS Indiana, VS New Jersey, and VS Cocal viruses was effective in neutralizing Isfahan virus, as well as, those viruses injected to prepare the antiserum.

Heterologous interference by DI particles has been used to assess relatedness among <u>Vesiculoviruses</u>. Crick and Brown (1973) tested the effect on the yield of infectious virus from cells infected with heterologous mixtures of DI and B particles employing the viruses VS Indiana, VS Argentina, VS Brazil, VS Cocal, VS New Jersy, Piry, and Chandipura. Employing Indiana DI particles, the yields of VS Indiana,

VS Argentina, VS Brazil, and VS Cocal from infected cells were depressed by about the same extents. A low level of inhibition of the growth of VS New Jersey was detected. Recently Reichmann et al (1978) have shown that Indiana DI particles interfere with VS NJ Concan reproduction but not with VS NJ Hazelhurst. In cells coinfected with VS New Jersey(M) DI particles and each of the test viruses, interference was seen only in the homologous mixture. Reciprocal mixtures of DI and infectious particles did not produce any heterologous interference among VS Indiana, VS New Jersey, Chandipura, and Piry except between VS Ind and VS NJ(M). From the interference studies, no relationship was detected between Piry and Chandipura, or with these viruses and the VS viruses, but the VS viruses were shown to be related to each other by this phenomenon of heterologus interference.

In vivo complementation of ts mutants from different serotypes was reported by Pringle and Wunner (1973). Complementation was detected between certain pairs of ts mutants from VS Indiana(C) and VS Cocal virus. VS Indiana mutants from group III and V which possess defects in their M and G proteins respectively were complemented by specific Cocal mutants. The complementation was not symmetrical since the VS Cocal γl, G protein defect could not be complemented by VS Indiana ts mutants. Interserotypic complementation was not observed between other combinations of ts mutants including those from Chandipura and VS New Jersey(M) (Pringle et al, 1971). An ability of wild type VS New Jersey (Concan) to restore the in vivo primary transcription capability of the VS Indiana(C) ts mutant G-1-114 at nonpermissive temperature has, however, been demonstrated (Repik et al, 1976).

Another comparison of the interconvertibility of viral constituents was achieved by *in vitro* reconstitution of transcription. It is possible to fractionate viral components required for *in vitro* transcription into a soluble component and a sedimentable component (Emerson and Wagner, 1972). Heterologous mixtures of the soluble enzymatic component containing L and NS proteins from VS Indiana, and the sedimentable RNP templates of VS Cocal, VS New Jersey, and Chandipura were assayed for transcription capability. RNA dependent RNA transcription was detectable in the VS Indiana, VS Cocal combination but not in combinations containing VS New Jersey or Chandipura (Bishop *et al*, 1974).

The functional studies just described give evidence of close relationships between certain members but one cannot necessarily conclude that those viruses, which do not have components which can interact productively or be recognized antigenically, are not ancestrally related (Zuckerkandl and Pauling, 1966). Other studies dealing with the structure of virion RNAs, as well as, viral proteins have been carried out. These studies indicate conserved features which are not antigenically detectable or demonstrable by reconstitution experiments.

Several studies have examined RNA homology by employing RNA hybridization, oligonucleotide fingerprinting and sequencing techniques. The RNA homology of VS Indiana, VS New Jersey, VS Cocal, Piry, Chandipura, Lagos bat virus, Mokola, Spring Viremia of Carp Virus (SVCV), Pike Fry Virus (PFV), and five rabies strains was examined by RNA-RNA hybridization (Repik *et al*, 1974; Bishop and Smith, 1977).

These workers hybridized [^{3}H]-labeled viral genomes to homologous and heterologous complementary RNA strands from infected cells. Cleavage with ribonuclease A or ribonuclease A and T_1 followed by TCA precipitation was used to determine the fraction of RNA existing as a double strand. Cleavage at the pyrimidine bases with ribonuclease A allowed, in every case, a greater percentage of RNA to be acid precipitable and thus represents less stringent conditions for hybrid detection. Only in the cases of VS Cocal and VS Indiana, and among the rabies strains was there evidence of homology under stringent conditions. However, under nonstringent conditions homology was detectable among rabies, Mokolo, and Lagos bat virus, and among Chandipura, VS Cocal, VS Indiana, VS New Jersey and Piry viruses. There was some indication that Piry and Chandipura viruses were somewhat less related to each other than were VS Indiana, VS NJ, and VS Cocal.

More recently the ribonuclease, T_1 digests of Rhabdovirus RNAs were analyzed by fingerprinting using two dimensional polyacylamide gel electrophoresis (Clewley et al, 1977). Oligonucleotide fingerprints were prepared for the four strains of VS Indiana: San Juan, Colorado, New Mexico and Indiana standard; five strains of VS New Jersey: Missouri, Hazelhurst, Concan, Ogden, and Guatemala; two isolates of Chandipura: Ibadan and Nagpur; and VS Cocal virus. The different isolates of VSV Indiana are distinguishable by their oligonucleotide fingerprints and thus represent strains, but, the majority of the characteristic oligonucleotides appear to be conserved. RNA fingerprints of the five VS NJ strains are easily distinguished from those of VS Indiana. Three of the VS NJ isolates are similar to each

other but different from the other pair which also resemble each other. The oligonucleotide pattern of the genome of VS Cocal virus is distinct from that of the closely related VS Indiana. The Nagpur (India) and Ibadan (Africa) isolates of Chandipura virus are unique from each other and the other viruses tested. The high resolution of oligonucleotide fingerprinting is suited for examining strain differences but may be too discriminatory for studying lower levels of relatedness. In the same paper, Clewley et al (1977) showed that the various VS NJ strains exhibit differing extents of RNA homology to each other and divide themselves into the same two groups as determined by examination of RNA finger-prints. Reichmann et al (1978) have subsequently classified the VS NJ strains into subtypes on the basis of several criteria, including sequence homology, as determined by hybridization of viral RNA with fractionated mRNAs employing the 13-18S and 30S size classes. Members within the same subtype exhibit homologies of 80-100%, whereas members of different subtypes cross anneal to an extent not exceeding 30%. Heterotypic interference by DI particles from VS Indiana HR occurs with VS NJ Hazelhurst but not with VS NJ Concan, further establishing the distinction of the two subtypes.

Reciprocal neutralization among the five VS NJ strains revealed cross-reactivity to a degree which readily permits relegating all five isolates to the same serotype but which allows division into two subtypes. Clewley et al (1977) also documented similar quantitative differences in reciprocal neutralization between the subtypes. On the basis of cross hybridization, oligonucleotide mapping, heterotypic interference and differential cross neutralization, the New Jersey

serotype of VSV has been further subdivided into the Concan subtype with members Concan, Ogden, and Guatemala strains, and the Hazelhurst subtype containing Hazelhurst and Missouri strains. This reclassification is rather important in light of the fact that most biological and biochemical characterization has employed Ogden and Concan strains, while all the current ts mutants of VS NJ are derived from the Missouri strain which is in a separate subtype.

Direct comparison of nucleotide sequence has been made for the leader sequence and 25% of the N protein mRNA for VS Indiana and VS NJ(M) (McGeoch et al, 1980). The 3' end of the viral RNA as well as the 3' end of the N mRNA were sequenced for a total of 406 nucleotides. Since the gene order commences with the N gene, sequencing of the 3' end of viral RNA encompasses the coding region of the N gene after the sequence specifying the leader and spacer. The aligned sequences demonstrated 70.8% homology overall. The predicted amino acid residues are identical in 63 out of 95 positions which represents 66.3% conservation of coding specificity. The sequence variability has resulted in conservation of protein structure, since the majority of changes are silent third position substitutions. Most of the amino acid substitutions (18/32) were of a homologous nature with residues of similar size, charge, and hydrophobicity replacing each other, such as Gln-Asn, Tyr-Phe, Lys-Arg, and Glu-Asp.

A nucleotide region was identified which had a very low conservation of sequence with several addition-deletion changes. The region was 20-23 residues long and was immediately distal to the N protein termination codon. This may represent a spacer region since it

is followed by a symmetric AU rich region which may be a processing control region.

Rhodes and Banerjee (1980) have sequenced 96 bases adjacent to the poly (A) of the N protein of VS NJ Ogden strain. Comparison of the coding portion of this sequence with that of VS Ind and VS NJ Missouri showed it to be highly conserved, with 82% and 85% homology respectively. Overall sequence homology of the 3' end regions of the N mRNA is higher between the VS NJ strains, Ogden and Missouri, (82%) than between VS NJ Ogden and VS Indiana (75%). This is consistent with the finding of McGeoch et al (1980) that 71.4% of the bases in the distal 206 bases of the N gene were conserved between VS NJ Missouri and Indiana. The Ogden N mRNA sequence adjacent to the coding region was the least conserved when compared with VS Indiana or VS NJ Missouri, in agreement with the speculation that this is a spacer region. The sequence comparisons show the VS NJ strains to be extensively homologous to each other and slightly less so with VS Ind.

9. PEPTIDE MAPPING: GENERAL

Most comparisons of protein structure employ digestion with proteoloytic enzymes and separation of the resulting peptides. Trypsin has been the enzyme of choice since it exhibits the highest degree of substrate specificity known for an endopeptidase (Kasper, 1975). Trypsin cleaves peptide bonds formed by the carboxyl group of lysine and arginine. There is a decreased rate of hydrolysis for susceptible peptide bonds adjacent to polar side chains. The peptide bond involving the imino nitrogen of proline is totally resistant to tryptic hydrolysis (Kasper, 1975). Chymotrypsin is a trace contaminant in trypsin

preparations, however, treatment with TPC^{K} specifically inhibits its activity (Kostka and Carpenter, 1963). Proteins are often labeled with [^{35}S]methionine prior to tryptic peptide mapping because of its high specific activity and the fact that the peptide maps are less complex, making them suitable for comparison. Peptides are fractionated by cation exchange chromatography or by two dimensional separation on thin layers using chromatography and electrophoresis.

Recently a technique of peptide mapping employing partial proteolysis and fractionation using SDS-PAGE has been described (Cleveland et al, 1977). Enzymatic proteolysis is carried out in the presence of SDS which restricts the number of susceptible sites. In spite of this, the patterns can be very complex since the complexity relates to the number of cleavage sites, n, by the relationship $(n+1)(n+2)/2$.

The partial proteolysis approach to mapping has been modified by using chemical agents for partial proteolysis since specific agents are available to hydrolyze peptide bonds of the rarest amino acids; methionine (Gross and Witkop, 1962), cysteine (Degani and Patchornik, 1974), and tryptophan, (Omenn et al, 1970; Schechter et al, 1976). Cleavage of peptide bonds with cyanogen bromide and 2-nitro-5-thiocyanobenzoic acid have been employed for partial proteolysis mapping (Nikodem and Fresco, 1979; Garoff and Soderlund, 1978). The development of N-chlorosuccinimide cleavage for partial proteolysis at tryptophan residues is described in this thesis (Brown and Prevec, 1979).

9.1 Peptide Mapping: Comparisons

Nucleotide sequence conservation can misrepresent amino acid

sequence conservation due to the degeneracy of the genetic code and the fact that 3 nucleotides code for each amino acid but substitution of 1 in 3 can change the coding. Synonomous substitutions allow conservation of protein structure in the presence of genetic variability. Peptide mapping procedures have been employed to measure structural similarity of Vesiculovirus viral proteins (Hayward et al, 1978; Doel and Brown, 1978; Brown and Prevec, 1978 and 1979; Burge and Huang, 1979). Doel and Brown (1978) examined structural relationships of the major structural proteins: N,G, and M among VS Brazil, VS NJ and VS Indiana. N proteins labeled in vivo with [^{35}S]methionine or post-translationally with [^{125}I] (bonded to tyrosine residues) were digested with trypsin and peptide mapped by two dimensional separation on thin layer sheets. The [^{125}I]-labeled maps demonstrate extensive conservation of structure in comparisons between VS Brazil and VS Indiana, but these maps have less in common with VS NJ. The same relationships are borne out by the [^{35}S]-labeled patterns, but VS NJ appears to have a simpler pattern than VS Ind or VS Brazil appearing less similar than the tyrosine-labeled maps would indicate. [^{125}I]-labeled peptide maps were also compared among G and M proteins of these viruses. The M protein tryptic maps demonstrated a more unified relationship among the three viruses with slightly less than half of the component spots in each pattern being common. The three glycoproteins differ considerably and there were few peptides which could reliably be considered common to two or three of the viruses. The ambiguity may stem in part from the fact that comparisons were made on separate peptide maps instead of mixing two trypsinized proteins and subsequently mapping the mixture.

Doel and Brown (1978) cite a personal communication from D.F. Summers who employed ion exchange chromatography to compare the methionine-containing tryptic peptides of G, NS, N, and M proteins of VS Indiana and VS New Jersey viruses. The method demonstrated 10% homology between G protein peptides but 90% between corresponding N, NS, and M patterns. This is in disagreement with the findings of Brown and Prevec (1978) and Doel and Brown (1978) who both detected less similarity.

Heyward et al (1978) compared N proteins of VS Ind standard strain wth ts G1-114 mutant from VS Ind(C), VS Ind New Mexico, VS Ind San Juan, and VS New Jersey Concan. The Indiana strains were almost identical when compared by cation exchange chromotography of [^{35}S]-methionine or [^{3}H]lysine-labeled tryptic digests. However, when VS New Jersey (C) N protein labeled with [^{35}S]-methionine was compared with the standard strain of Indiana, the patterns were very distinct, not only in elution position, but also in the number of peptides.

Recently, a rapid method of protein comparison has been devised by Cleveland et al (1977) which employed non-limit proteolysis in the presence of SDS during SDS-PAGE. Burge and Huang (1979) employed this technique to compare the five proteins of VS Indiana standard, VS Indiana(C), VS Indiana(SJ) and VS NJ(C). The partial proteolysis profiles were identical among the three strains of Indiana for L, M, N, and NS proteins, but some minor differences were detectable in the G protein pattern. The G protein pattern of VS NJ was completely distinct from the Indiana strains but the L, M, N, and NS proteins appeared to have part of their features in common with the corresponding patterns derived from the VS Indiana strains. Doel and Brown (1978) reported

that in preliminary experiments employing the same technique for comparative analysis of N proteins of VS Brazil, VS Indiana, VS NJ, VS Argentina, Chandipura, VS Cocal, Piry and rabies, similar patterns were obtained for all viruses except rabies. They also found that the New Jersey and Chandipura patterns were more similar to each other than they were to any of the other protein patterns.

Taken together, the biological and structural data show close homology among VS Indiana, VS Cocal, VS Argentina, and VS Brazil, less with VS New Jersey and some detectable homology with Piry and Chandipura. The only data regarding the classification of Isfahan virus is serological and indicates a closer relationship to Piry and Chandipura than to the other Vesiculovirus members (R.B. Tesh as reported by Karabatsos, 1978). A summary of the qualitative relationships of VS Indiana to those viruses employed in this thesis is shown in Table 2.

10. VS NEW JERSEY (M) D1 ts MUTANT

Temperature-sensitive mutants of VS NJ fall into six complementation groups, A-F (Pringle et al, 1971). The only member of the D complementation group, D1, is unique in possessing two aberrant polypeptides when analyzed by SDS-PAGE (Wunner and Pringle, 1974). The G and N proteins of VS NJ D1 have increased electrophoretic mobility in SDS gels corresponding to decreases in apparent molecular weights of 3.5K and 1K respectively. Generally ts mutants do not possess proteins which are detectably different by SDS-PAGE, though a study of FMDV ts mutants showed many of them to possess proteins with altered isoelectric points (King and Newman, 1980).

TABLE 2 SUMMARY OF RELATIONSHIPS OF VESICULOVIRUSES TO THE PROTOTYPE VS INDIANA

Virus	Method of Comparison				
	Serological	RNA-RNA Hybridization	Heterologous Interference	Reconstitution of Transcription	Interserotypic Complementation
VS Cocal	+	+	+	+	+
VS New Jersey	+	+	+	–	–
Piry	+	+	–	Not Done	–
Chandipura	+	+	–	–	–

The ts lesion may reside in the Dl N gene since most ts^+ revertants have N proteins with wild type electrophoretic mobility, but not all revertants possess this characteristic (Wunner and Pringle, 1974; Evans et al, 1979). The defect in the Dl G protein may prevent normal glycosylation since it has a lower ratio of carbohydrate to protein as determined by labeling with [^{3}H]fucose, [^{3}H]glucosamine, and [^{35}S]methionine (Wunner and Pringle, 1974).

The increased mobility of N protein on SDS gels suggests that it may be smaller than wild type by ~9 amino acid residues (Wunner and Pringle (1974). The ts phenotype reverts at a frequency of $\sim10^{-5}$ often with a restoration of wild type mobility to the N protein. The fact that the N protein reverts to wild type mobility, regardless of whether or not this phenomenon is linked with the ts phenomenon, is suggestive that it is due to a point mutation and not a deletion. Point mutations can result in a specific type of deletion protein product as a result of premature termination in translation. If the mutation results in a stop codon in the coding portion of the gene, a protein smaller than the wild type will result. These mutants which result in premature termination have been termed nonsense mutants since they arise from the conversion of a sense codon to a nonsense (termination) codon.

If VS NJ Dl N protein is the result of a nonsense mutation, it would mean that the truncated molecule is still functional but at a lower temperature than wild type. There is an example of a nonsense mutation in T4 phage which also confers temperature sensitivity (Nivinskas et al, 1978). Nonsense mutants of eukaryotic systems have been characterized by in vitro suppression of mutations in adenovirus-

SV40 hybrid virus (Gesteland *et al*, 1977), herpes simplex virus (Cremer *et al*, 1979), and mouse L cells (Capecchi *et al*, 1977).

A suppressible conditional lethal virus would be invaluable as a tool for screening for suppressor carrying mammalian cells. Suppressor carrying mammalian cells could then be used to select suppressible nonsense mutants of mammalian viruses which would simplify and advance genetic analysis. The advantage of suppressible nonsense mutants is that the altered gene product is identified and related to its function. Suppressor carrying yeast have been isolated by Kohli *et al* (1979), but no mammalian cells expressing appreciable levels of suppressor tRNA are presently available (Geller and Rich, 1980).

11. AREA OF CONCERN

Comparisons of peptide structure were employed in an attempt to detect conserved peptide sequences and thus clarify relationships among *Vesiculoviruses*. This was particularly important in the case of Piry and Chandipura viruses which share a tenuous relationship to other members of the genus.

Classical tryptic peptide mapping analysis was employed to compare polypeptide structure along with two novel modifications to the Cleveland approach to peptide mapping. Partial proteolysis products were generated by chemical cleavage of peptide bonds of internally and also of terminally labeled proteins. The results obtained allowed maps of cleavage sites to be derived and compared.

A distinct area of concern was the elucidation of the structural alteration of the VS NJ(M) DI N protein abnormality which is characteristic of a suppressible nonsense mutation.

MATERIALS AND METHODS

MATERIALS

1. SOURCE OF CELLS

The cell line used routinely in these studies was a subline, L60, of Earle's mouse L cells which were obtained originally from Dr. A.F. Howatson, University of Toronto. Primary chick embryo fibroblasts were a kind gift of Dr. S. Bachetti.

2. SOURCE OF VIRUSES

The VS Indiana standard strain was obtained from Dr. T. Nakai. VS New Jersey Missouri strain was provided by Dr. D.H.L. Bishop, Birmingham Alabama. The VS New Jersey Concan strain was obtained from Dr. J. Campbell, University of Toronto. VS Cocal, Piry, and Chandipura viruses were provided by Dr. J. Obijeski, Atlanta, Georgia. Newcastle disease virus was obtained from G. Cleland, Hamilton, Ontario, and subsequently passaged in eggs by D. Legault. The NJ(M) Dl temperature sensitive mutant was obtained from Dr. C.R. Pringle, Glasgow, Scotland.

3. RADIOCHEMICALS

New England Nuclear supplied [^{32}P]phosphoric acid, carrier free; [^{3}H]-labeled mixture of 16 amino acids; [^{3}H]lysine, 78.1 Ci/mmole; [^{3}H]-arginine, 21.4 Ci/mmole; [^{3}H]proline, 3.7 Ci/mmole and [^{3}H]-water, 5 Ci/ml. Radioactive [^{35}S]methionine, 1200-1300 Ci/mmole specific activity, was from Amersham Radiochemical Division.

4. BIOCHEMICALS

TPCK trypsin (247 U/mg) and alpha chymotryspin (49 U/mg) were products of Worthington Biochemical Corp. DFP treated carboxypeptidase A (60 U/mg), DFP treated carboxypeptidase B (180 U/mg), ribonuclease A, PMSF trypsinogen, crystalline BSA, ovalbumin, β lactoglobulin, and lysozyme were purchased from Sigma Chemical Co., St. Louis, Missouri. Staphylococcal V8 protease was from Miles Research Corp. Protein A sepharose CL4B was a product of Pharmacia, Sweden. Wheat embryo tRNA was a kind gift of Dr. S.T. Bayley. Cell culture media and solutions were products of Grand Island Biochemical Co. The dipeptides α-L-Glu-α-L-Lys and α-L-Asp-α-L-Lys were products of Bachem Biochemicals, California and Vega Biochemicals, Arizona, respectively. Freund's complete adjuvant was from Difco Chemical. Rabbit reticulocyte translation extract was purchased from New England Nuclear.

5. REAGENTS

N-hydroxysuccinimide, N-chlorosuccinimide, amino acids, iodoacetic acid were purchased from Sigma Chemical Co. N-chlorosuccinimide was recrystallized from ethyl acetate prior to use. NN'-dicylohexylcarbodiimide was obtained from Aldrich Chemical Co. Ampholyte solution was bought from LKB, Sweden. Thin layer sheets on plastic backing were purchased from Brinkman Instrument Ltd., Toronto. Amberlite IR 120 sulfonated polystyrene beads were a product of BDH Chemicals and were treated prior to use. Phenol, pyridine and acetic anhydride were distilled from reagent grade stock at 182°C, 115.5°C, and 136.4°C respectively. N-ethylmorpholine was a product of Eastman

Organic Chemicals and was distilled at 138^{0}C before use. BNPS-skatole cyanogen bromide, p-toluenesulfonic acid monohydrate (sequenal grade) and 3(2-aminoethyl)indole HCl were purchased from Pierce Chemical Co. Ultra-pure urea was a product of Swartz-Mann. Constant boiling HCl for acid hydrolysis was made by collecting the distillate at 108^{0}C from a solution of 5.5 M HCl.

6. SOLUTIONS

6.1 Cell Culture Media

Eagle MEM and Joklik modified MEM were prepared by dissolving powdered medium along with $NaHCO_3$ in water followed by filter sterilization.

Joklik modified MEM containing 1/40 of the normal concentration of amino acids was prepared by mixing concentrates of Earle's salts, MEM-vitamins, MEM-antibiotics and MEM-amino acids.

Joklik modified MEM lacking methionine or lysine was prepared by supplementing a dehydrated medium lacking lys, val, leu, and met. L-lysine HCl, L-valine, and L-leucine were added to final concentrations of 72.5, 46, and 52 mg/l respectively in the rehydrated medium to produce MEM lacking methionine. To fabricate Joklik MEM lacking L-lys, L valine and L leucine were added to the same concentration as above and L-methionine was added to produce a 15 mg/l concentration.

6.2 Dulbecco's Phosphate Buffered Saline (Dulbecco and Vogt, 1954)

KCl	0.2g
KH_2PO_4	0.2g
NaCl	8.0g

$Na_2HPO_4 \cdot 7H_2O$	2.16g

made up to 1 l with water and filter sterilized.

6.3 Solutions for Discontinuous SDS-PAGE

6.3.1 Stock Acrylamide Solution

Stocks contained different concentrations of NN'-methylene-bisacrylamide a) 0.4% b) 0.8% c) 1.62%

acrylamide	30 g
NN'-methylenebisacrylamide	a) 0.4 g
	b) 0.8 g
	c) 1.62 g

the solutions were made up to 100 ml with water.

6.3.2 Stock Resolving Gel Buffer (pH 8.9)(8X)

tris	36.6 g
1 M HCl	48 ml

add water to 100 ml.

6.3.3 Stock Stacking Gel Buffer (pH 6.7)(8X)

tris	5.98 g
1 M HCl	48 ml

add water to 100 ml.

6.3.4 Electrode Buffer (pH 8.9)

tris	6.32 g
glycine	3.99 g

SDS	1.0 g

add water to 1 l.

6.3.5 Resolving Gel Solution

Different amounts of stock acrylamide were added to produce gels of different concentrations.

solution 6.3.1

for a 10% acrylamide solution	33.3 ml
for a 15% acrylamide solution	50.0 ml
solution 6.3.2	12.5 ml

water was added to give a total volume of 98.6 ml and the solution was degassed by applying a vacuum.

to the degassed solution add:

10%(w/v)SDS	1 ml
10%(w/v)AP	0.3 ml
TEMED	0.05 ml

6.3.6 Stacking Gel Solution

solution 6.3.3	1.25 ml
solution 6.3.1	1.0 ml
water	7.65 ml

the solution was degassed before adding

10%(w/v)SDS	0.01 ml
10%(w/v)AP	0.1
TEMED	0.02 ml

6.3.7 Sample Buffer (2X)

solution 6.3.3	1 ml

20%(w/v)SDS	2.5 ml
glycerol	1 ml
urea	7.2 g
2-mercaptoethanol	0.5 ml
2% (w/v) Bromphenol blue	0.1 ml

the solution was made up to 10 ml with water

Samples were solubilized by adding an equal volume of sample buffer and heating for 1 min. in boiling water.

6.3.8 Fixative for Polyacrylamide gels

acetic acid (glacial)	7 ml
methanol	50 ml
water	50 ml

6.4 Continuous Phosphate Buffered SDS-PAGE

6.4.1 0.2 M Sodium Phosphate Buffer (pH 7.4)

0.2 M Na H_2PO_4	190 ml
0.2 M Na_2HPO_4	810 ml

6.4.2 7.5% Acrylamide Gel (pH 7.4)

solution 6.4.1	25 ml
solution 3.1(a)	16.6 ml
4 M urea	6.25 ml

This solution was degassed in a vacuum before the addition of:

10%(w/v)SDS	0.5 ml
10%(w/v)AP	0.35 ml
TEMED	0.065 ml

6.4.3 Electrode Buffer

solution 6.4.1	1 l
SDS	2 g

volume increased to 2 l with water.

6.4.4 Sample Lysis Buffer (2X)

solution 6.4.1	1 ml
10%(w/v)SDS	2 ml
4 M urea	2.5 ml
2-mercaptoethanol	0.2 ml
0.2%(w/v)BPB in saturated sucrose	1.5 ml
water	2.8 ml

An equal volume of this buffer was added to protein samples prior to heating in boiling water for 1 min.

6.5 Scintillation Fluids

6.5.1 Triton-Xylene Based Fluors

xylene	750 ml
PPO	4.94 g
POPOP	.063 g
after solubilization mix with:	
triton X114	250 ml

6.5.2 Toluene Based Fluors

PPO	4 g
POPOP	0.3 g
toluene	1 l

6.6 Performic Acid (Celma and Ehrenfeld, 1975)

formic acid	1.9 ml
30% (w/v) hydrogen peroxide	0.1 ml

incubate at room temperature for 1 hr.

6.7 50 mM Ammonium Bicarbonate (pH 8.5)

28-30% (w/w) ammonia	6.58 ml

add water to 1 l and bubble CO_2 through the solution until the pH is reduced to 8.5.

6.8 Pyridine Acetate Buffer (pH 3.5)

pyridine	1 ml
acetic acid (glacial)	10 ml
water	100 ml

6.9 0.2 M Sodium Citrate (pH 2.2)

$Na_3C_6H_5O_7.2H_2O$	58.8 g
water	500 ml

the pH was adjusted to 2.2 with 6 M HCl and the volume made 1 l by adding water.

6.10 Synthesis of N-Formyl -[^{35}S]Methionyl-tRNA$_i$ Met

6.10.1 Amino Acylation Buffer

0.1 M KCl	1.5 ml
0.2 M tris HCl (pH 7.6)	2.5 ml
1 M magnesium acetate	0.1 ml
0.1 M ATP	0.5 ml
0.05 M CTP	0.1 ml

make up to 10 ml with water

6.10.2 Amino Acylation Reaction Mix

solution 6.10.1	120 µl
wheat embryo tRNA 10 mg/ml	60 µl
[^{35}S]Met 1200-1300 Ci/mmole	600 µCi
E coli amino acyl synthetase 100 U/ml	12 µl

add water to produce 600 µl total volume.

6.10.3 Triethylamine Acetate Buffer (pH 8.0)

1 M $MgCl_2$	2.0 ml
triethylamine	2.8 ml
water	150 ml

adjust pH by adding acetic acid and then add water to 200 ml.

6.10.4 Preparation of N-Hydroxysuccinimide Formyl Ester

The method was a modification of Gillam *et al* (1968)

a) formic acid 99% (w/w)	35 µl
dry dioxane	0.5 ml
b) N-hydroxysuccinimide	115 mg
dry dioxane	1 ml
c) N-N'-dicyclohexylcarbodiimide	206 mg
dry dioxane	3.5 ml

to a conical centrifuge tube add a and b, then with stirring add c. Seal with a stopper and incubate at room temperature for 3 hr. Centrifuge twice, 2K rpm for 10 min. to remove dicyclohexylurea crystals.

6.11 Cell Free Translation in Cytoplasmic Extracts of L Cells (Ghosh *et al*, 1977)

6.11.1 Cell Washing Buffer (pH 7.6)

NaCl	12.8 g
tris	6.36 g

1 M HCl was added to lower the pH to 7.6 and water was added to 1 l. The solution was autoclaved to sterilize.

6.11.2 Extraction Buffer (pH 7.5)

Hepes	9.53 g
KCl	17.9 g
$(CH_3COOH)_2Mg.4H_2O$	2.15 g
water	1.5 l

the pH was adjusted with 1 M KOH and the volume increased to 1.992 l. The solution was autoclaved and cooled before adding:

2-mercaptoethanol	8.41 ml

6.11.3 Component A of Premix

1 M Hepes (pH 7.0)	9.4 ml
2 M KCl	0.6 ml

6.11.4 Component B of Premix

0.1 M ATP (pH 7.0)	0.2 ml
0.05 M GTP (pH 7.0)	0.025 ml
20% (w/v) creatine phosphate	0.1 ml
0.06 M spermine (pH 7.0)	0.013 ml
wheat embryo tRNA 100 OD/ml	0.02 ml
water	0.042 ml

6.11.5 Premix

solution 6.11.3	50 μl

solution 6.11.4	40 µl
1 M dithiothreitol	5 µl
creatine kinase (powder)	one crystal
water	5 µl

6.11.6 Cell Free Translation Mix

solution 6.11.5	5 µl
20 µM each of 19 amino acids without Met	5 µl
[^{35}S]Met 1 µCi/µl	10 µl
infected L cell cytoplasm	15 µl
20 mM magnesium acetate	variable

water was added to produce a total volume of 50 µl

6.11.7 1 mM Hemin Stock (Weber et al, 1975)

Hemin	65.2 mg
1 M KOH	1 ml

dissolve then add:

20 mM Hepes (pH 7.4)	60 ml
water	39 ml

6.12 Isoelectric Focusing (O'Farrell, 1975)

6.12.1 Ampholyte Solution

40% (w/v) ampholytes pH 5-7	4 ml
40% (w/v) ampholytes pH 3-10	1 ml

6.12.2 Ampholyte Containing Gel Solution

solution 6.3.1(c)	2.66 ml
urea	11 g
solution 6.12.1	1 ml

water	7.54 ml
deaerate the solution by evacuation and add:	
NP40	0.4 ml
10% (w/v) AP	0.04 ml
TEMED	0.028 ml

6.12.3 Lysis Buffer

urea	57 g
NP40	2 ml
solution 6.12.1	5 ml
2-mercaptoethanol	5 ml

made up to 100 ml with water and stored frozen

6.12.4 Sample Overlay Buffer

urea	10.8
solution 6.12.1	0.5 ml

made up to 20 ml with water

6.13 Reduction Buffer for Alkylation

EDTA 50 mg/ml	0.75 ml
1.44 M tris HCl (pH 8.6)	7.5 ml
2-mercaptoethanol	0.25 ml
20%(w/v) SDS	0.3 ml
water	21.2 ml

6.14 Immunoprecipitation Solutions

6.14.1 Cell Lysing Buffer

NP40	1 ml
glycerol	10 ml

0.2 M tris HCl (pH 9)	5 ml
1 M NaCl	6.85 ml
0.1 M $CaCl_2$	0.5 ml
0.05 M $MgCl_2$	0.5 ml

made to 100 ml with water

6.14.2 <u>Washing Buffer</u>

1 M tris HCl (pH 9)	50 ml
1 M LiCl	250 ml
2 mercaptoethanol	5 ml

made to 500 ml with water

METHODS

1. GROWTH OF L CELLS

L cells were grown in suspension culture at a titre between 3 and $7x10^5$ cells/ml. The cell concentration was monitored by counting in a hemocytometer and adjusted by dilution with 37^0C Joklik Modified MEM supplemented to 5% NBCS, 100 ug/ml streptomyim and 100 units/ml penicillin. Cells were maintained in suspension in stoppered flasks at 37^0C by stirring with a teflon coated magnetic stirring bar.

2. GROWTH AND PREPARATION OF VIRUS

2.1 Titration of Virus Stocks: Plaque Assay

To assay the plaque titre, serial 10 fold dilutions of virus suspensions were made in PBS and 0.1 ml aliquots of diluted virus were applied to L cell monolayers. L cell monolayers were formed by incubation of $5x10^6$ L cells in 60 mm culture dishes for one hr at 37^0C in a 5% CO_2 in air atmosphere.

Virus was allowed to adsorb for 1/2 hr before overlaying with MEM containing agar. The agar overlay was Joklik MEM supplemented to 0.9% noble agar, 5% NBCS, 100 ug/ml streptomyim and 100 units/ml penicillin. The overlay solution was prepared by mixing equal volumes of 1.8% noble agar at 48^0C and 2 times concentrated Joklik MEM plus supplements at 37^0C.

The infected monolayers were incubated at an appropriate temperature until plaques were visible.

The monolayers were fixed by flooding with 5 mls of Carnoy's fixative, consisting of ethanol and acetic acid, (3:1) for 1/2 hour after which the agar overlays were flushed out with water and the plates air dried.

2.2 Cloning by Plaque Purification

The object of plaque purification is to isolate the virus yielded from a single plaque which arose from a single virion. Infected L cell monolayers containing fewer than 10 plaques were chosen from plates infected with serial dilutions of virus and any individual spatially distinct plaque was selected for isolation. The agar overlay immediately above the plaque area was excavated using a pasteur pipette fitted with a suction bulb. The agar chunks were transferred into 1 ml of 0.1% BSA in PBS and broken up by repeated pipetting. After a 1 hr incubation at 4^0C to allow diffusion of virus from agar pieces, the virus suspension was titrated to generate plaques again. The second plaque isolate was used to make stock virus.

2.3 Selection of Revertants of ts Mutants

Temperature-sensitive virus stock was titrated at nonpermissive temperature (39.5^0C) and plates containing the fewest plaques were chosen for plaque isolation. Isolated plaques were replaqued for a total of three times. The virus yield of the third plaque was used to make virus stock.

2.4 Preparation of Stock Virus

Serially plaque purified virus was amplified by 3 serial low moi (0.1 pfu/cell) passages in L cells. The first two passages were in L

cells growing in monolayers. L cell monolayers were freed of growth medium by aspiration and subsequently infected by a virus suspension. Adsorption was for 1/2 hr at the appropriate temperature (wt and ts^{+} revertants at 37°C, ts mutants at 32°C) and then MEM containing 2% NBCS and antibiotics was added and incubation was continued until cytopathic effect was observed. The growth medium was then collected and centrifuged at 200xg for 10 minutes to sediment cells and cell debris.

After two passages in L cell monolayer, virus suspension from the second passage was used to infect cells in suspension for stock preparation. Cells were collected by sedimentation of 67xg for 10 min. and diluted in virus suspension supplemented with MEM to result in $1\text{-}2 \times 10^{7}$ cells/ml. Adsorption proceeded with spinning for 1/2 hr before dilution to 1×10^{6} cell/ml with MEM containing 2% NBCS and antibiotics. The culture medium was collected after 20-24 hr incubation, clarified by centrifugation as before and stored at -70°C.

2.5 Virus Purification

Infected cell culture medium was freed of cellular debris by centrifugation at 8K rpm for 20 min in a Sorvall GSA rotor. The virus was sedimented at 18K rpm for 165 min in a Beckman T19 rotor. The virus pellets were suspended in PBS.

Linear 5-40% sucrose gradients in PBS were overlaid with virus suspension and centrifuged at 24K rpm for 35 min in a SW27 rotor. The opalescent region which contained virions was removed by side puncture with a needle and syringe and was centrifuged at 39K rpm for 30 min in a SW50 rotor. The virus pellet was resuspended in PBS or water.

3. PREPARATION OF RADIOACTIVE VIRAL PROTEINS

3.1 Isotopic Labeling of Infected Cells

L cell monolayers in 60 mm culture dishes were infected at an moi of 10-20 employing the conditions specified for virus production. At 3.5-4 hr pi the monolayers were aspirated free of medium and rinsed twice with PBS or phosphate free MEM prior to labeling with radioactive amino acids or phosphate respectively.

3.1.1 [^{32}P]-Labeling

Infected monolayers were rinsed at 3.5 hr pi and overlaid with 1 ml of phosphate free MEM containing 2% NBCS, antibiotics and 50 uCi[^{32}P] (carrier free) phosphoric acid. The labeling period was 1.5 hr.

3.1.2 Labeling with Amino Acids

Infected monolayers were rinsed at 4 hr pi with PBS and then overlaid with MEM lacking one amino acid or containing 1/40 of the standard concentration of each amino acid but supplemented with an isotopically labeled precursor. Labeling was for 1 hr unless specified otherwise. All labeling solutions were supplemented with NBCS and antibiotics as in the case of [^{32}P]-labeling.

Cells to be labeled with [^{35}S]methionine were overlaid with 1 ml of MEM lacking Met or 1/40 amino acid MEM both of which were supplemented with 30-50 uCi[^{35}S]Met.

Cells to be labeled with [^{3}H]lysine were overlaid with 1 ml of MEM lacking lysine supplemented with 50 uCi[^{3}H]lysine.

Labeling with [^{3}H]arginine was carried out using 1/40 amino acid concentration MEM supplemented with 50 uCi [^{3}H]Arg. Cells were labeled

with a mixture of 16 [^{3}H]amino acids by overlaying with 100 uCi [^{3}H]-amino acid mixture in 1 ml of 1/40 amino acid MEM. Amino acid mixtures received in HCl solutions were neutralized with KOH prior to use.

3.2 Isotopic Labeling of Virus

Labeling of virions with [^{35}S]methionine was carried out in suspension. Cells were infected at an moi of 20 and subsequently sedimented by centrifuging at 67xg for 10 min. The infected cell pellet was suspended to 10^6 cells/ml in MEM lacking Met supplemented with NBCS to 2% and antibiotics and then incubated with stirring. At 1.5 hr pi [^{35}S]Met was introduced to give a concentration of 1 uCi/ml and incubated for a further 18 hr. The virus yield was then purified.

Virus was also labeled with a mixture of 16 [^{3}H]amino acids in infected L cell monolayers contained in 32 oz Brockway bottles. Cells ($2\text{-}3 \times 10^7$/bottle) were infected at an moi of 20 and then overlaid with 5% NBCS in MEM supplemented with antibiotics. At 1.5 hr pi the medium was decanted and the infected monolayer rinsed with PBS before an overlay was applied consisting of 10 ml of 1/40 amino acid MEM supplemented with NBCS to 2%, antibiotics, and 250 uCi [^{3}H]amino acid mixture. Incubation was continued until 3.5 hr pi, when an additional 40 ml of this medium without [^{3}H]amino acids was added and incubation continued until virus harvest at 20 hr pi.

3.3 N-Formyl-[^{35}S]Met-Labeling of Proteins by Cell Free Translation

3.3.1 Synthesis of f[^{35}S]Met-tRNA$_1^{Met}$

3.3.1.1 Aminoacylation of tRNA$_1^{Met}$

Wheat embryo initiator tRNA$_1^{Met}$ was amino acylated in a

mixture containing [^{35}S]Met, unfractionated E coli aminoacyl-tRNA synthetases, unfractionated wheat embryo tRNAs, tris HCl (pH 7.6), KCl, magnesium acetate, ATP and CTP. Incubation was for 15 min at 37°C and then 4 volumes of 2% potassium acetate (pH 5.4) were added followed by hot phenol extraction.

3.3.1.2 Hot Phenol Extraction of RNA

The RNA solution was mixed with equal volumes of water saturated phenol and chloroform containing 4% isoamyl alcohol. The mixture was heated at 50°C for 2 min with occasional shaking. The phases were separated by centrifugation at 2.5K rpm for 10 min in a clinical centrifuge. The aqueous phase was removed and the phenol phase was reextracted with another volume of 2% potassium acetate (pH 5.4). The aqueous phases were pooled and extracted with ether before the RNA was precipitated by the addition of 2.5 volumes of cold ethanol and incubation at -20°C overnight and collected by centrifugation at 8K rpm for 20 min in a Sorvall GSA rotor.

3.3.2 Chemical Formylation

The formyl ester of N-hydroxysuccinimide was prepared in a manner similar to the procedure of Gillam et al (1968), by combining 35 ul of formic acid in 0.5 ml of dry dioxane with 1 ml of 1 M N-hydroxysuccinimide in dry dioxane followed by the addition with stirring of 3.5 ml of 0.285 M NN'-dicylohexylcarbodiimide in dry dioxane. These compounds were allowed to react for 3 hr at room temperature in a sealed tube followed by the removal of crystalline dicyclohexylurea by two cycles of centrifugation, 2K rpm for 10 min. The formylating reagent

solution prepared in this manner was kept on ice and used shortly there after.

The [^{35}S]Met-tRNA$_1^{Met}$ ethanol precipitate was dissolved in 1 ml of 0.1 M triethylamine acetate (pH 8.0) and then 0.5 ml of the formylating reagent was added followed by a predetermined volume of 0.2 M KOH to adjust the pH to 8.0. The reaction was complete after 10 min at 0^{0}C when 0.2 ml of 20% potassium acetate (pH 5.4) was added, followed by collection of tRNA by ethanol precipitation.

3.3.3 Determination of the Extent of Formylation

The method was a modification of the one described by Smith and Marcker (1970). Aliquots of formylated and nonformylated [^{35}S]-Met-tRNA$_1^{Met}$ were digested with equal volumes of 1 mg/ml ribonuclease A in water for 10 min at room temperature. The digested samples were then subjected to thin layer electrophoresis to separate the formyl-methionine oligonucleotide from the methionyl oligonucleotide. Electrophoresis was in pyridine acetate (pH 3.5) on a cellulose thin layer for 2 hr at 500 V. Labeled components were detected by autoradiography and quantitated, following elution, by liquid scintillation counting. The extent of formylation was found to vary from 89-99% among different preparations.

3.3.4 Cell Free Translation

Cell free translation was employed to synthesize proteins containing formyl -[^{35}S]methionine at their amino terminus. The first translation system employed infected L cell extract (Ghosh et al, 1973) but was not efficient at incorporating f[^{35}S]Met. Subsequently,

commercial rabbit reticulocyte translation extracts were used in conjunction with L cell extracts.

3.3.5 Preparation of Cytoplasmic Extracts from Infected L Cells (Ghosh *et al*, 1973)

Glassware was cleaned by soaking in sulphuric acid:nitric acid (3/1) followed by baking at 350^0F overnight, in addition to washing with detergent. New disposable glassware was baked prior to use.

L cells growing in exponential phase at $5x10^5$ cells/ml were infected at an moi of 25 and incubated in MEM supplemented with NBCS to 5% and antibiotics for 4 hr for VS Ind, VS Cocal, and Piry and for 7 hr for VS NJ D1R4. A total of 10^9 cells were employed at a concentration of 10^6/ml. At the end of incubation, the infected cell suspension was chilled rapidly to 0^0C by immersion of the culture flask in iced water and the addition of 1/2 volume frozen cubes of PBS. All preparative procedures following this were carried out on ice or in a 4^0C environment. The cooled cell suspension was sedimented at 67xg for 5 min and then washed three times in diminishing volumes of cold washing buffer: 250, 100, and 10 ml. The final cell pellet was suspended in 2 volumes of cold extraction buffer and was disrupted by 15 slow strokes in a tight fitting 7 ml dounce homogenizer. The disrupted cell suspension was centrifuged at 4Kxg for 10 min, and the supernatant was collected and dialyzed against extraction buffer for 5 hr. The dialized extracts were aliquoted, frozen in liquid nitrogen, and stored at -70^0C.

3.3.6 Cell Free Translation Employing Cytoplasmic Extracts

A mixture containing infected L cell extract was incubated at

32^0C for 1 hr. The mixture consisted of 0.3 volume of infected L cell extract, 0.1 volume of premix, 0.1 volume of 19 amino acids minus Met, with the remaining 0.5 volume made up of magnesium acetate solution, water and [^{35}S]Met or f[^{35}S]Met-tRNA$_1^{Met}$. The relative proportion of these three components depend on the optimum magnesium ion concentration and the volume of the isotope containing solution. When labeling with f[^{35}S]Met-tRNA$_1^{Met}$, methionine was added to give 10^3 fold excess over the quantity of methionine added as radioactive precursor.

3.3.7 Rabbit Reticulocyte Cell Free Translation

The translation system was made by New England Nuclear Corp. according to the procedure of Pelham and Jackson (1976). The reaction mixture contained 0.4 volume of reticulocyte lysate, 0.08 volume of 1 M potassium acetate, 0.08 volume of cocktail, 0.02 volume of 32.5 mM magnesium acetate and the remaining 0.42 volume was made up of water, mRNA solution, and labeled methionine. When infected L cell extracts were employed as a source of mRNA, the magnesium acetate was omitted. Labeling with the f[^{35}S]Met-tRNA$_1^{Met}$ precursor was carried out in the presence of 10^3 fold excess of Met.

3.3.8 Quantitating Radioactive Incorporation in Cell Free Extracts

Two methods of sample preparation were employed. The first was that of Hunt et al (1972) where sample aliquots were transferred to 1 ml of ice-cold water followed by the addition of 0.1 N NaOH and incubation at 37^0C for 15 min. The protein was precipitated with 2.5 volumes of

10% TCA and collected on cellulose acetate or glass fibre filters, rinsed with TCA solution, and dried at 80^0C for 20 min. The filters were suspended in toluene based scintillation cocktail and counted in a liquid scintillation counter.

Alternatively, the samples were applied to Whatman 3 M filter paper wedges (1 cm^2), dried and placed in 10% TCA (Roberts and Paterson, 1973). After 10 min. the wedges were transferred to 5% TCA and boiled in a water bath for 10 min, rinsed twice with 5% TCA followed by 2 alcohol rinses and drying. The radioactivity was determined by liquid scintillation counting. A blank filter paper wedge was included with the sample wedges to provide a blank value.

4. DETECTION AND QUANTITATION OF RADIOACTIVITY

4.1 Liquid Scintillation Counting

Dry samples on filters or paper were counted in toluene based scintillation cocktail and aqueous samples were counted in 10 volumes of triton X114-xylene based scintillation cocktail.

4.2 Fluorography of Polyacrylamide Gels

Two scintillants were used for polyacrylamide gels: PPO (Bonner and Laskey, 1974) or salicylate. PPO was used for fluorography of analytical gels and salicylate was used for preparative gels labeled with [^{3}H]. Both types of fluors resulted in similar autoradiographic image enhancement but salicylic acid fluoring resulted in a loss of resolution which was not desirable in most applications.

4.2.1 Impregnation of Polyacrylamide Gels with PPO

Polyacryamide gels were fixed and then dehydrated by immersions with shaking in two changes of dimethyl sulfoxide (DMSO), 1 hr each. Impregnation was achieved by immersion in a solution of 22.2% (w/v) PPO in DMSO for 3 hr. Before drying the gels were immersed in water for 1 hr to remove DMSO and precipitate the PPO.

4.2.2 Impregnation of Polyacrylamide Gels with Salicylate

Unfixed preparative gels were immersed in a solution of 1 M sodium salicylate (pH 7) for 5 min prior to drying. Fixed gels were washed in water prior to impregnation since acidification of the fluor solution results in precipitation. Increased fluorogenic image enhancement could be achieved by increasing the impregnation time to 1 hr.

4.3 Fluorography of Thin Layers

Two methods as outlined below were employed: impregnation of the thin layer with PPO plus POPOP in acetone or impregnation with napthalene containing PPO (Bonner and Stedman, 1978). The latter method enhanced fluorographic images 15 times as effectively as PPO plus POPOP but was not discovered until most of the thesis was complete.

4.3.1 Impregnation of Thin Layers with PPO plus POPOP

A solution of 7% PPO:POPOP (40:3) in acetone was applied to thin layers by capillary action. Thin layers were placed in a chromatography tank containing a 1 cm deep layer of this solution until the sheets were

completely wet. The sheets were removed, dried, and subsequently fluorographed.

4.3.2 Impregnation of Thin Layers with PPO in Naphthalene

A mixture of 8% PPO in naphthalene was melted in a pyrex tray on a hot plate until fuming. The thin layer sheets were dipped quickly into the solution and allowed to drain until solidified. This method was superior to PPO plus POPOP however, the optimum concentration of PPO is 0.4% according to Bonner and Stedman (1978).

5. PROTEIN FRACTIONATION

5.1 Discontinuous SDS-PAGE

The method was similar to that outlined by Laemmli (1970) with some modification. Gels were cast in the form of slabs in two portions. The resolving gel was poured to a level 4 cm from the base of the notch in the glass plate. Immediately prior to use, a stacking gel was formed over the resolving gel with a toothed insert in place to produce sample slots. The plate assembly was sealed to the apparatus with molten 1.8% agar. Electrophoresis was carried out at 100-200 V until the BPB tracker die reached the bottom of the gel, then the gel was removed and treated in a manner dependent on the type of sample or its purpose.

Analytical gels were dried after fixation, whereas preparative gels were dried without prior fixation. Unfixed gels could be stored by freezing at -70^0C. Gels were dried under vacuum in steam heat for 1-2 hr.

5.1.1. Gradient Polyacrylamide Gels

Gels containing a linear gradient of polyacrylamide

concentration were formed by using a Buchler gradient maker. The higher concentration acrylamide solution was made 10% in glycerol to stabilize the resulting gradient. The concentration of TEMED was decreased 4 fold to increase the time required for polymerization.

5.1.2 Continuous SDS-PAGE

The gels were cast in one stage with the toothed insert in place to form sample wells. Once polymerized, the gel was manipulated in an identical manner to discontinuous SDS gels. Both the gel and electrode tanks contain 0.1 M sodium phosphate (pH 7.4) plus 0.1% SDS. Samples were disrupted in phosphate buffered lysis buffer. Electrophoresis was carried out at 60 V until the BPB tracker dye reached the bottom of the gel.

5.1.3 Fixing and Staining Gels

Fixation was accomplished by shaking gels in 10-20 volumes of fixative for 1 hr. Staining required shaking of gels in fixative supplemented to 0.1% with coomassie brilliant blue for 1-2 hr. Gels were then destained by shaking with several changes of fixative until the gel background was free of stain.

5.2 Isoelectric Focusing of Proteins

The method was that of O'Farrell (1975) except that the polyacrylamide gels were cast as a slab instead of tubes. The ampholyte containing slab gel was cast between glass plates with a sample well spacer in position. Once polymerized, the sample spacer was removed and the wells were filled with lysis buffer. After 1-2 hr, fresh lysis buffer was added before generating the pH gradient by electrophoresis.

The cathode solution was 20 mM NaOH made by adding 100x stock, dropwise, into degassed water in the upper tank of the electrophoresis apparatus. The anode tank was filled with 10 mM phosphoric acid before subjecting the ampholyte containing gel to an electric field. The sequence was begun with 200 V for 15 min, followed by 300 V for 30 min, and concluded by 400 V for 30 min. Samples were then introduced into the sample wells, overlaid with 10 ul sample overlay buffer and subjected to electrophoresis at 400 V until 5,000 to 10,000 volt hr had been consumed; the procedure being completed by a final hour at 800 V. The gel was removed and prepared for either autoradiography or separation by SDS-PAGE in a perpendicular dimension.

5.2.1 Second Dimensional Separation by SDS-PAGE

After isoelectric focusing, sample channels were excised with a razor blade and marked at one end by piercing with a pin coated with India ink. The gel strip was then equilibrated in SDS-PAGE sample buffer with shaking for 30 min and then affixed to the top of a stacking gel with molten 1% agar in sample buffer. The spacer gel was 5 cm deep and instead of sample wells, it had a level surface.

5.2.2 Sample Preparation for Isoelectric Focusing

Radioactive protein samples in water were lyophilized and then dissolved in lysis buffer. Solubilization in lysis buffer was accomplished by 3 cycles of freezing and warming to 80^0C.

5.2.3 Determining the pH Gradient Profile of Isoelectric Focusing Gels

A strip 5 mm wide was cut from an isoelectric focusing slab gel was sectioned into 5 mm pieces and each section was introduced into 0.5

ml of degassed water. After equilibration of the gel sections by shaking for 6-8 hr, the pH was determined using a Fisher pH meter.

6. PROTEIN ISOLATION

6.1 Protein Purification

Protein purification employed fractionation by SDS-PAGE followed by detection and elution. Preparative scale SDS-PAGE was used to fractionate the protein and radioactively labeled material was located by autoradiography. Unlabeled protein bands were located by staining. Preceding autoradiography, reference points were marked on the Whatman 3M paper backing with radioactive ink composed of [^{35}S]Met in India ink.

6.1.1 Protein Extraction from Gels

Two methods of elution were employed: electrophoretic and diffusion (Anderson et al, 1973; Crawford and Gesteland, 1973). Electroelution resulted in a protein extract in a relatively small volume of buffer (~3 ml). Diffusion was more suited to protein extraction from wet gel fragments.

When extracting protein from gel by diffusion, the gel was first cut into small pieces and shaken with 3 changes of buffer containing SDS over a 36 hr period at 37°C. The buffer consisted of 0.1% SDS, 0.1% (v/v) 2-mercaptoethanol and 50 mM tris HCl (pH 8.9). The gel fragments were removed by filtration.

Electroelution employed a tube gel electrophoresis apparatus. Dry gel strips were inserted into tubes plugged at their lower end with 1.8% noble agar and capped with a dialysis bag containing SDS-PAGE

electrode buffer. The tubes were inserted into the apparatus which was filled wtih the same buffer, and electrophoresis was carried out at 100-200 V for 12-24 hr. The apparatus was cooled to ~8^{0}C by means of water circulated through a water jacket.

6.2 Spectrophotometric Determination of Protein Concentration

The method was that of Warburg and Christiana as described by Layne (1957). The optical densities at 260 nm and 280 nm were measured and the protein concentration determined as mg/ml by the calculation:

1.54 (OD at 280nm) - 0.76 (OD at 260nm) = protein concentration.

7. TRYPTIC PEPTIDE MAPPING

7.1 Tryptic Hydrolysis

Two methods of trypsinization were employed initially, and one was chosen for routine use. Proteins fractionated by SDS-PAGE were eluted prior to trypsin hydrolysis in the one method (Crawford and Gesteland, 1973). The other method involved trypsinization *in situ* without prior extraction (Knipe *et al*, 1975).

7.1.1 *In situ* Trypsinization

Protein bands in fixed and washed gels were located by autoradiography, excised, and immersed in 50 ug/ml TPCK trypsin in 50 mM ammonium bicarbonate (pH 8.5). Digestion proceeded for 24 hr at 37^{0}C with shaking before removing the trypsin solution and replacing it with a fresh portion and incubating for another 6 hr. The trypsin solutions were filtered and then lyophilized, followed by two cycles of dissolving and lyophilization before oxidation with performic acid and then 2 more cycles of dissolving and lyophilization.

7.1.2 Tryptic Hydrolysis of Extracted Protein

Protein eluates were precipitated by making the samples 20% TCA by adding 100% TCA. If the protein concentration was less than 100 ug/ml, BSA was added to supplement to this level prior to precipitation. Precipitation proceeded for 20 min on ice before the precipitate was collected by centrifugation at 10K rpm for 20 min in a Sorvall GSA rotor. The protein pellet was dissolved in 0.1 M NaOH before reprecipitation with an equal volume of 40% TCA. A total of four precipitations were employed for the results presented in Section I but subsequent work employed only one TCA precipitation. The final TCA precipitate was washed with several changes of cold acetone to remove residual TCA. The protein precipitate was then oxidized before lyophilization followed by two washing and lyophilization steps. Tryptic digestion was achieved by adding 20% (w/w) TPCK trypsin in two equal portions. The first portion of 1 ug/ul TPCK trypsin in 50mM ammonium bicarbonate (pH 8.5) was added and incubated 1 hr at 37^{0}C before the second portion of trypsin was added and incubation continued for 3 hr until the reaction was stopped by lyophilization. The tryptic peptides were subsequently dissolved and lyophilized twice before dissolving in electrophoresis buffer and application to thin layer plates.

7.2 Oxidation of Proteins or Peptides (Celma and Ehrenfeld, 1975)

Dry samples were dissolved in 100-300 ul of fresh performic acid for 3 hr at 0^{0}C. The performate was then diluted 4 fold with water and removed by lyophilization.

7.3 Two Dimensional Peptide Mapping

Tryptic digests were dissolved in pH 3.5 pyridine acetate buffer such that 1-5 ul contained sufficient activity for a single peptide map. The samples were applied as a spot or a narrow band perpendicular to the direction of the first separation.

Tryptic map comparisons were made by mapping two samples in parallel on one 20x20 cm thin layer plate, as well as mapping the reference tryptic digest in parallel with a mixture of the sample plus the reference (Hendrix and Casjens, 1974). After spotting, the plastic backed thin layer sheets were affixed to a glass backing with vacuum grease. Tryptic peptide maps produced on MN 400 cellulose thin layers were prepared by separation with TLE in the first dimension followed by TLC in the second. The second dimensional separation was preceded by an overnight drying step. Samples which produced poor resolution maps on cellulose thin layers due to streakiness when TLE preceded TLC, would generally yield improved peptide maps by reversing the order and employing TLC before TLE. Silica G thin layers produced best results when electrophoresis was carried out first.

7.3.1 Thin Layer Electrophoresis of Tryptic Peptides

Thin layer electrophoresis required an initial wetting of the thin layer with pyridine acetate (pH 3.5) buffer accomplished by spray application using an atomizer in conjunction with a suitable plexiglass shield to cover the sample area. Once the thin layer was wet enough to produce a dull sheen and the sample zone has accumulated buffer by capillary flow, the thin layer was placed on the cooling block of a

Brinkman thin layer electrophoresis apparatus and connected to the electrode tanks by filter paper wicks. A minimum volume of buffer was placed in the electrode tanks and cooling was by circulating water (~8^{0}C). Electrophoresis was at 450 V until a coloured marker had migrated a specific distance. With MN 400 cellulose thin layer sheets, electrophoresis was continued until a picric acid marker had migrated 5 cm but with Silica G thin layers, electrophoresis was continued until a phenol red marker had migrated 10 cm. After electrophoresis the thin layer was dried in a stream of cold air followed by a dry 37^{0}C atmosphere overnight.

7.3.2 Thin Layer Chromatography of Tryptic Peptides

Thin layer sheets were inserted into a stainless steel wire rack inside a chromatography tank lined on both sides with Whatman 3M paper soaked in the solvent to be employed. The tank lid was sealed with vacuum grease and following a 30 min equilibration period, chromatography solvent was introduced sufficient to contact the thin layer sheet. Development proceeded until the solvent had ascended to the top of the thin layer and then the thin layers were removed and dried.

7.4 Ninhydrin Staining of Thin Layers

Dry thin layer sheets were flooded with a solution of 0.1% (w/v) ninhydrin in acetone. After the acetone had evaporated, the thin layer was placed in an oven at 80^{0}C for 10-15 min or left at room temperature overnight. All amino acids appeared as bluish spots and proline appeared yellow.

7.5 Fluorescamine Staining of Thin Layers (Felix and Jiminez, 1974)

After ninhydrin staining, the thin layers were reacted with fluorescamine which resulted in the detection of several extra spots. The procedure involved 3 spraying steps:

1. Spray with a solution of 10% (v/v) triethylamine in methylene chloride.
2. After several seconds, spray with a solution of 0.05% (w/v) fluorescamine in acetone and air dry several seconds.
3. Repeat step 1.

After air drying spots were visualized under UV illumination.

8. PARTIAL PROTEOLYSIS MAPPING USING SDS-PAGE

8.1 Cleavage of Proteins with V8 Protease

The method was essentially that of Cleveland *et al* (1977) with modifications made by Nathanson and Hall (1979). In this procedure, protein samples in gel pieces were fragmented with proteolytic enzymes during SDS-PAGE.

Sample containing gel pieces were equilibrated in 2 times concentrated stacking gel buffer containing 0.1% (w/v) SDS for 15 minutes, before being placed on an SDS polyacrylamide slab gel along with a solution of discontinuous SDS-PAGE sample buffer lacking urea but containing 1% agarose and V8 protease. The stacking gel was 5 cm deep. The protease was employed at different concentrations to control the extent of proteolysis. Electrophoresis was carried out at 100 V and was interrupted for 15 min when the BPB tracker die was 1 cm from the resolving gel and then continued until the BPB had traversed the gel.

8.2 Cleavage of Tryptophan Bonds in Proteins with BNPS Skatole

Protein samples were dissolved in one volume of 70% (v/v) acetic acid followed by the addition of 1/5 volume of BNPS skatole in glacial acetic acid (Omenn et al, 1970). BSA was added as carrier if the protein concentration was low. Incubation was at 37^{0}C in darkness for 24 hr, when protein digestion products were precipitated by the addition of 5 volumes of cold acetone and overnight storage at -20^{0}C. The resulting precipitate was collected by centrigation at 10K rpm for 20 min and then dissolved in water and lyophilized.

8.3 Cleavage of Tryptophan Bonds in Protein with N-Chlorosuccinimide

Proteins were fractionated by SDS-PAGE, eluted electrophoretically and lyophilized. The lyophilizate was dissolved in 1 ml of 0.5 M acetic acid followed by the addition of 0.25 ml of 75 mM N-chlorosuccinimide in dimethyl formamide. Incubation at room temperature with shaking was stopped after 2 hr by the addition of an equal quantity on a molar basis of methionine to NCS. Carrier BSA was added if the protein concentration was low. Cleavage products were precipitated by the addition of 5 volumes of cold acetone and then collected by centrifugation as described for BNPS skatole and immediately dissolved in SDS-PAGE sample buffer or dissolved in water and lyophilized.

8.4 Cleavage of Protein with CNBr

The method was that of Gross and Witkop (1962) using the modification of Steers et al (1965). Lyophilized protein preparations were dissolved in 0.2 ml of 2 mg/ml CNBr in 70% (v/v) formic acid. Incubation without agitation was carried out at ambient temperature in

darkness for 4 hr. When incubation was complete, 4 volumes of water were added followed by lyophilization. The dried samples were dissolved in SDS-PAGE sample buffer.

9. C-TERMINAL DETERMINATION

9.1 Carboxypeptidase Treatment

9.1.1 Reduction and Alklyation of Proteins Prior to Carboxypeptidase Treatment

Proteins were reduced and subsequently carboxymethylated by reaction with iodoacetic acid by a modification of Crestfield *et al* (1963) and Schlesinger *et al* (1972).

Protein samples were reduced in 3 ml of 5.4 mM EDTA, 360 mM tris HCl (pH 8.6), 120 mM 2-mercaptoethanol and 1% (w/v) SDS by incubation at 37^0C for 4 hr under a nitrogen barrier.

The reduced sample was alkylated by the addition of 0.25 ml of 1.44 M iodacetic acid in 1 M NaOH and incubated for 15 min in the dark at 37^0C. Reagents were removed by dialysis against 300 volumes of SDS-PAGE sample buffer overnight in darkness at 4^0C. The protein mixture was then fractionated by SDS-PAGE and eluted electrophoretically followed by dialysis against 3 changes of 100 volumes of 0.2 M N-ethylmorpholine acetate (pH 8.0) over a 24 hr period at 4^0C. The dialyzate was then suitable for reaction with carboxypeptidase.

9.1.2 Preparation of Carboxypeptidase A

DFP treated CPA was obtained as a suspension in water and prepared as described by Potts *et al* (1962). An aliquot of CPA crystals was washed in water, sedimented by centrifugation, and then dissolved in

2 M ammonium bicarbonate to produce a concentration of 8 ug CPA/ul. This solution was diluted to 1 ug CPA/ul before use. DFP treated CPB was used as supplied in a solution of 10% NaCl.

9.1.3 Enzymatic Hydrolysis with CPA and CPB

Reduced and carboxymethylated protein samples in a uniform volume of 0.2 M N-ethylmorpholine acetate (pH 8.0) were used as substrate. Non-radioactive amino acids were introduced for ninhydrin detection and [^{3}H]-proline was introduced as an internal standard for quantitation.

CPA and CPB were added in specific amounts and allowed to act for a specified period of time. The reactions were stopped by heating in boiling water for 5 min.

9.1.4 Analysis of [^{3}H]-Labeled Amino Acids Released by Carboxypeptidases

After the carboxypeptidase reaction was complete, the samples contained a mixture of liberated amino acids and the residual protein molecule. Before fractionation and quantitation, the amino acids were separated from the undigested protein. This served two functions: the amino acids were desalted which made them more suitable for thin layer separation and it yielded the undigested protein fraction free of labeled amino acids for further analysis.

9.1.5 Extraction of Amino Acids

Amino acids were bound to sulfonated polystyrene beads and subsequently eluted by the method of Ambler (1967).

Amberlite IR 120, H^+ form sulfonated polystyrene beads (1/2 volume) were placed in 5 ml disposable syringes plugged with pyrex wool and a glass fibre filters. The amino acid solution was added to the beads and adsorption proceeded with shaking for 20 min. The unbound protein in solution was collected and the polystyrene beads were washed with 4 bead volumes of water followed by elution with 6 bead volumes of 5 M ammonia. The amino acid eluate was lyophilized and concentrated before fractionation by two dimensional chromatography.

9.1.6 Preparation of Amberlite IR 120, H^+ form

The amberlite IR 120 was received in the H^+ form but was unsuitable for concentrating amino acids for chromatographic separation. I speculate that it was due to minute particles of sulfonated polystyrene since pronounced yellow streaks were observable on the thin layer chromotograms.

The beads were first washed in several changes of water and the light particulates were decanted. The washed beads were transferred to a filter paper lined buchner funnel and treated with 2 litre volumes of 3 M NaOH, 7.5 M ammonia and 6 M HNO_3 with water rinsing between treatments. The last washing was extensive and was carried out by placing the beads in a column and passing 20-30 l of water through the column over a period of 24 hours.

9.1.7 Fractionation of Amino Acids by 2 Dimensional Chromatography

Separation of amino acids was by 2 dimensional TLC according to the procedure of von Arx and Neher (1963) as described by Brenner and Niedewieser (1967). All amino acids were resolved from each other

except leucine and isoleucine. The thin layers were MN 300 or EM cellulose which were prewashed by ascending chromatography with water and subsequently dried before sample application. Thin layer chromotography in the first dimension employed the solvent, n-butanol: acetone:diethylamine:water (10:10:2:5) (pH 12), and was followed by drying and TLC in the second dimension with the solvent, isoproponal: formic acid:water (40:2:10) (pH 2.5). Separation was most effective in a nonsaturated chamber without equilibration.

9.1.8 Detection of Labeled Amino Acids

Isotopically labeled amino acids were located by isotope dilution (Laurenson, 1969), where sufficient non radioactive amino acids were added to allow detection by ninhydrin staining. A mixture of 10 nmoles each of the 20 amino acids normally encountered in protein was added prior to sample fractionation. Amino acid mixtures to be extracted from solution with cationic resin beads were augmented with this mixture prior to adsorption. The ninhydrin staining procedure was as described earlier.

9.1.9 Elution and Quantitation of Amino Acids from Thin Layers

Individual amino acid spots detected by ninhydrin staining were asperated into 200 ul Eppendorf pipette tips plugged wtih pyrex glass wool. The amino acids were subsequently eluted with 3 aliquots of 170 ul of 10 mM HCl. Each aliquot was introduced into the pipette tip and drained through the plug of thin layer sorbent under centrifugal force (1K rpm for 3 min on a clinical centrifuge) into disposable 6 ml scintillation vials.

Radioactivity was quantitated by liquid scintillation counting after mixing each sample with 10 volumes of triton X114:xylene based scintillation cocktail. All radioactivity values were subtracted with a blank value activity and all physical losses were corrected from the recovery of the internal standard [^{3}H]proline.

9.2 Tritium Labeling of the C-Terminus

The method is that of Matsuo and Narita (1975), where protein is reacted with acetic anhydride and pyridine in the presence of tritiated water. This reaction results in [^{3}H] incorporation into any C-terminal amino acid. The C-terminal imino acid, proline, is not labeled by this procedure.

The protein for reaction was dissolved in 70% formic acid and transferred to a heavy walled reaction tube which was also used for subsequent acid hydrolysis. The protein was lyophilized prior to the addition of reagents. Each sample employed 100 mCi of tritiated water which required that the work be carried out under strict containment conditions. The maximum extent of labeling expected was 15-50k cpm/nmole. The pyridine and acetic anhydride used for reaction were distilled from reagent grade stocks prior to use.

The protein sample was dissolved in 20 ul T_2O (5 Ci/ml) and 40 ul of pyridine, placed on ice and then 40 ul of acetic anhydride was added with mixing. After 5 min incubation at 0^0C, incubation was continued for 15 min at ambient temperature. The sample was then placed on ice and additional pyridine, 80 ul, and acetic anhydride, 80 ul, were added. After 5 min on ice and 60 min at ambient temperature, 20 ul of

water was added and incubated for 60 min at ambient temperature. The reaction was then complete and reaction products were removed by vacuum distillation.

Vacuum distillation involved connection of the sample tube to a system of cold traps and a vacuum pump. The sample tube was frozen in a dry ice: acetone bath and evacuated, followed by warming to transfer solvents into the cold trap. Exchangeable tritium was removed by five cycles of suspension in 10% acetic acid (100 ul), and lyophilization.

The labeled amino acid was liberated by acid hydrolysis, separated by 2 dimensional chromatography and detected by autoradiography or quantitated by elution and LSC.

10. DETERMINATION OF AMINO ACID CONTENT

10.1 Acid Hydrolysis of Proteins into Constituent Amino Acids

Pure protein extracts were prepared for acid hydrolysis by precipitation with 20% TCA and subsequent acetone washing. The protein was dissolved in 70% formic acid and then divided into 1 mg aliquots and placed into acid washed hydrolysis tubes. Protein samples were lyophilized and then washed and lyophilized again before addition of 0.3 ml of double distilled constant boiling HCl (6.02 M) or 3 M p-toluenesulfonic acid with 0.2% (w/v) 3-(2-aminoethyl)-indole. The former is the classical method (Moore and Stein, 1963), but results in the destruction of tryptophan, whereas the latter method (Liu and Chang, 1971) spares tryptophan.

Hydrolysis was carried out at 105°C under reduced pressure (20-30 um Hg) in a nitrogen atmosphere for 26 and 46.5 hr in the case of HCl

and 26 and 72 hr in the case of p-toluenesulfonic acid. After hydrolysis with HCl, the samples were taken to dryness by heating at 80^0C in a stream of air. The dry samples were dissolved in 0.2 M sodium citrate (pH 2.2) in preparation for amino acid analysis. The p-toluenesulfonic acid samples were mixed with 2 volumes of 1 M NaOH before amino acid analysis.

10.2 Amino Acid Analysis

Amino acid analysis was carried out on a Beckman 121 amino acid analyzer by Mr. C. Yu of the Dept. of Biochemistry, University of Toronto. Data were produced as a ninhydrin absorbance profile of the cation exchange column effluent. The peaks were integrated by multiplying the height of the peak in adsorbance units by the width of half the height. The quantity of amino acid in a given peak was calculated by dividing its area by the appropriate constant, C, (height x width per micromole) which is a function of the colour yield of the amino acid and the dimensions of the adsorption cell and is determined by calibrating the apparatus with a synthetic mixture of amino acids. A table of C values was made available by Mr. C. Yu for the specific machine employed. The quantities of each amino acid were calculated and then the amino acid content was determined for each amino acid by solving the equation:

$$\text{no. of moles } i/\text{mole protein} = \frac{\text{weight of mole protein}}{\sum\limits_{\substack{x\ i-j \\ y\ i-j}} (x_i y_i)} \cdot x_i \text{ (moles)}$$

where x is the amount of amino acid i, y is the residue MW of i, and i-j are the component amino acids. The amino acid content values were not corrected for destruction of serine, threonine, and tyrosine, or for slow hydrolysis of valine and isoleucine (Blackburn, 1968). The correction factors are 5-15% for a 22 hr hydrolysis but since different times of hydrolysis were employed, no corrections were made.

Cysteine content was not determined since it requires prior conversion to cysteic acid by means of performic acid oxidation.

11. IMMUNOLOGICAL PROCEDURES

11.1 Antibody Neutralization of Viral Infectivity

Stock virus was incubated with an equal volume of 1/10 diluted antiserum in PBS for 30 min at 37^0C. Subsequent to this, samples were titrated for infectivity using the plaque forming assay. Control virus was incubated with PBS.

11.2 Antiserum Production

Rabbits were injected with purified virus yield of 10^9 L cells. The virus was purified by velocity centrifugation in sucrose gradients, sedimented, suspended in 1 ml of PBS, and divided into 3 aliquots. The first aliquot was injected intramuscularly in the thigh, as an emulsion formed by mixing with an equal volume of Freund's complete adjuvant. At 2 week intervals, the remaining 2 aliquots were injected subcutaneously. Two weeks after the last injection, the rabbits were bled and the serum collected. The serum was heat treated at 56^0C for 30 minutes to inactivate complement.

11.3 Immunoprecipitation

The technique was carried out as described by Schauffhauser *et al* (1978) employing protein A sepharose beads.

The antigen preparations were isotopically labeled infected L cells which were solubilized in lysing buffer. The insoluble cell components were removed by sedimentation at 8K rpm for 10 min. The supernatant was saved and 0.75 ml of it was mixed with 10 ul of antiserum and 40 ul of protein A sepharose CL 4B and incubated at 4 ^{0}C for 1 hr. The sepharose beads were prepared for use by equilibration in lysing buffer overnight. After adsorption, the beads were centrifuged, 800 rpm for 1 min, and the supernatant removed. The beads were then washed 6 times with cold washing buffer before adding SDS-PAGE sample buffer and heating in preparation for SDS-PAGE analysis.

RESULTS

Section I: Peptide Map Comparisons of Vesiculovirus Proteins

1. ASSESSMENT OF VIRUS STOCKS BY ANTIBODY NEUTRALIZATION

The uniqueness of the viruses employed in this study was determined firstly, by antibody neutralization with hyperimmune antiserum and secondly, by the pattern of their proteins on SDS-PAGE.

Viruses used to derive stocks were obtained from single plaques on infected L cell monolayers. The virus yield from a plaque after two plaque purification steps was amplified by 3 serial passages in mouse L cells at an moi of 0.1. Virus titres were assayed by plaque formation as described in methods.

The neutralization of infectivity of virus suspensions by exposure to hyperimmune antisera was carried out as described in methods. Table 3 lists the log reduction in infectivity as plaque forming ability of virus suspensions treated with homologous and heterologous antisera relative to untreated virus suspensions. All of the viruses: VS Ind(T), VS NJ(C), VS NJ(M), VS Cocal and Piry were neutralized by homologous antisera, but no cross-neutralization between heterotypic mixtures of virus and antiserum was observed.

The Concan and Missouri strains of the VS NJ serotype showed quantitative differences in the extent of neutralization by antiserum to VS NJ(C). The homologus mixture resulted in 5.3 log reduction in infectivity of VS NJ(C) and only a 2.0 log reduction in infectivity of VS NJ(M). This is one of the detectable differences between the subtype

TABLE 3

NEUTRALIZATION OF INFECTIVITY OF INTACT VIRUS[a,b]

Virus	Antiserum (1/10 dilution)				
	Control[c] (pfu/ml)	Anti-Ind(T)	Anti-NJ(C)	Anti-Cocal	Anti-[d] Piry
VS Ind(T)	8.9	4.8	-0.4	0.0	-0.1
VS NJ(C)	8.3	-0.2	5.3	-0.3	-0.4
VS NJ(M)	8.8	-0.1	2.0	-0.1	0.0
VS Cocal	8.6	0.3	-0.1	4.4	0.1
Piry	7.8	0.0	0.0	0.0	3.9
Chandipura	7.6	-0.1	-0.6	-0.1	ND

(a) Stock virus of the indicated serotype was mixed with an equal volume of 1/10 diluted rabbit antiserum or mouse ascitic fluid and kept at 37 C for ½ hr.
(b) Values are log reduction in infectivity titre (pfu/ml) relative to control.
(c) No antiserum was added.
(d) The source of antibody was hyperimmune anti-Piry ascitic fluid from a mouse.
(ND) not done

members of VS NJ as noted in the introduction. From antibody neutralization it was clear that each virus preparation was distinct.

2. ASSESSMENT OF VIRUS STOCKS BY SDS-PAGE

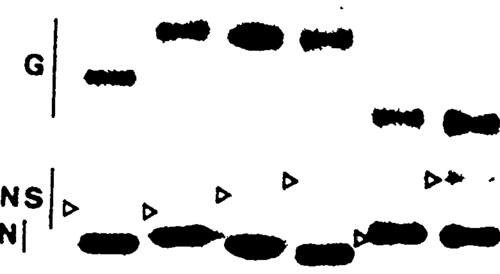
I analyzed preparations of [^{35}S]Met labeled viruses by SDS-PAGE to determine if the protein patterns were distinct and characteristic. Viruses purified by differential centrifugation and velocity centrifugation in sucrose gradients were prepared for SDS-PAGE as described in methods. The analytical polyacrylamide slab gels were routinely 10% acrylamide and 0.13% NN'-methylenebisacrylamide. An autoradiogram of [^{35}S]Met labeled viral proteins after SDS-PAGE of VS Ind(T), VS NJ(C), VS NJ(M), VS Cocal, Piry and Chandipura viruses is represented in Figure 1. As seen in Figure 1, each virus preparation can be distinguished by the pattern of electrophoretic mobilities of their five constituent polypeptides. The G and M proteins have the most varied and thus characteristic mobilities on SDS gel electrophoresis. The VS NJ strains, Concan and Missouri, are distinguishable by small mobility differences in G, N and M proteins and a large difference in the mobility of NS proteins.

3. IDENTIFICATION OF NS PROTEINS BY [^{32}P]LABELING

The NS proteins were located by [^{32}P]labeling for each virus except Chandipura and the location is marked by a triangle in Figure 1. The Chandipura NS protein position was not confirmed but since there is only one protein band between N and G, it was assumed to be NS. This was not a critical point since the Chandipura NS protein was not the subject of peptide mapping comparisons.

Figure 1. <u>SDS-PAGE analysis of [^{35}S]Met-labeled purified virus of different serotypes or subtypes</u>. Virus was produced by infection of suspension cultures of L cells in the presence of [^{35}S]Met. Virus was purified by differential and velocity centrifugation in sucrose gradients. Aliquots containing 15 K cpm [^{35}S]Met were analyzed. Electrophoresis was at 150 V and the protein bonds were detected following fluorography at -70°C on Kodak RPR 14 xray film. The protein designation and the range of mobilities covered by each is indicated with vertical bars. The positions of the NS proteins are marked by open triangles. The viruses analyzed in each lane are indicated: (IND), VS Indiana (T) standard; (COC), VS Cocal; (PIR), Piry; (CHA), Chandipura; [NT(C)], VS New Jersey Concan; and [NJ(M)], VS New Jersey, Missouri.

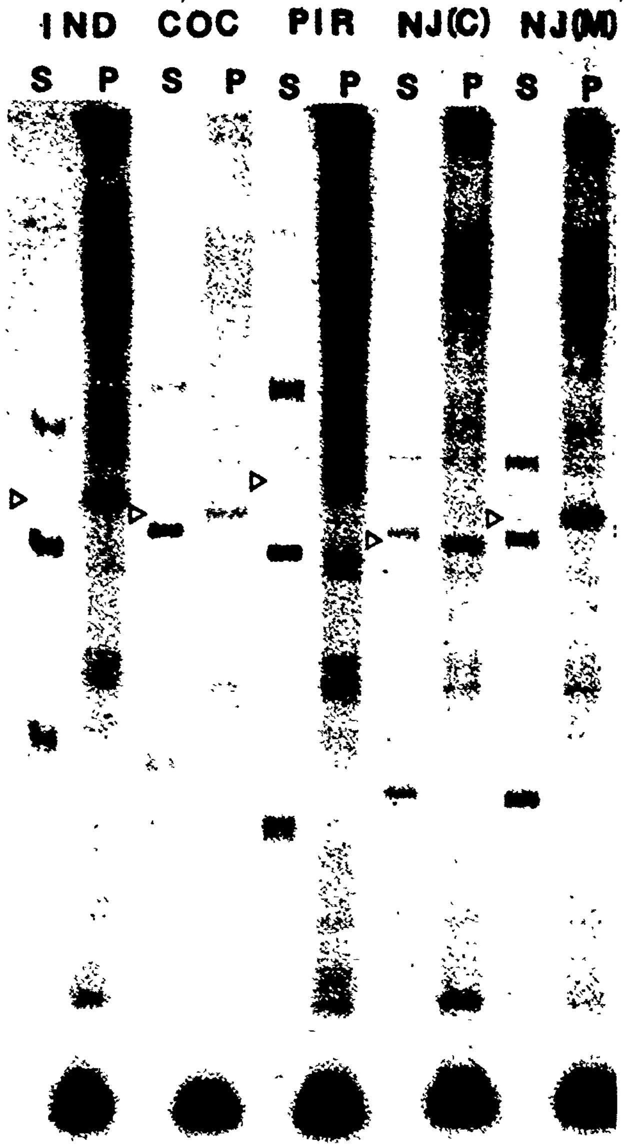
IND
COC
PIR
CHA
NJ (C)
NJ (M)
L

G
NS
N

M

The NS protein is a less abundant constituent of the virion and as a result is less prominent and thus more difficult to detect in autoradiograms. It is, however, a phosphoprotein (Sokol and Clark, 1973) and can be labeled by growing infected cells in the presence of [^{32}P]phosphate.

Infected cells were labeled with [^{32}P]phosphate as described in methods and then analyzed by SDS-PAGE (Figure 2). The same [^{35}S]Met virus preparation employed in Figure 1 were electrophoresed in adjacent sample slots in order to reference the position of NS to the other viral proteins. The NS protein appeared as a prominent labeled band in the [^{32}P]labeled protein channels (P). It was confirmed that the NS protein mobilities of VS NJ(C) and VS NJ(M) were markedly different. VS NJ(C) NS protein was adjacent to the N protein in the SDS-PAGE profile in Figure 2 but was not detectable in Figure 1, presumably because it was masked by the N protein. This situation prevented the isolation of pure NS protein and required that SDS-PAGE conditions be altered to provide adequate separation of NS and N proteins. I found that fractionation of VS NJ(C) proteins on polyacrylamide gels made from 30% acrylamide stock containing 0.8% NN'-methylenebisacrylamide resulted in enhanced separation of N and NS proteins (data not shown). VS NJ Concan NS protein migrates faster than N protein in gels made from this stock acrylamide solution. All other viruses contained NS proteins which resolved away from their respective N proteins. A band below the NS protein in [^{35}S]Met labeled Piry virus, may be the NS precursor described by Bell and Prevec (1979).

Figure 2. Autoradiogram of SDS-PAGE analysis of [^{35}S]Met-labeled viruses of different serotypes and [^{32}P] orthophosphate labeled cells infected with different serotypes. The [^{35}S]Met-labeled virus preparations were purified by differential centrifugation and velocity centrifugation in sucrose gradients. Cells infected at an moi of 10 pfu/cell were labeled with [^{32}P] orthophosphate from 3.5 to 5 hr pi. Samples containing 20K cpm of [^{35}S]Met or 5×10^4 infected L cells labeled with [^{32}P] orthophosphate were introduced into sample cells. Electrophoresis was at 150 V and the labeled protein bands were detected by autoradiography. Samples were arranged in pairs representing [^{35}S]Met-labeled virus (S) and [^{32}P]-labeled infected cells (P) of each virus preparation. Open triangles mark the position of NS protein bonds. The viruses analyzed are labeled with the same abbreviations as Figure 1.


IND COC PIR NJ(C) NJ(M)
S P S P S P S P S P

Although the M protein is phosphorylated (Clinton *et al*, 1979), it is present in this form in small amounts and was not observed by [^{32}P]labeling in Figure 2.

4. PURIFICATION OF PROTEINS

Purified [^{35}S]Met-labeled virions were fractionated on SDS-PAGE of 7.5 or 10% acrylamide concentation made from a 30% acrylamide stock slab gels solution containing 0.4% NN'-methylenebisacrylamide or 0.8% NN'- methylenebisacrylamide when fractionating VS NJ(C) proteins.

Purified virus from 5x10^{8} infected L cells was disrupted in SDS-PAGE sample buffer by heating as described in methods. Each virus preparation was subjected to SDS-PAGE and the gels dried without fixation. The protein bands were located by autoradiography and excised.

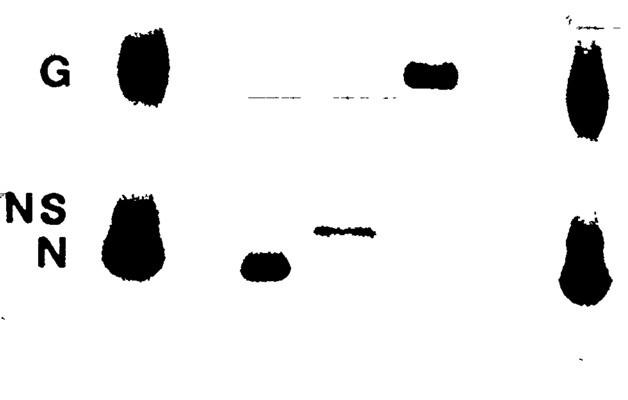
Extraction of proteins from the gel was by diffusion into a buffered 0.1% (w/v) SDS solution as described in methods. The protein concentrations were determined by the spectrophotometric method of Warburg and Christians as described by Layne (1957), and BSA was added as carrier if the concentration was less than 100 ug/ml. When the five VS Ind(T) proteins isolated by this means were assayed by SDS-PAGE (Figure 3) single discreet bands were observed with the same electrophoretic mobility as the corresponding viral protein, thus demonstrating purity and intactness.

5. TRYPTIC PEPTIDE MAPPING

5.1 Comparison of Tryptic Hydrolysis Conditions

Two methods of tryptic digestion were compared: tryptic

Figure 3. Autoradiogram of SDS-PAGE analysis of purified [^{35}S]Met labeled proteins of VS Ind(T). Protein extracts in SDS containing solutions were mixed with SDS PAGE sample buffer and heated prior to electrophoresis. Aliquots from the viral preparation from which the individual proteins were extracted are also included for reference, labeled here as Ind. Samples containing 0.5 to 10 K cpm were applied to each channel and subjected to electrophoresis at 150 V. Proteins were detected by fluorography after impregnation with PPO. Protein designations are indicated at the left and sample designations are noted at the top of each lane.


IND
M
N
NS
G
L
IND
L
G
NS
N
M

digestion of extracted protein and tryptic digestion of protein in gel fragments without prior extraction. The latter method was attractive from the standpoint of simplicity and decreased losses of protein by omitting the manipulations required in extraction.

The [^{35}S]Met labeled N protein of NJ(M) D_1R1 was separated from virions by SDS-PAGE and subsequently digested with trypsin in situ or following extraction; all as described in methods. The resulting maps of tryptic peptides produced by the two procedures were different, as seen in Figure 4, panels A and B. Several peptides are in similar positions and are indicated by arrows but several peptides are in different positions. The state of oxidation of Met and Cys was probably not the basis for the differences since both preparations were oxidized with performic acid.

For later tryptic map analysis, I chose to extract protein before trypsinizatin because it is the more classical approach and because oxidation can precede trypsin treatment. Prior extraction also avoids the possibility that specific fragments will not dissociate from the gel.

5.2 Demonstration of the Need for Oxidation

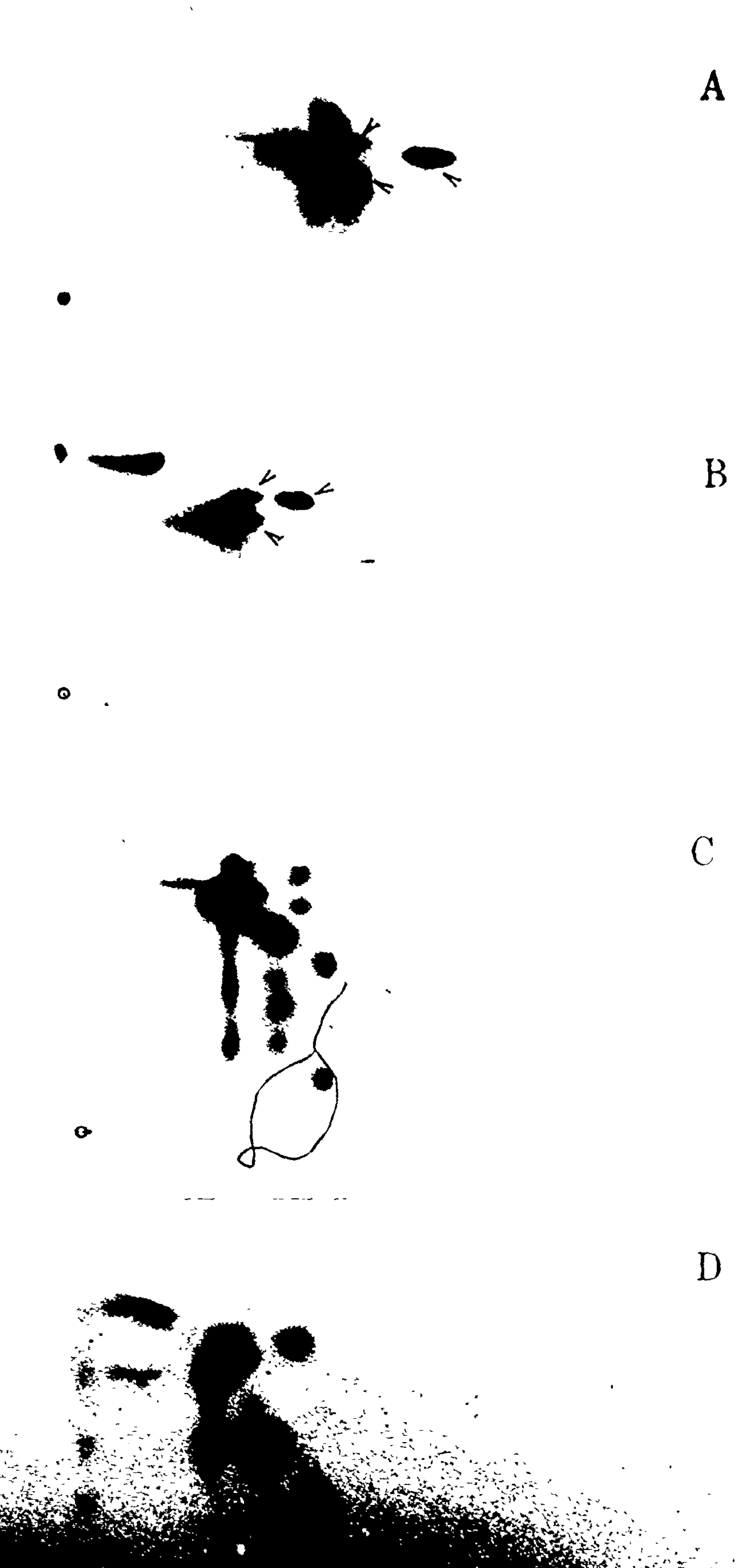
Tryptic peptides which exist in more than one chemical form will increase the complexity of peptide maps. This is a serious consideration when analyzing methionine containing peptides since air oxidation of methionine produces methionine sulfoxide. When [^{35}S]Met labeled Ind(T) protein was tryptic peptide mapped with and without

Figure 4. Autoradiogram of tryptic peptide maps of [^{35}S]Met-labeled viral proteins produced by different procedures. Thin layer electrophoresis was at 450 V at 8°C in pyridine acetate buffer (pH 3.5) in the horizontal dimension. Ascending thin layer chromatography was carried out in a saturated chamber with n-butanol: water: pyridine: acetic acid (204:143: 143:50). Samples contained ~5x10^{4} cpm of [^{35}S] and detection was by autoradiography. The origins are marked by open circles.

Panel A - Nucleocapsid protein of VS NJ (M) D1R1 was trypsinized by immersion in a solution of 50 ug TPCK trypsin/ml 50 mH NH_4^+ HCO_3 (pH 8.5). The resulting peptides were oxidized with performic acid prior to analysis.

Panel B - N protein of VS NJ(M) D1R1 was extracted by diffusion into SDS containing solution. The protein was obtained by preparative SDS PAGE. TCA precipitation, acetone washing and performic acid oxidation preceded trypsinization as described in methods.

Panel C and D - VS Ind (T) N protein labeled with [^{35}S]Met was obtained by SDS-PAGE fractionation of purified virus and subsequent extraction by diffusion. The sample was divided into two portions. One portion was TCA precipitated and acetone washed prior to trypsinization (Panel C), whereas the other portion was TCA precipitated, acetone washed and, in addition, oxidized with performic acid prior to trypsinization (Panel D).


A
B
C
D

performic acid oxidation, it was found that the oxidized protein had a much less complex pattern (Figure 4, panels C and D). This is presumably due to the fact that the non oxidized sample possessed peptides containing methionine in alternate states of oxidation, whereas the oxidized sample possessed peptides containing the stable oxidation product, methionine sulfone. Cysteine and cystine residues are also converted to the stable oxidation product, cysteic acid, as a result of performic acid oxidation. Oxidation of [^{35}S]Met labeled protein was therefore done routine prior to tryptic hydrolysis.

5.3 Tryptic Peptide Map Comparisons

The fingerprints of the methionine containing tryptic peptides of N protein from each of VS NJ(M), VS NJ(C), VS Cocal and Piry virus were compared to the N protein of the prototype virus, VS Ind(T). A peptide with the same map position as a peptide of the Indiana serotype was designated by the spot number characteristic of the VS Indiana peptide, preceeded by the name of the virus serotype; for example, a VS Cocal peptide which is indistinguishable from VS Ind(T) peptide number 1, was termed Cocal 1.

The peptide mapping procedure is described in detail in methods. Proteins of purified virus labeled with [^{35}S]Met were fractionated by SDS-PAGE, eluted by diffusion, alternately TCA precipitated and dissolved 4 times, oxidized in performic acid and trypsinized. The peptides were separated in 2 dimensions, by thin layer electrophoresis (TLE) on cellulose thin layers in pyridine acetate (pH 3.5) buffer

followed by thin layer chromatography (TLC) in n-butanol:pyridine: acetic acid:water (204:143:50:143).

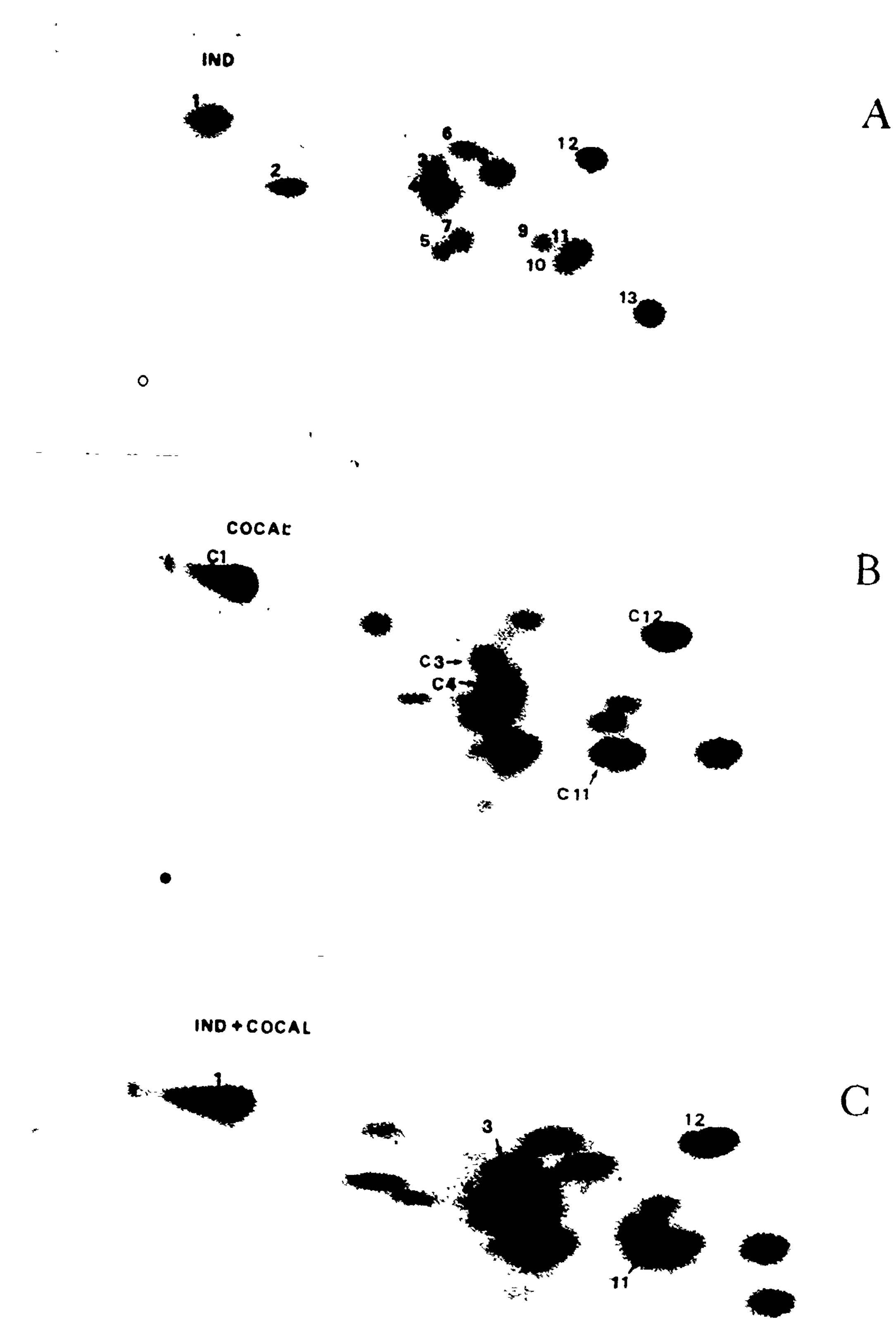
5.3.1 VS Ind(T) N Protein

As seen in Figure 5, the autoradiogram of the tryptic peptides of purified VS Ind(T) N protein after separation in two dimensions by TLE and TLC contains 13 spots. It is evident that tryptic spots have different autoradiographic intensities and either contain different amounts of methionine or are present in different amounts.

The relative radioactivities in these spots based on excision and liquid scintillation counting in toluene based scintillation cocktail fall into 3 groups: six spots (labeled 3,5,6,7,9,10) containing nominally one "unit" of radioactivity, six spots (1,2,8,11,12,13) containing 2 "units" and one spot (4) with 4 units (Table 4). If one "unit" of radioactivity represents 1 methionine residue, then there are 22 methionines in VS Indiana(T) N protein with six tryptic peptides containing 1 methionine, 6 containing 2 methionines, and 1 containing 4 methionines. It would seem unlikely, however, that a 50K MW protein would possess 22 methionines and also that the majority of tryptic peptides would contain more than one methionine residue.

The expected number of methionine labeled tryptic peptides is equal to or less than the number of constituent methionine residues, depending on the number of peptides containing more than one methionine. I wanted to know if the methionine content of VS Ind(T) N protein was

Figure 5. Autoradiogram of tryptic peptide maps for comparison of nucleocapsid proteins of [^{35}S]Met-labeled VS Ind(T) and VS Cocal viruses. Proteins isolated from purified viruses were oxidized with performic acid prior to trypsinization. Samples containing ~8×10^4 cpm of [^{35}S]Met were applied to MN400 cellulose sheets and detection of labeled peptides was by autoradiography. Electrophoresis was at 450 V in pyridine acetate (pH 3.5) and chromatography employed the solvent n-butanol:Pyridine:water:acetic acid (204:143:143:50). The tryptic peptide map of N protein from the VS Ind(T) virus is presented in Panel A. The component peptide spots are numbered. Panel B shows an autoradiogram of [^{35}S]Met-labeled tryptic peptide map of VS Cocal N protein. Peptide spots common with VS Ind(T) N protein peptides are numbered. Panel C represents an autoradiogram of a tryptic peptide map of a mixture of tryptic peptides from VS Ind(T) and VS Cocal N protein. Peptides of VS Ind(T) and VS Cocal which comigrate are numbered according to the designation of the VS Ind(T) peptide.



IND
1
2
3
4
5
6
7
9
10
11
12
13
A
COCAL
C1
C12
C3
C4
C11
B
IND + COCAL
1
3
12
11
C

TABLE 4

QUANTITY OF $[^{35}S]$METHIONINE IN PEPTIDE FRAGMENTS OF VS IND(T) N TRYPTIC DIGESTS

Peptide number[a]	$[^{35}S]$ (cpm)[b]
1	1920
2	1870
3	760
4	3850
5+7[c]	2080
6	1050
8	2230
9	1000
10	1100
11	1910
12	2020
13	2350

(a) Peptide spots were located by autoradiography, excised and quantitated by LSC using toluene based scintillation fluid.
(b) Cumulative values from four tryptic maps and have been corrected for background.
(c) Peptide spots not resolved in most fractionations.

consistent with the [^{35}S]Met-labeled tryptic map complexity. To this end, I analyzed the amino acid content of VS Ind(T) N protein.

5.3.1.1 Amino Acid Analysis of VS Ind(T) N Protein

Purified N protein from purified virus was hydrolyzed by using two different agents: 6 M hydrochloric acid or 3 M p-toluenesulfonic acid. The classic HCl hydrolysis results in the destruction of tryptophan, whereas p-toluenesulfonic acid in the presence of 2-(3-aminoethyl) indole spares tryptophan. Horse heart cytochrome c from Sigma was subjected to hydrolysis with p-toluenesulfonic acid as a control. The calculated amino acid contents found for cytochrome c were in good agreement with the accepted values as seen in Table 5. The values for VS Ind(T) N protein were from two complete determinations after p-toluenesulfonic acid digestion and two partial determinations of HCl hydrolyzed protein. In the latter, the amino acids fractionated on the acidic cation exchange column were poorly resolved and could not be quantified.

The methionine content of VS Ind(T) N protein, assuming a MW of 50K, was calculated to be 13.3±1.4 residues per molecule. This is in good agreement with the value of 12 reported recently by Heyward et al (1978). This value is approximately 1/2 of the methionine content of 22 derived from the radioactivities of the tryptic peptides. If one methionine residue is considered to possess 2 "units" of radioactivity instead of one, then 6 peptide spots contained 1 methionine, 1 peptide spot contained 2 methionines and since a peptide spot cannot contain a fractional content of methionine, it must be concluded that 6 peptide

TABLE 5

AMINO ACID COMPOSITION OF VS IND(T) N PROTEIN

Amino acid	Cytochrome-c[a] control				VS Ind(T) N[b] protein				
	43½hr PTSA	71hr PTSA	$\bar{X}$±SD	Exp-[a] ected	26hr HCl	46½hr HCl	26hr PTSA	72hr PTSA	$\bar{X}$±SD
Lys	21.0	22.2	21.6±.8	19	43.2	38.6	48.9	36.8	41.9±5.4
His	4.0	3.7	3.9±.2	3	7.1	6.9	12.0	7.9	8.5±2.4
NH_4OH	9.3	8.0	8.7±.9	8	ND	ND	ND	ND	ND
Arg	3.1	2.6	2.9±.4	2	29.0	29.6	41.3	28.4	32.1±6.2
Asp	7.7	7.4	7.6±.3	8	48.3	49.4	41.0	49.0	46.9±4.0
Thr	7.2	6.9	7.1±.2	10	ND	ND	17.8	20.4	19.1±1.8
Ser	0.7	0.5	0.6±.1	0	ND	ND	27.1	22.1	24.7±3.5
Glu	10.6	11.2	10.9±.4	12	ND	ND	34.6	35.2	34.9±0.4
Pro	5.8	5.8	5.8± 0	4	ND	ND	23.3	25.2	24.3±1.3
Gly	11.2	10.8	11.0±.3	12	ND	ND	30.4	35.7	33.1±3.7
Ala	6.5	6.4	6.4±.1	6	ND	ND	27.8	32.7	30.3±7.4
Cys	ND	ND	ND		ND	ND	ND	ND	ND
Val	3.8	3.5	3.7±.2	3	ND	ND	24.2	31.2	27.7±4.9
Met	1.9	1.7	1.8±.1	2	13.2	13.9	11.3	14.7	13.3±1.4
Ile	5.0	5.3	5.2±.2	6	23.4	23.2	15.5	21.4	20.9±3.7
Leu	5.7	5.5	5.6±.1	6	41.1	40.5	25.2	38.2	36.3±7.5
Tyr	4.0	3.8	3.9±.1	4	23.2	24.2	20.0	21.5	22.0±1.9
Phe	3.9	4.0	4.0±.1	4	19.3	21.0	15.8	19.2	18.8±2.2
Trp	0.2	0.1	0.2±.1	1	ND	ND	9.2	4.4	6.8±3.4 (11.9)[c]

Purified VS Ind(T) N protein was hydrolyzed with 6 M HCl or 3 M p-toluenesulfonic acid (PTSA).

(a) The MW of horse heart cytochrome c (11,702) and the amino acid composition are from Margoliash et al (1961).
(b) The MW value of 50K was used for VS Ind(T) N protein.
(c) Value determined by extrapolation to 0 time.

spots were present in less than molar concentration. Peptides would be present in submolar amounts if they were partially chemically modified and thus existed as more than one species or if a peptide were incompletely cleaved by enzymic hydrolysis or incompletely recovered after enzymic hydrolysis. The method of protein purification makes it unlikely that the low intensity peptides resulted from contaminant protein. It is most likely that some of the spots in the VS Ind(T) N peptide map represent peptides which were partially altered chemically (as a result of protein preparation and oxidation) or were only partially released by proteolysis.

It is known that performic acid oxidation results in destruction of tryptophan residues (Finlayson, 1969) and also production of halotyrosines in the presence of halogen (Hirs, 1967). Deamidation may have occurred to decrease the yield of specific peptides (Robinson et al, 1970). Some tryptic peptides may be present in lower yields as a function of incomplete cleavage due to the effect of adjacent polar residues (Kasper, 1975; Ambler et al, 1967).

The fact that a peptide is present in less than molar abundance does not decrease its value as a feature of comparison, as long as identical conditions are employed in preparing different samples. The fact that a peptide is present in a lower relative amount is useful in that it represents a phenotypic characteristic. That is; identical peptides should be subject to the same chemical modifications and would be expected to be consistently present as major or minor intensity components.

5.3.2 Comparison of Tryptic Peptide Maps of N Proteins

Figure 5 shows tryptic peptide maps of N protein from VS Cocal and VS Ind(T). These maps contain a similar number of major and minor intensity features but are easily distinguishable. In maps of a mixture of peptides from VS Cocal and VS Ind(T) it was seen that at least 5 of the Cocal peptides comigrate with VS Ind(T) peptides: 1,3,4,11, and 12. Peptide 6 also appeared to comigrate with a VS Cocal peptide in Figure 5, panel C but they have been resolved in other similar analyses (data not shown).

As a further check that the identically migrating tryptic peptides represented identical sequences, I separated these peptides in another chromatography system. When VS Ind(T) and VS Cocal N protein tryptic peptides were chromatographed using n-proponal: 2.5 ammonia (70:30) after TLE, the spots 1,4,11, and 12 as well as a spot which may have been number 3 of each serotype were again indistinguishable (Figure 6). These five peptides probably represented identical sequences in the two viruses since they comigrated in 3 separation systems.

Tryptic peptide maps of VS NJ(C) N protein contained only 4 major intensity [^{35}S]methionine-labeled peptides, making them much less complex than the coresponding VS Ind(T) N peptide maps (Figure 7). VS Indiana(T) peptides 1,3,4, and 7 migrate to positions identical to those of 4 peptides from NJ(C) protein, as evidenced by comigration when mapped in combination. As also shown in Figure 7, the NJ(M) N protein tryptic peptide map was almost identical to that of the Concan strain.

Figure 6. Autoradiogram of tryptic peptide maps for comparison between [^{35}S]Met-labeled tryptic peptide maps of N proteins of VS Ind(T) and VS Cocal employing the chromatography solvent, n-proponal: 2.5 M NH_4OH (70:30). Purified, oxidized, [^{35}S]Met-labeled N proteins of VS Ind(T) and VS Cocal were digested with trypsin and subsequently peptide mapped. Approximately $8x10^4$ cpm of [^{35}S]Met radioactivity was applied to MN 400 cellulose thin layer sheets before fractionation by TLE in pyridine acetate (pH 3.5) and TLC in n-propanol: 2.5 M NH_4OH. Autoradiograms of the resulting tryptic peptide maps are presented in Panels A and B. Peptides with identical map positions are numbered. Panel A contains the tryptic peptide map of VS Ind(T) N protein and Panel B contains the tryptic peptide map of VS Cocal N protein.

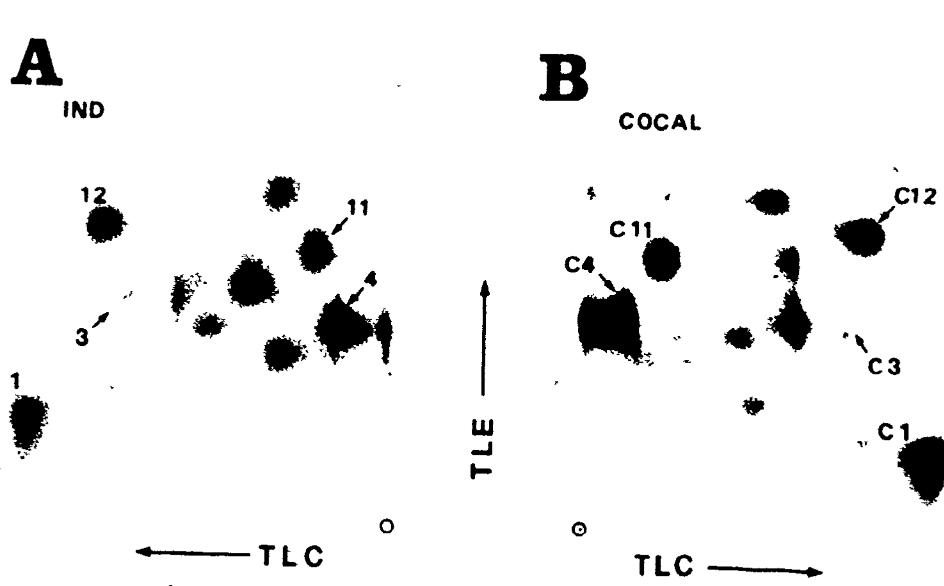
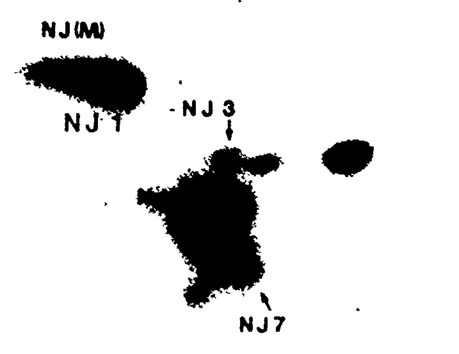

A
IND
12
11
4
3
1
B
COCAL
C12
C11
C4
C3
C1
TLE
TLC
TLC

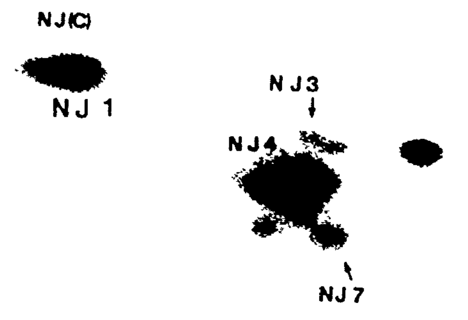
Figure 7. Autoradiogram of tryptic peptide map comparisons of nucleocapsid proteins of [^{35}S]Met-labeled VS NJ(M), VS NJ(C) and VS Ind(T) viruses. Proteins isolated from purified viruses were tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~8×10^4 cpm [^{35}S]Met radioactivity and detection was by autoradiography. The origins are marked by open circles. Peptides with identical map positions to VS Ind(T) peptides are numbered.

Panel A – Tryptic peptide map of N protein from VS NJ(M)
Panel B – Tryptic peptide map of N protein from VS NJ(C)
Panel C – Tryptic peptide map of a mixture of tryptic peptides from N proteins of VS Ind(T) and VS NJ(C).



O

B



O

C

IND + NJ (C)

O

The same four peptides of VS NJ(M), 1,3,4, and 7, were in common with VS Ind(T).

The Piry N protein tryptic map was easily distinguishable from the VS Ind(T) pattern and was less complex possessing 6 major intensity spots (Figure 8). One peptide of the Piry N tryptic peptide map occurs at an identical position with peptide 3 of the VS Ind(T) N protein.

5.3.3 Comparison of Tryptic Peptide Maps of M Proteins

The prototype VS Ind(T) tryptic map possessed five major intensity methionine containing spots and several minor intensity spots labeled 1-10 on Figure 9. Amino acid analysis data of Kendal and Cohen (1976) indicated 6.6 methionine residues but 13 were predicted from the M mRNA sequence of J. Rose (personal communication). The tryptic peptide map complexity could not discriminate between the two methionine contents.

The VS Cocal M protein tryptic map consists of only two major methionine containing peptides neither of which comigrates with the reference peptides of the prototype (Figure 9). Four minor spots coincided with reference tryptic peptides 1,3,8, and 9, but peptide 3 of Ind(T) is a high intensity spot and thus is probably different from the low intensity peptide Cocal 3.

The VS NJ strains Missouri and Concan produce simple peptide maps of 5 major intensity spots (Fig. 10), four of which are indistinguishable when co-run (data not shown). The VS NJ(M) M protein peptide map contained 3 peptides, 5,6, and 9, in common with the

Figure 8. Autoradiogram of tryptic peptide map comparison of nucleocapsid proteins of [^{35}S]Met-labeled Piry and VS Ind(T) viruses. Proteins isolated from purified virus preparations were tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~8x10^{4} cpm [^{35}S]Met radioactivity and detection was by autoradiography. The origins are marked by open circles. Tryptic peptides with identical map positions to VS Ind(T) are numbered.

Panel A - Tryptic peptide map of N protein from Piry virus.

Panel B - Tryptic peptide map of a mixture of tryptic peptides derived from N proteins of VS Ind(T) and Piry virus.

PIRY
A
P3

IND + PIRY
B
3

Figure 9. Matrix protein tryptic peptide map comparisons of VS Ind(T) and VS Cocal. Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~$8x10^4$ cpm of [^{35}S]Met radioactivity and was detected by autoradiography. Peptides with identical map positions to VS Ind(T) peptides were numbered. The origins are marked by open circles.

Panel A - Tryptic peptide map of M protein from VS Ind(T). Peptides are numbered for reference.

Panel B - Tryptic peptide map of M protein from VS Cocal.

Panel C - Tryptic peptide map of a mixture of tryptic peptides derived from M protein of VS Ind(T) and VS Cocal.

IND

A

2

1

8

9 4 3 5

6

7

COCAL

B

C9

C1 C8

C3

IND + COCAL

C

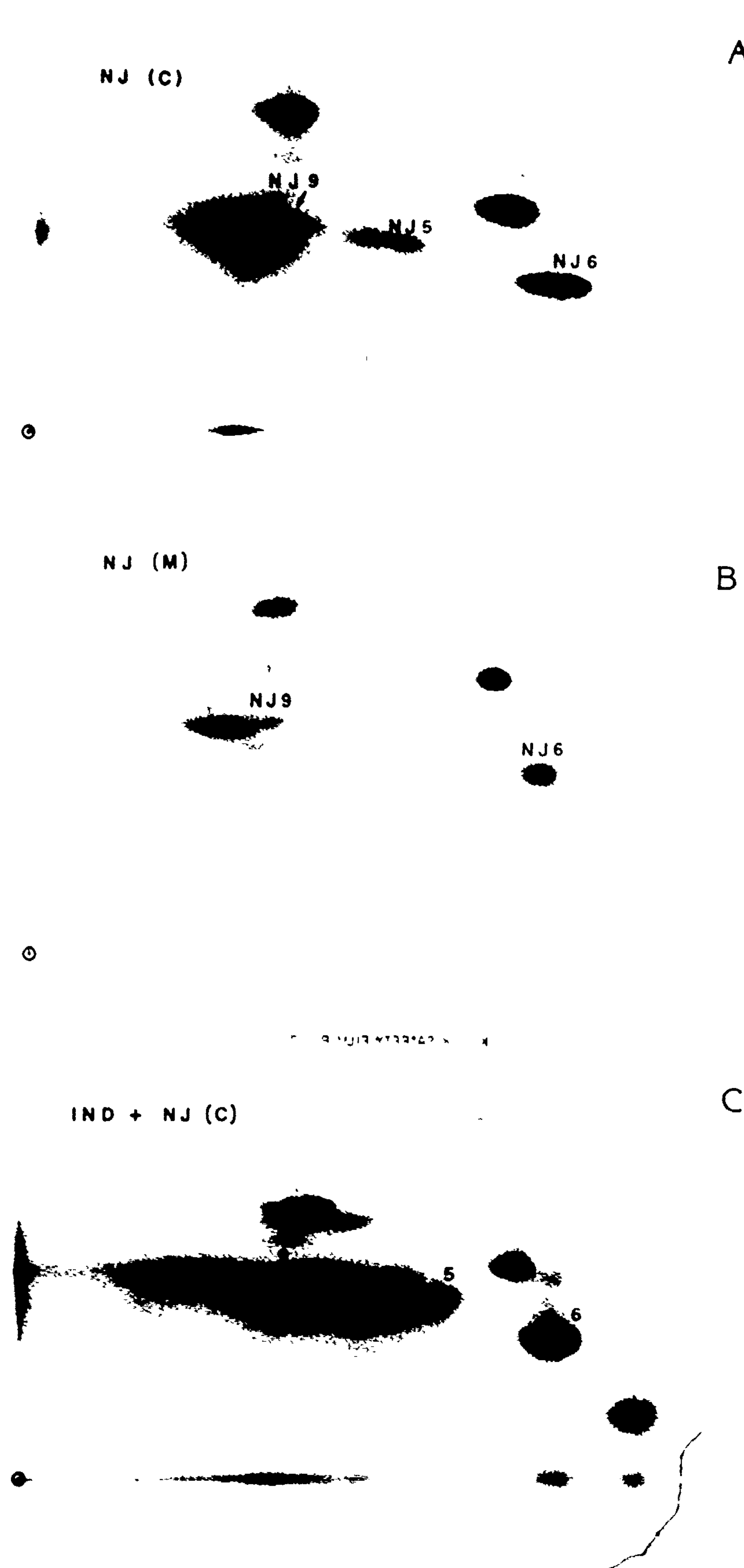
9

3

1 8

Figure 10. Autoradiograms of [^{35}S]Met-labeled matrix protein tryptic peptide maps for comparison of VS Ind(T), VS NJ(C) and VS NJ(M). Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~8x10^{4} cpm of [^{35}S]Met radioactivity and was detected by autoradiography. Peptides with identical map positions to VS Ind(T) peptides were numbered. The origins are marked by open circles.

Panel A - Tryptic peptide map of M protein from VS NJ(C)
Panel B - Tryptic peptide map of M protein from VS NJ(M)
Panel C - Tryptic peptide map of a mixture of tryptic peptides derived from M proteins of VS Ind(T) and VS NJ(C).

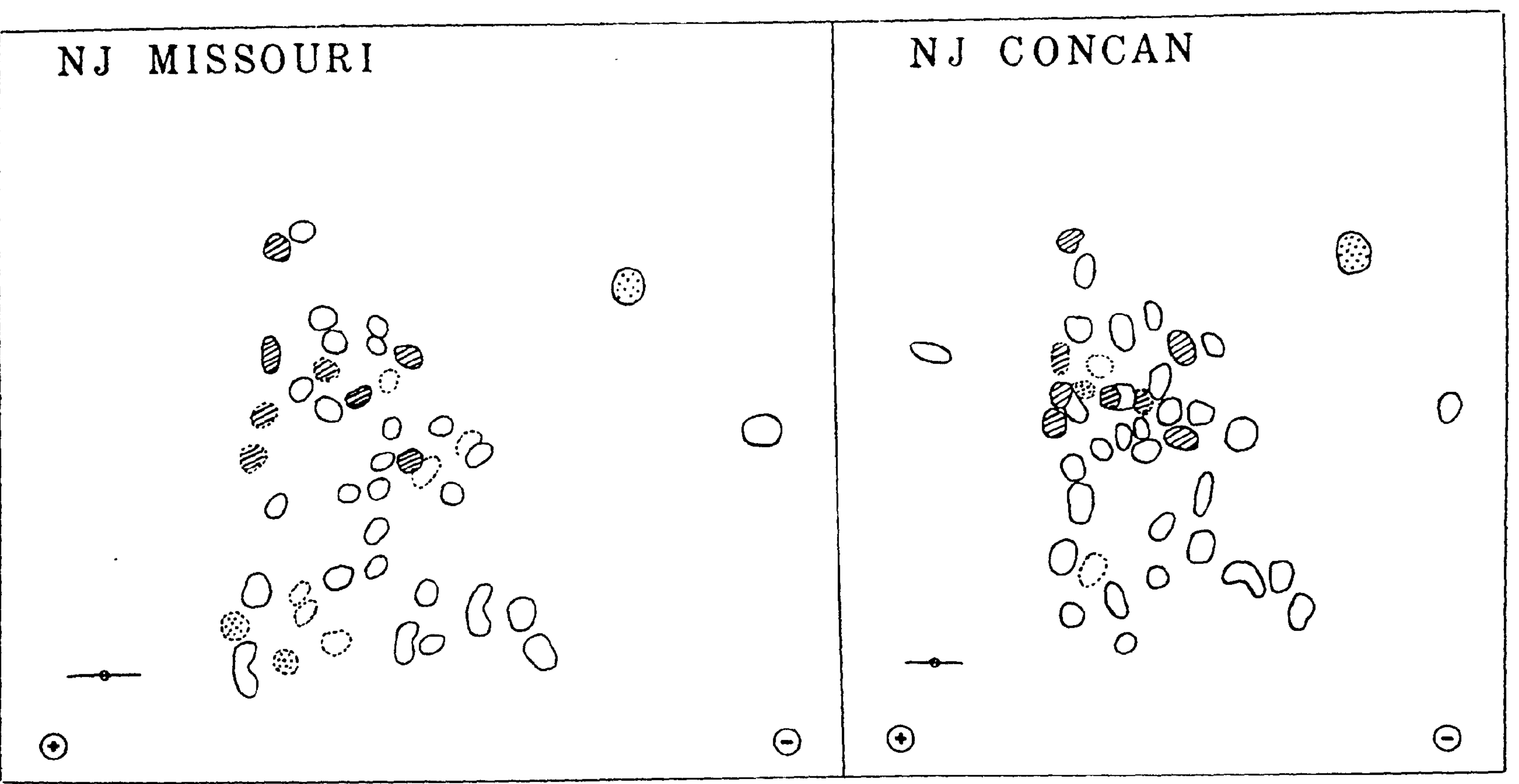

A
NJ (C)
NJ9
NJ5
NJ6
B
NJ (M)
NJ9
NJ6
C
IND + NJ (C)
5
6

reference pattern and VS NJ(C) M protein possessed 2 peptides, 6 and 9, in common with the prototype M protein.

To determine whether the degree of homology demonstrated by the methionine containing subset of the tryptic peptides was representative of the total tryptic peptide homology, tryptic peptide maps were prepared for M protein of VS NJ strains, Missouri and Concan, by ninhydrin and fluorescamine staining. Both of these agents react with primary amines to yield coloured and fluorescent products respectively (von Arx and Neher, 1963; Weigele et al, 1972). Detection of peptides by ninhydrin or fluorescamine staining requires ~10 nmoles of material which was 0.25 mg in the case of the 25K MW M protein. The same protein digests used in Figure 9 were used to prepare peptide maps for staining. Approximately 0.5 mg of tryptic peptides were used in each peptide map. The tryptic peptide maps were stained with ninhydrin and the resulting spots were marked. The same thin layer sheets were then stained with fluorescamine and any additional spots detected under ultra violet illumination were scored. The peptide maps were then autoradiographed to locate [^{35}S]Met containing peptides.

Figure 11 represents the composite tracings of tryptic peptide maps of ninhydrin and fluorescamine stained M proteins from VS NJ strains, Missouri and Concan. The stained tryptic peptide maps are several fold more complex than the [^{35}S]Met labeled tryptic peptide maps but comparison of the Missouri and Concan peptide maps shows them to be distinguishable but generally similar. While the exact relationship of peptides is difficult to determine without cochromatography of samples,

Figure 11. Composite tracing of ninhydrin and fluorescamine staining tryptic peptides of VS NJ(M) and VS NJ(C). Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and subsequently tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Peptide maps were stained with ninhydrin and then fluorescamine as described in methods. The [^{35}S]Met containing peptides were located by autoradiography. Peptide spots are indicated by circling with solid lines for prominently stained spots and broken lines for lightly stained spots. Spots detected by fluorescamine staining are stipled, whereas, spots labeled with [^{35}S]Met are indicated by hatch marks.


NJ MISSOURI
NJ CONCAN

it would appear that the relationship between the methionine-labeled maps is representative of the extent of homology between the total tryptic peptide maps. Half of the stained tryptic peptides may be common between M proteins of VS NJ(M) and VS NJ(C).

Piry M protein tryptic maps were unique and featured four major component spots (Figure 12). None of the [^{35}S]Met labeled peptides copurified with peptides of the reference map (Figure 12).

5.3.4 Comparisons of Tryptic Peptide Maps of Glycoproteins

It can be seen in Figure 13 that the [^{35}S]Met labeled tryptic peptide map of VS Ind(T) G protein consists of 4 or 5 spots of dominant intensity. This number of labeled tryptic components is consistent with the methionine content of 5.7 per molecule calculated by Schloemer and Wagner (1975), considering a MW of 62K for the polypeptide portion of the molecule, but is less consistent with the findings of Doel and Brown (1978) who calculated 9.6 methionines per glycoprotein.

The Cocal G tryptic map was of similar complexity to the reference map but produced a distinguishable pattern. Three tryptic peptides of VS Ind(T) G protein labeled 5,6, and 7, coincide with three VS Cocal G tryptic peptides as evidenced in Figure 13.

Figure 14 represents glycoprotein peptide maps of VS NJ(C), VS NJ(M), and a mixture of VS NJ(C) with the VS Ind(T) reference protein. The VS NJ(M) and VS NJ(C) G protein peptide pattern as seen in Figure 14 are totally distinct from the VS Ind(T) reference peptide map. The VS NJ strains were distinguishable but did contain four peptides in common: a,b,c, and d.

Figure 12. Autoradiograms of [^{35}S]Met-labeled matrix protein tryptic peptide maps for comparison of VS Ind(T) and Piry. Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~8x10^{4} cpm of [^{35}S]Met radioactivity. The origins are marked by open circles.

Panel A - Tryptic peptide map of M protein from Piry virus.

Panel B - Tryptic peptide map of a mixture of tryptic peptides derived from M proteins of VS Ind(T) and Piry.

PIRY

A

O

IND + PIRY

B

Figure 13. Autoradiograms of [^{35}S]Met-labeled glycoprotein tryptic peptide maps for comparisons of VS Ind(T) and VS Cocal. Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~5×10^4 cpm of [^{35}S]Met radioactivity. Tryptic peptides with identical map positions to VS Ind(T) peptides are numbered. The origins are marked by open circles.

Panel A - Tryptic peptide map of G protein from VS Ind(T)
Panel B - Tryptic peptide map of G protein from VS Cocal
Panel C - Tryptic peptide map of a mixture of tryptic peptides derived from G proteins of VS Ind(T) and VS Cocal.

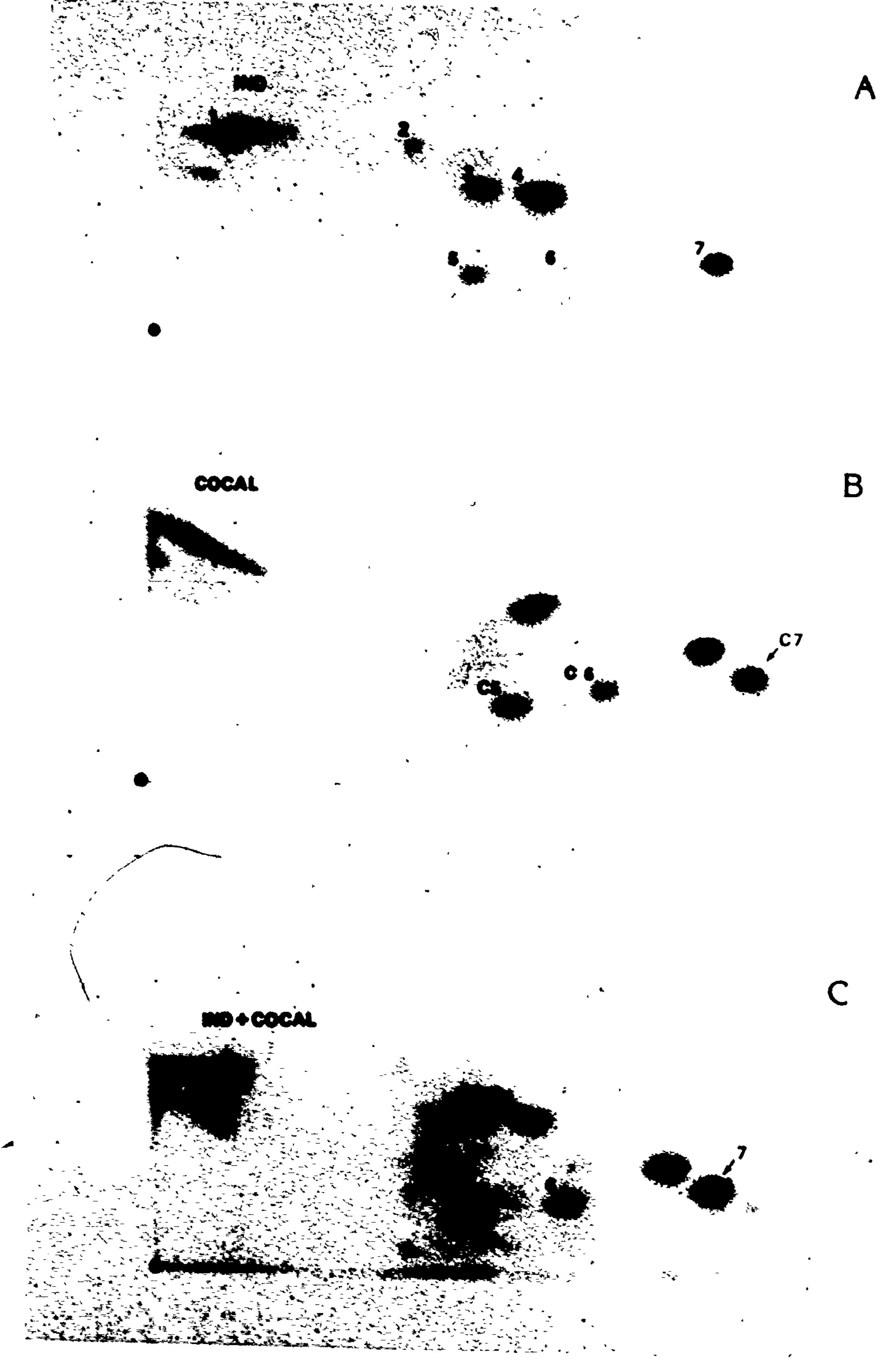
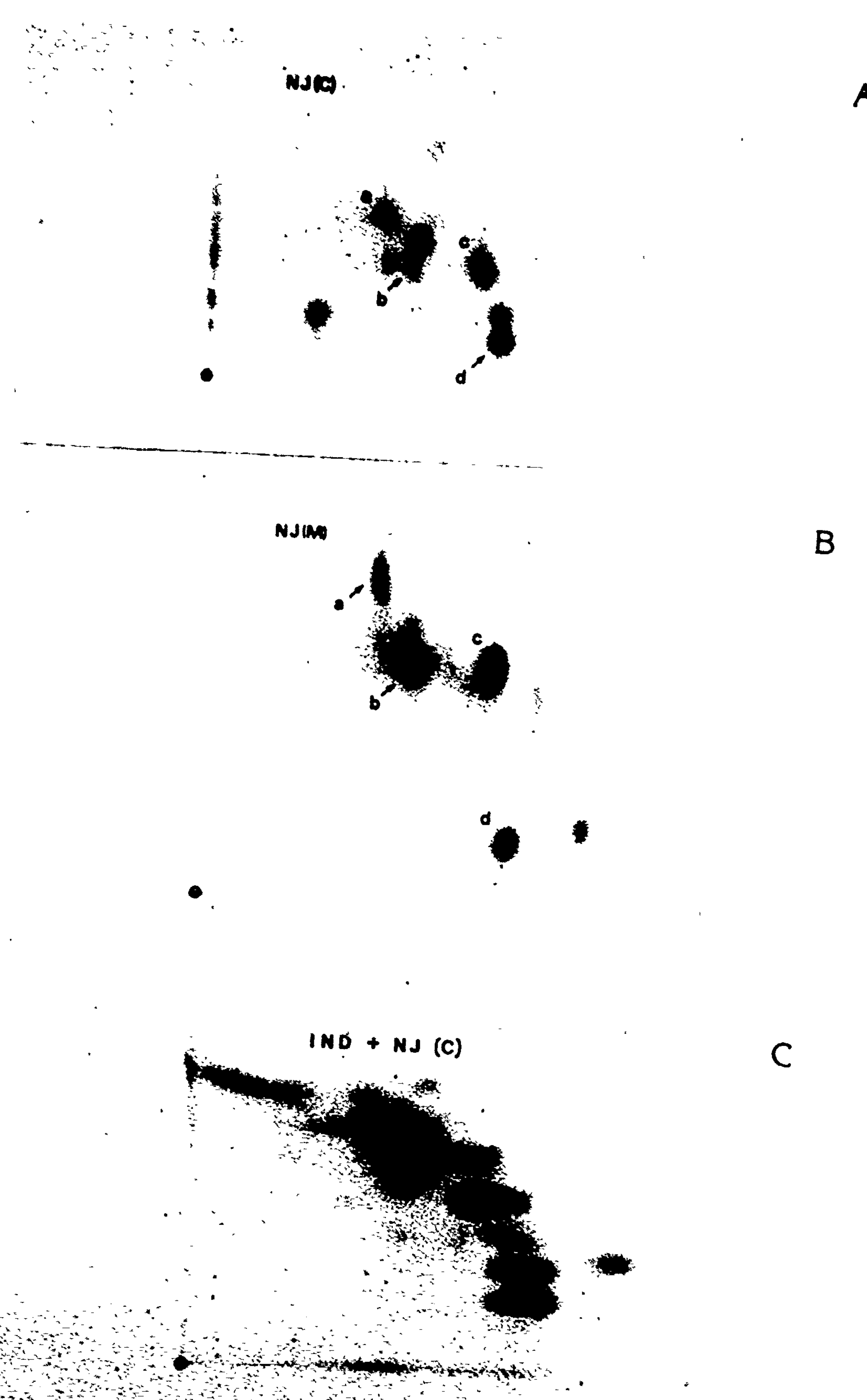

A
IND
2
4
5
6
7
B
COCAL
C7
C 6
C
IND + COCAL
7

Figure 14. Autoradiograms of [^{35}S]Met-labeled glycoprotein tryptic peptide maps for comparison of VS Ind(T), VS NJ(C), and VS NJ(M). Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~5×10^4 cpm of [^{35}S]Met radioactivity. The origins are marked by open lines.

Panel A - Tryptic peptide map of G protein from VS NJ(C). Peptides in common with VS NJ(M) are marked with letters, a-d.

Panel B - Tryptic peptide map of G protein from VS NJ(M). Peptides in common with VS NJ(C) are marked with letters, a-d.

Panel C - Tryptic peptide map of a mixture of tryptic peptides derived from G proteins of VS Ind(T) and VS NJ(C).


NJ(C)
A
a
b
c
d
NJ(M)
B
a
b
c
d
IND + NJ (C)
C

The tryptic peptide pattern of Piry G protein is distinct as demonstrated in Figure 15. This peptide map did not possess any peptide features in common with the reference pattern as evidenced by mapping in combination with the VS Ind(T) reference protein.

5.3.5 Tryptic Peptide Maps of Vesiculovirus L and NS Proteins

Tryptic peptide maps of [^{35}S]Met labeled NS and L proteins are presented here but they were not compared in the same manner as the proteins N,M, and G due to the relative paucity of these proteins in virions and, in the case of L protein, the high level of complexity of the resulting tryptic maps.

Tryptic peptide maps of [^{35}S]Met labeled L and NS proteins of each virus type were produced and are represented in Figures 16 and 17. Tryptic digests were not mapped in combination with the VS Ind(T) reference protein thus preventing detailed comparison of individual peptide spots, but general pattern features were compared.

Amino acid analysis of VS Ind(T) NS protein by Heyward et al (1978) indicated 6 methionine residues but J. Rose has predicted 3 methionine-containing tryptic peptides on the basis of nucleotide sequencing of the NS gene (personal communication). The VS IND(T) NS tryptic peptide map has 3 major intensity and one minor intensity spot which would suggest 3 methionine residues.

The reader should be reminded at this point that the viral NS protein can be fractionated into several forms by DEAE cellulose chromatography and SDS-urea-PAGE (Kingsford and Emerson, 1980). The NS peptide maps presented here are, therefore, mixtures of different forms

Figure 15. Autoradiograms of [^{35}S]Met-labeled glycoprotein tryptic peptide maps for comparison of VS Ind(T) and Piry viruses. Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained ~$2x10^4$ cpm of [^{35}S]Met radioactivity. The origins are marked by open circles.

Panel A - Tryptic peptide map of G protein from Piry virus.

Panel B - Tryptic peptide map of a mixture of tryptic peptides derived from G proteins of VS Ind(T) and Piry.

PIRY A

IND + PIRY B

and may offer a further reason for some submolar intensity peptides, however none of the [^{35}S]Met labeled peptides of cellular NS protein appear to contain phosphate (Appendix I).

Of the five NS protein maps, the VS NJ(C) and VS NJ(M) patterns are the most similar (Figure 16). NS tryptic peptide maps of VS Ind(T), VS Cocal, and Piry are unique but there may have been some pattern overlap. However, it is not possible to accurately determine if component spots were coincident without mapping in mixture with the reference protein.

The L tryptic peptide pattern of VS Ind(T) was more complex than could be predicted by the methionine content of 12 residues reported by Heyward et al (1978), and suggested at least twice as many methionines. Visual comparison of the 5 L protein tryptic peptide maps in Figure 17 demonstrated a similar trend of relationship as in the NS patterns, with the highest degree of similarity detectable between the Missouri and Concan strains of VS NJ. A group of 6 peptide spots exist in identical conformation in both VS NJ(C) nad VS NJ(M) and are indicated by arrows in Figure 17. Piry, VS Ind(T), and VS Cocal L tryptic maps were unique but the extent of pattern overlap could not be ascertained. The Piry L protein pattern was much less complex than the other L proteins tested.

5.3.6 Tryptic Peptide Maps Other Proteins Isolated from Virions

In addition to the 5 virus specific proteins normally detected by SDS-PAGE analysis of virions, other minor intensity bands are always present. I have prepared tryptic peptide maps of several of these [^{35}S]Met-labeled extra bands and have presented autoradiograms of these

Figure 16. Autoradiograms of [^{35}S]Met-labeled NS protein tryptic peptide maps of different serotypes. Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sample contained 1-2x10^4 cpm of [^{35}S]Met radioactivity. The origins are marked by open circles. An open triangle is included in each panel as a reference. Its position relative to the origin relates to the extent of electrophoresis and chromatography. NS proteins tryptic peptide maps are displayed in the different panels: VS Ind(T), (A); VS Cocal, (B); Piry, (C); VS NJ(C), (D); and VS NJ(M), (E).

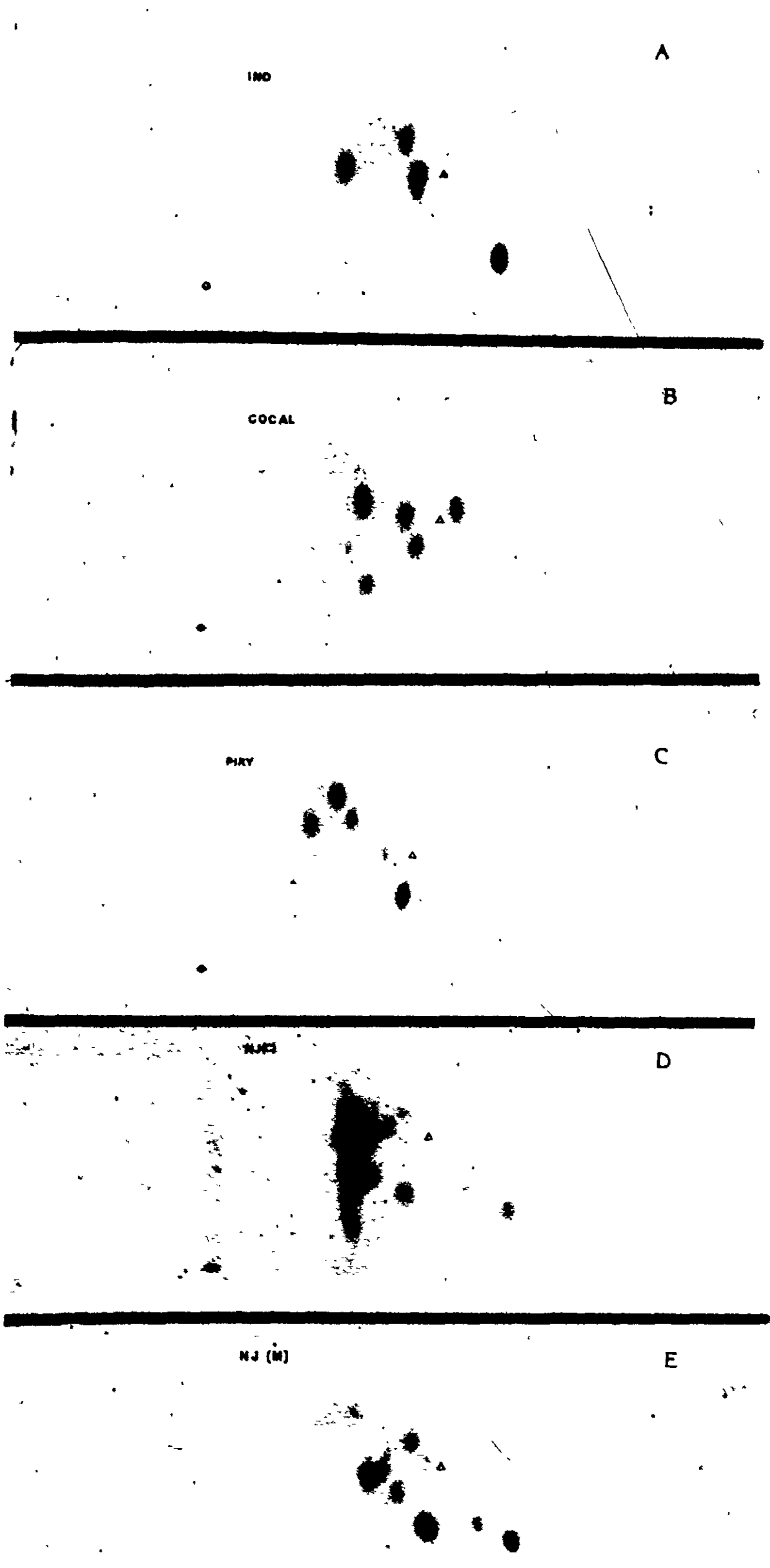
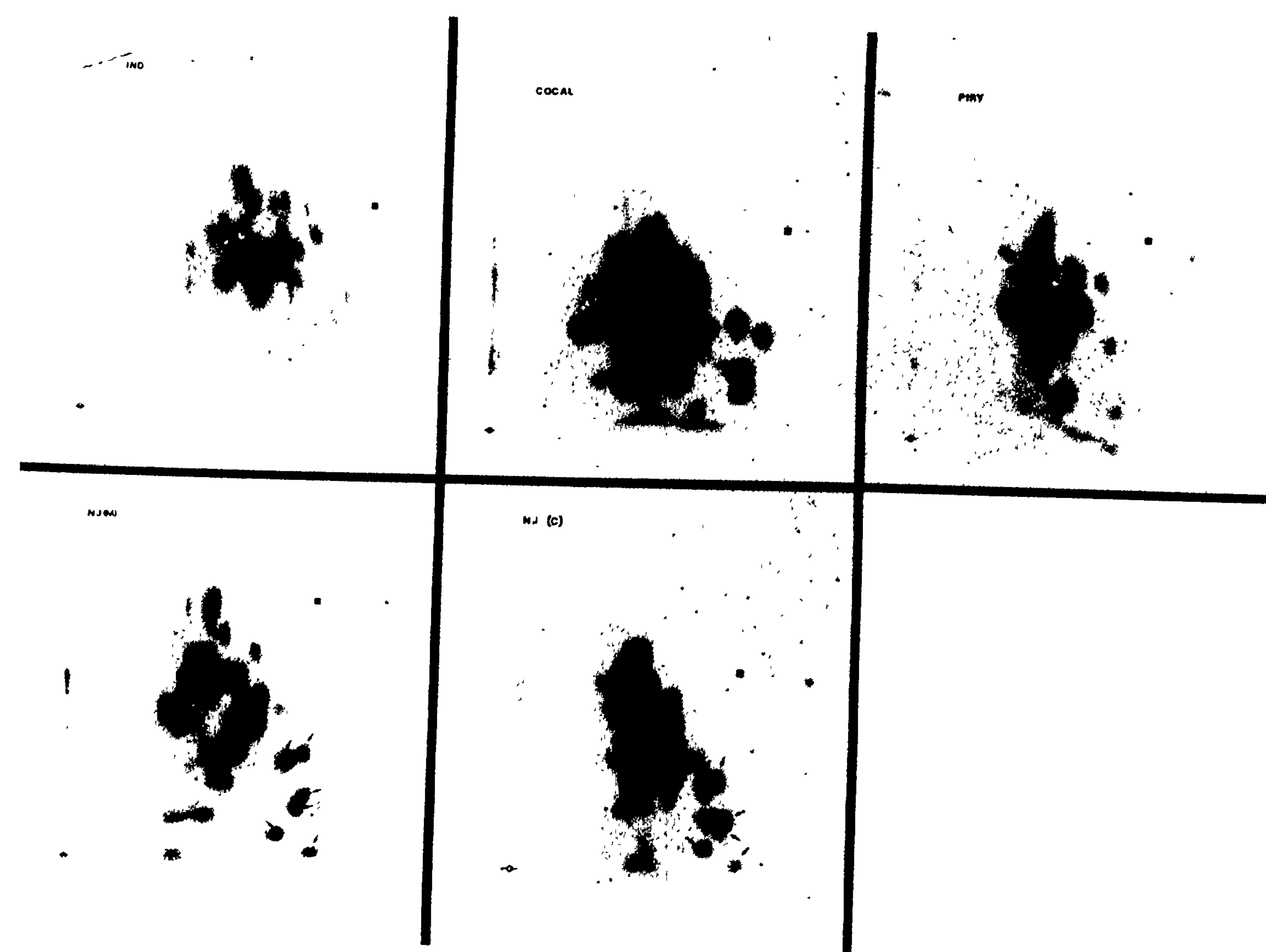

A
IND
B
COCAL
C
PIRY
D
E
NJ (M)

Figure 17. Autoradiograms of [^{35}S]Met-labeled L protein tryptic peptide maps of different serotypes. Proteins labeled with [^{35}S]Met were isolated from purified virus preparations and tryptic peptide mapped as described in methods employing MN400 cellulose thin layer sheets. Each sampl- contained ~1x10^{5} cpm of [^{35}S]Met radioactivity. The origins are marked by open circles. A closed square serves as a reference point in each panel. The position of the square relative to the origin relates to the extent of electrophoresis and chromatography. L protein tryptic maps of different viruses are displayed in the panels: VS Ind(T), VS Cocal, Piry, VS NJ(M) and VS NJ(C).



IND
COCAL
PIRY
NJ (C)

in Appendix I. Most of these peptide patterns are partially or totally identical to patterns from G and M proteins from the corresponding virus. The tryptic peptide map of a protein band subjacent to VS Cocal N protein possessed a similar pattern to its NS protein. Recently two host specified proteins have been detected in VS Ind but these were not detected when cells were labeled after infection (Lodish and Porter, 1980). However, Bishop and Roy (1972) have noted the existence of two other proteins subjacent to N protein on SDS-PAGE profiles and have termed them A and B.

6. PEPTIDE MAPPING BY PARTIAL PROTEOLYSIS

6.1 Partial Proteolysis of N Proteins with V8 Protease and Analysis by SDS-PAGE

The method of Cleveland *et al* (1977) as modified by Nathanson and Hall (1979) was used. The method involves SDS-PAGE analysis of a protein sample in the presence of protease and SDS. Proteolysis occurs in the stacking gel during electrophoresis and subsequently the proteolysis products are separated in the resolving gel. I employed *S. aureus* V8 protease which primarily cleaves glutamoyl peptide bonds (Houmard and Drapeau, 1972).

N protein of VS Ind(T), VS Cocal, VS NJ(M) and Piry virus were separated by SDS-PAGE from [^{35}S]Met-labeled infected cells. The dried gel fractions were equilibrated in sample buffer and then applied to a 15% polyacrylamide gel in the presence of different concentrations of V8 protease in agar containing SDS sample buffer. Electrophoresis was

carried out at 100 V with a 15 min interruption when the BPB marker was 1 cm from the resolving gel to allow proteolysis.

Figure 18 represents an autoradiogram of N proteins reacted with 0, 0.001, 0.01, and 0.1 ug of V8 protease and fractionated by SDS-PAGE. Each N protein pattern while unique did share similarities which were demonstrable after different extents of cleavage. VS Ind(T), VS NJ(M), and VS Cocal N proteins possessed similar patterns and peptide mobilities when treated with 0.001 ug of V8 protease. The Piry N pattern deviated the most from the other three patterns under that condition but there was some pattern overlap with VS NJ(M) protein. The peptide patterns produced from VS Cocal and VS NJ(M) with the highest enyzme concentration were very similar. The VS Ind(T) pattern resulting from 0.1 ug of V8 protease was simple but contained some features coincident with Cocal.

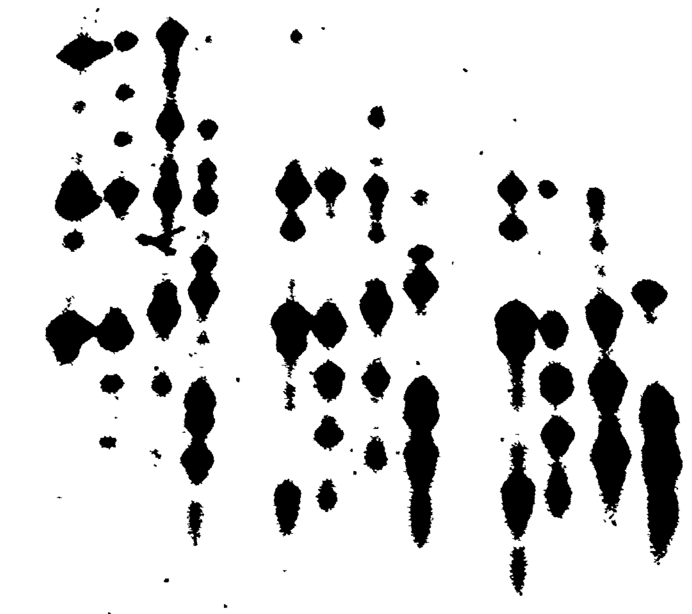
To encapsulate the findings of this approach, the viruses VS Ind(T), VS Cocal, and VS NJ(M) were the most similar with less similarity to Piry.

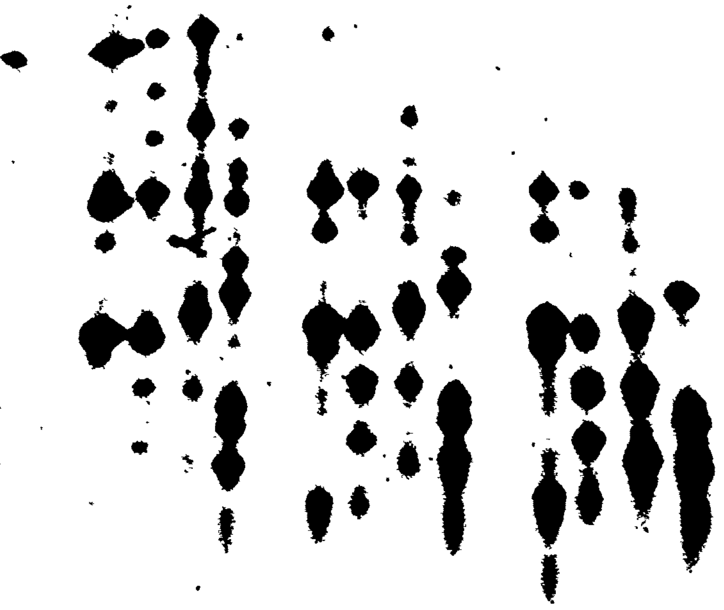
6.2. <u>Adaptation of Partial Proteolysis Mapping to Chemical Cleavage of Tryptophanyl Peptide Bonds.</u>

Partial enzymatic proteolysis and peptide separation by SDS-PAGE provides a convenient and adaptable procedure for the comparison of viral proteins. I adapted the general approach to employ chemical cleavage at tryptophan residues. Two major advantages are gained by this. All the tryptophan residues in a molecule will be susceptible to

A B C D

I C N P I C N P I C N P I C N P




A B C D
I C N P I C N P I C N P I C N P

partial cleavage and contribute to the resulting peptide pattern; contrary to enzymic proteolysis in SDS which restricts the specificity to a subset of the bonds normally defined to be susceptible to the protease. Furthermore, tryptophan is an ideal site of comparison because it is both the rarest and evolutionarily the most highly conserved amino acid (Dayhoff *et al*, 1972).

BNPS-skatole and N-chlorosuccinimide (NCS) are mild oxidants which cleave peptide bonds formed by the carboxyl group of tryptophan residues with complete specificity (Omenn *et al*, 1970; Schechter *et al*, 1976). Both reagents are suited for partial proteolysis since reaction with large excess of reagent results in partial cleavage of Trp bonds.

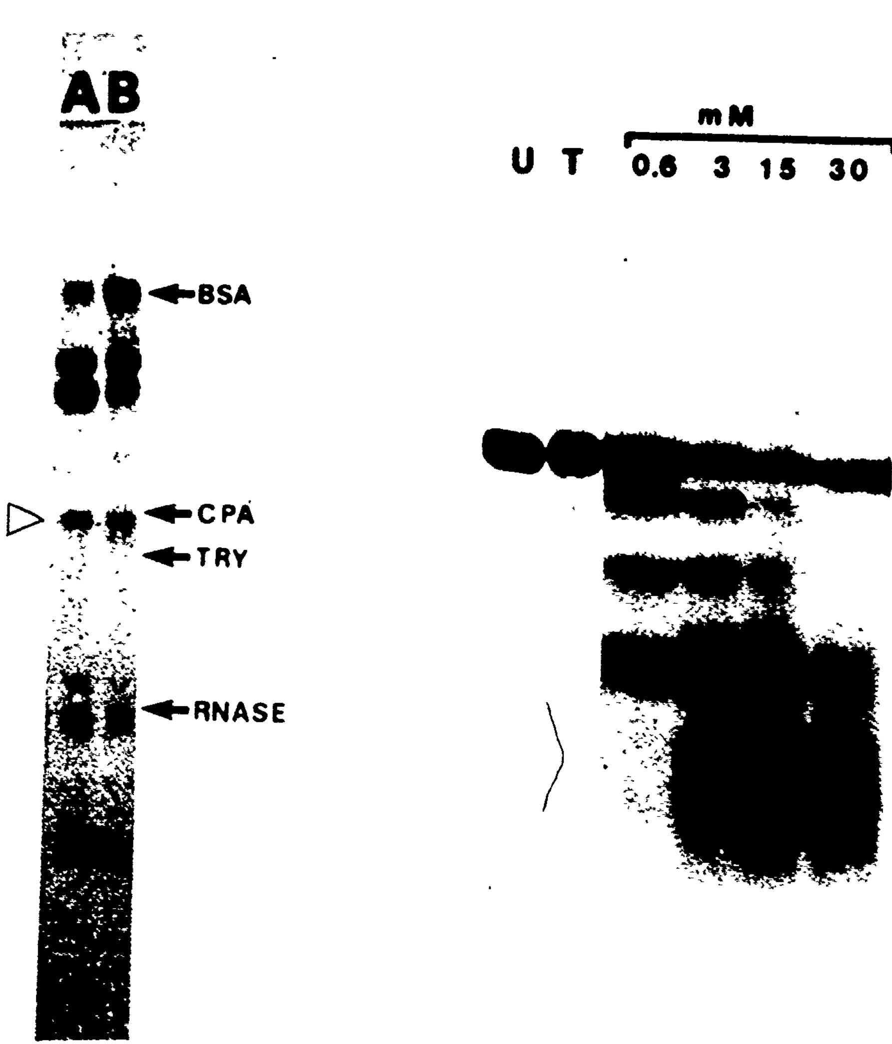
BSA which possesses 2 tryptophan residues was used to compare the two reagents. BSA samples were reacted with NCS by dissolving in 4 volumes of 50% acetic acid, followed by the addition of 100 equivalents of NCS in one volume of dimethyl formamide, and incubation for 2 hr with shaking at ambient temperature. For reaction with BNPS-skatole, BSA was dissolved in 14 volumes of 70% acetic acid and then 100 equivalents of BNPS-skatole in 1 volume of glacial acetic acid were added and incubated at 37°C for 24 hr. Reaction products were collected by acetone precipitation in each case.

Treatment of BSA with either NCS or BNPS-skatole generated seven identical peptide fragments on SDS-PAGE analysis as seen in Figure 19, left panel. From the sequence position of the two Trp residues in BSA (Dayhoff, 1976) six of the seven fragments were predicted. The

Figure 19. SDS-PAGE analysis of the peptides generated by cleavage of bovine serum albumin (BSA) and Piry virus M protein with N-chlorosuccinimide (NCS) or BNPS-skatole.

Left Panel - To crystalline BSA (Sigma Chemical Co.) in 70% glacial acetic acid was added 100 equivalents of BNPS-skatole in glacial acetic acid and reacted for 24 hr at 37°C. The peptide were collected as described in methods. Another portion of BSA was treated with 100 equivalents of N-chlorosuccinimide for 2 hr at room temperature with agitation. One volume of NCS in DMF was added to 4 volumes of a solution of BSA in 50% acetic acid. Reaction products were collected and dissolved in SDS-PAGE sample buffer. Samples containing 20 ug of cleaved BSA were fractionated by SDS-PAGE in a 15% polyacrylamide gel containing 0.2% NN'-methylenebisacrylamide. The peptides were detected by staining with CBB. Lane A contained BNPS-skatole cleaved BSA and lane B contained NCS cleaved BSA. The location of MW markers are indicated: BSA (bovine serum albumin), MW 66,000; CPA (α-bovine carboxypeptidase A) MW 35,000; TRY (PMSF-trypsinogen), MW 24,000; RNase (pancreatic ribonuclease), MW 13,700.

Right Panel - Piry virus M protein labeled with [^{35}S]Met was extracted by electroelution from a preparative polyacrylamide gel and then dialyzed against water. Samples of protein were made 0.2 M in acetic acid and reacted with NCS at the centration indicated in the right panel. Two control samples are included: (U), an untreated control and (T), a control sample treated in an identical manner to the cleaved samples but with NCS omitted. The controls were analyzed by SDS-PAGE on a 15% acrylamide 0.2% NN'-methylene-bisacrylamide gel. The NCS cleaved M protein samples were fractionated on a gel formed from a gradient of acrylamide, 15-20% acrylamide and 0.2-0.26% NN'-methylenebisacrylamide. Samples containing 10K cpm of [^{35}S]Met were subjected to electrophoresis at 150 V. The gel was subsequently impregnated with PPO and subjected to fluorography.



AB
BSA
CPA
TRY
RNASE
mM
U
T
0.6
3
15
30

unexpected fragment, indicated by an open arrow, is present in NCS cleavage patterns of reduced and carboxymethylated BSA suggesting that it is not the result of disulfide bond formation (data not shown). The extra band was also present when BSA was cleaved with NCS in the presence of 2.5% (w/v) SDS or 8 M urea.

Protein samples required prior denaturation in order to be susceptible to NCS cleavage. When BSA was denatured with SDS prior to NCS cleavage, mildly acidic conditions (0.2 M acetic acid) could be employed to produce the same cleavage map as in Figure 19, which employed 8.8 M acetic acid (data not shown). NCS was chosen for routine use since it reacts more rapidly and under less acidic conditions than BNPS-skatole

The effective concentration range of NCS was determined by cleavage of [^{35}S]methionine-labeled Piry virus M protein and then fractionation on a gradient acrylamide gel (15-20% acrylamide) as shown in Figure 19, right panel. From the peptide products of cleavage with 0.6, 3, 15, and 30 mM NCS in 0.2 M acetic acid, it appeared that two intermediate concentrations, 3 and 15 mM, were the most suitable for generating partial cleavage maps since they produced fragments covering the entire range of sizes (Figure 19, right panel). Complete cleavage was not obtainable at the highest concentration of NCS employed (30 mM).

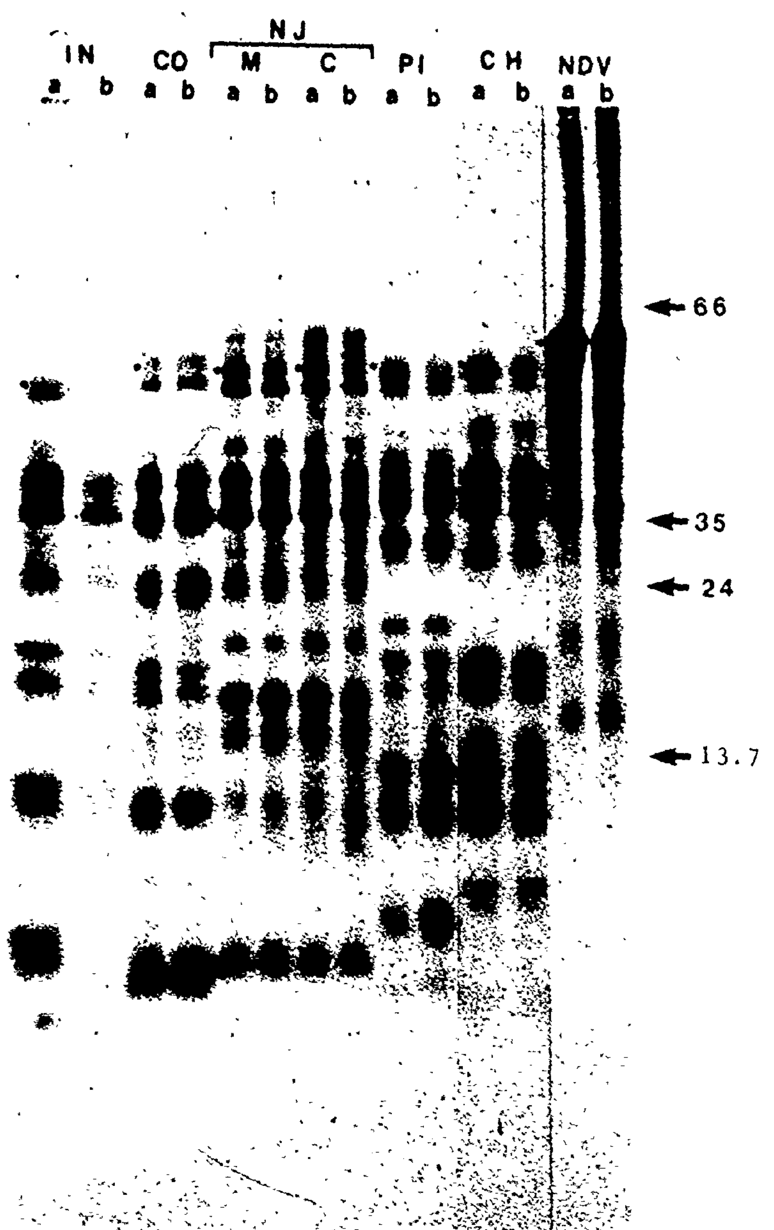
6.2.1 Comparisons of the NCS Cleavage Pattern of Vesiculovirus N Protein

N protein from several *Vesiculovirus* members and the analogous NP protein from Newcastle Disease virus were mapped after NCS cleavage.

Nucleocapsid proteins were isolated from [^{35}S]Met-labeled virions of VS Ind(T), VS NJ(C), VS NJ(M), VS Cocal, Piry and Chandipura viruses and from infected chick embryo fibroblasts in the case of the Paramyxovirus, Newcastles Disease virus. Protein was fractionated by SDS-PAGE, eluted electrophoretically, dialyzed against water, and lyophilized. The protein samples were suspended in 0.2 M acetic acid and treated with two concentrations of NCS, 3 and 15 mM as described in methods. NCS cleavage products were fractionated by SDS-PAGE on 15% acrylamide gels and visualized following fluorography.

Nucleocapsid proteins of all serotypes of the Vesiculovirus genus showed a broad range of hydrolysis products at both NCS concentrations (Figure 20), the higher concentration resulting in a detectable enhancement of hydrolysis in most cases. Comparing the partial proteolysis products, it was evident that all the Vesiculovirus N proteins examined contained a number of common features particularly with respect to the larger fragments. The fragment pattern of VS Cocal N proteins was very similar to that of VS Indiana(T) N protein although the electrophoretic mobilities of some corresponding fragments were different. Except for slight shifts in one or two bands, the patterns from the two VS NJ strains, Concan and Missouri, were indistinguishable. This result suggested that the tryptophan residues were identically located in both proteins. The NCS cleavage pattern of Piry and Chandipura N proteins and the actual electrophoretic mobilites of the cleavage fragments showed extensive similarity to each other. The N

Figure 20. Fluorogram of SDS-PAGE analysis of peptides generated by partial NCS cleavage of [^{35}S]Met-labeled N proteins of members of the Vesiculovirus genus and the NP protein of Newcastle disease virus. Radiolabeled N protein was prepared from purified [^{35}S]Met-labeled viruses: VS Indiana Toronto strain (IN), VS Cocal (CO), VS New Jersey serotype-Missouri strain [NJ(M)] or Concan strain [NJ(C)], Piry (PI), and Chandipura (CH). Radiolabeled NP protein from Newcastles disease virus (NDV) was isolated from secondary chick embryo fibroblasts. Proteins were fractionated by SDS-PAGE and subsequently electroeluted. The protein samples were dialyzed against water prior to cleavage with NCS at two concentrations: 3 mM (a) and 15 mM (b). The cleavage products (5-15,000 cpm) were analyzed by SDS-PAGE on 15% acrylamide gels containing 0.2% NN'-methylene-bisacrylamide. Bands were visualized by fluorography after impregnation with PPO. All samples shown in the figure were analyzed on the same slab gel, the panels resulting from the use of different exposure times to obtain roughly equivalent image intensity for all proteins. A dot marks the position of the intact protein for each represented virus. Protein bands of slower mobility were aggregation products. The position of MW markers are indicated with arrows and the MW value x 10^{-3}. The MW standards were BSA 66K MW, CPA 35K MW, and pancreatic ribonuclease 13.7K MW.


NJ
IN
CO
M
C
PI
CH
NDV
a b a b a b a b a b a b a b
66
35
24
13.7

protein NCS patterns of these viruses also show general similarity to the patterns of the other Vesiculovirus N proteins.

In contrast, the nucleocapsid, NP, protein of Newcastle Disease virus (NDV) showed a totally distinct NCS cleavage pattern under the same treatment conditions. The NDV, NP protein was less susceptible to fragmentation by NCS than the other nucleocapsid proteins. This could be due to fewer tryptophan sites or lower cleavage yields per site.

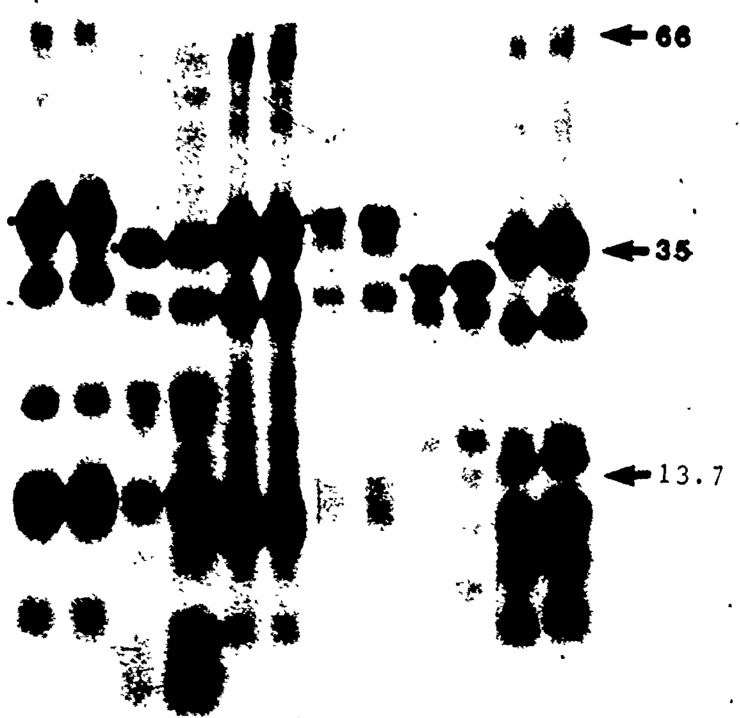
6.3 NCS Cleavage Pattern Comparisons of M Proteins

Matrix proteins were isolated from [^{35}S]Met-labeled purified virus preparations and reacted with NCS in the same manner as for the N protein.

The fluorograph of the cleavage products of Vesiculovirus M proteins is presented in Figure 21. The patterns were simpler than the coresponding N protein patterns and those of VS Ind(T), VS NJ(C), VS NJ(M), VS Cocal and Piry virus show similar pattern arrangements. The Chandipura M protein NCS cleavage pattern shows some resemblance to the pattern of Piry M protein but is more distinct from the other M protein patterns. The extent of cleavage was not appreciably different for the two concentrations of NCS employed. The large amount of uncleaved protein and the less complex patterns suggest that there were fewer cleavage sites in the M proteins than in the N proteins.

The NCS cleavage patterns of M protein from the VS NJ strains were identical but the Missouri pattern components had a higher mobility in proportion to the higher mobility of their intact M proteins.

Figure 21. Fluorogram of SDS-PAGE analysis of peptides generated by NCS cleavage of [^{35}S]Met-labeled M proteins of members of the Vesiculovirus genus. M proteins labeled with [^{35}S]Met were isolated from purified virions of the various serotypes: VS Indiana (T) standard strain (IND), VS Cocal (CO), VS New Jersey serotype-Missouri strain [NJ(M)] or Concan strain [NJ(C)], Chandipura (CHA), and Piry (PIR). Protein samples were extracted from polyacrylamide gels by electroelution and dialyzed against water prior to treatment with NCS at two concentrations 3 mM (a) and 15 mM (b). Peptides were fractionated on a 15% acrylamide gel containing 0.2% NN'-methylenebisacrylamide by electrophoresis at 150 V. Peptides were detected by fluorography after impregnation of the gel with PPO. The position and sizes (MW x 10^{-3}) of molecular weight standards are indicated by arrows: BSA 66K MW, CPA 35K MW, and pancreatic ribonuclease 13.7K MW.



NJ
IND
COC
M
C
CHA
PIR
b a b a b a b a b a b a
66
35
13.7

6.4 NCS Cleavage Pattern Comparisons of G Proteins

The glycoprotein of VS Ind, VS Cocal, VS NJ(M), and Piry viruses were isolated from infected cells. The NCS cleavage conditions were as described in methods employing a concentration of 15 mM NCS.

The G protein NCS cleavage products after SDS-PAGE fractionation as presented in Figure 22, showed pattern similarities between VS NJ(M) and VS Cocal especially in the arrangement and position of peptides with faster mobility than the 35K MW standard. The VS Ind(T) G pattern differed from VS Cocal and VS NJ(M) in that several extra bands exist in this region, however, the lower MW peptide pattern of VS Cocal and VS NJ(M) peptides formed a subset of the VS Ind(T) pattern.

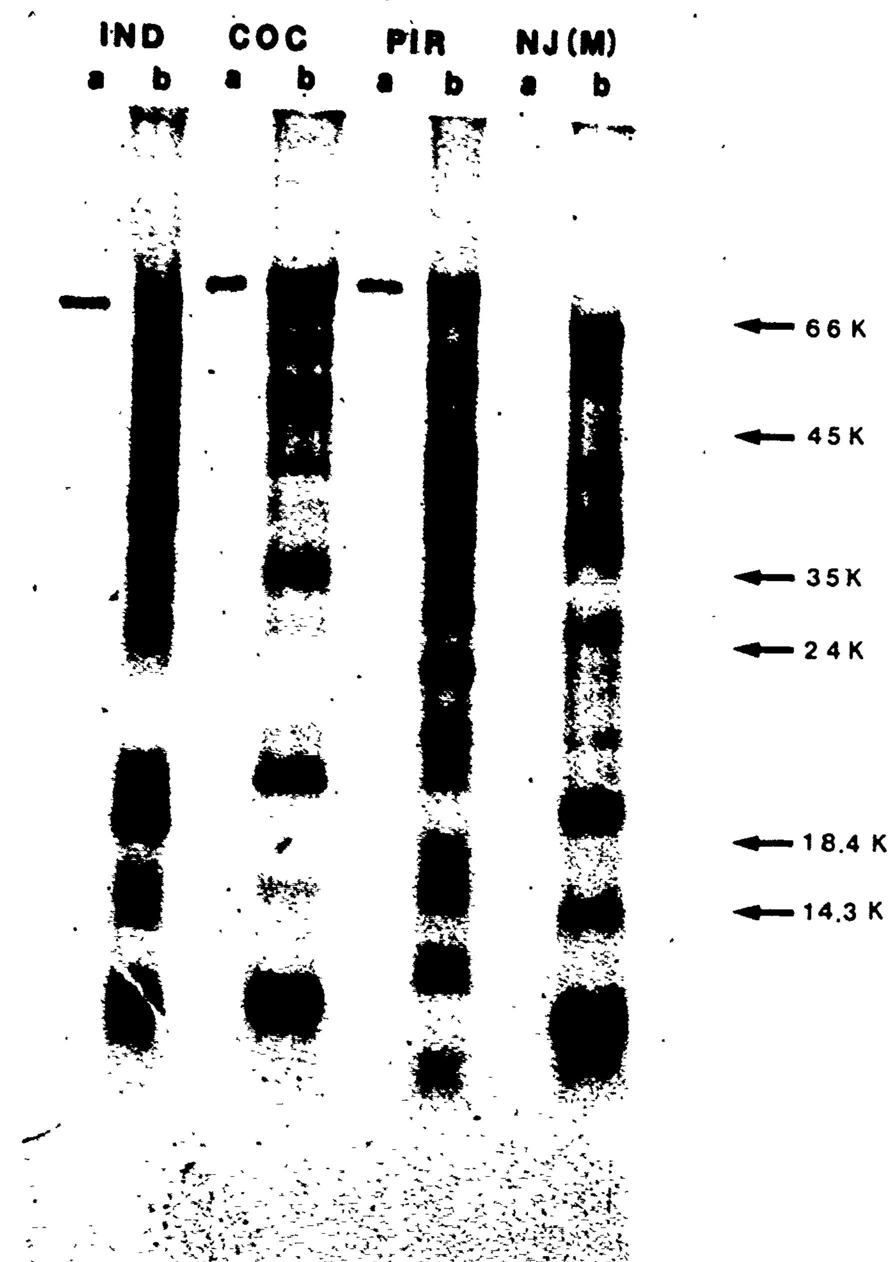
Piry virus G protein NCS cleavage products were the most distinct and could not be related to the other three G proteins by pattern or position of component peptides.

6.5 Linear Oriented Maps of Tryptophan Positions

Limited cleavage of terminally labeled proteins and subsequent size separation were employed to derive linear oriented arrangements of tryptophan residues in N and M proteins. The experimental rationale was analogous to Maxam and Gilbert's (1977) sequencing of DNA. I chose NCS to specifically cleave tryptophan peptide bonds of proteins. Proteins were labeled at the amino terminus with formyl -[^{35}S]methionine (f[^{35}S]Met) by cell free translation in the presence of f[^{35}S]Met-$tRNA_1^{Met}$.

Initiator wheat embrpyo $tRNA_1^{Met}$ was specifically acylated by the action of unfractionated E coli amionacyl-tRNA synthetases on

Figure 22. Fluorogram of SDS-PAGE analysis of peptides generated by NCS cleavage of [^{35}S]Met-labeled G proteins of several members of the Vesiculovirus genus. G proteins labeled with [^{35}S]Met were isolated from infected L cells by SDS-PAGE fractionation and electroelution. Samples were reacted with 15 mM NCS as described in methods (b) or applied without NCS treatment (a). NCS treated samples contained 25K cpm [^{35}S] radioactivity and untreated samples contained 1K cpm except NJ(M) which contained 0.2K cpm. G proteins from the serotypes VS Indiana Toronto strain (IND), VS Cocal (COC), Piry (PIR), and VS New Jersey Missouri strain [NJ(M)] were fractionated by SDS-PAGE at 150 V. The gel was 15% acrylamide, 0.2% NN'-methylenebisacrylamide. Detection was by fluorography after PPO impregnation. The position of molecular weight standards were indicated with values x 10^{-3}: BSA 66K MW, ovalbumin 45K MW, CPA 35K MW, PMSF trypsinogen, 24K MW, β lactoglobulin 18.4K MW, and lysozyme 14.3K MW.


IND
COC
PIR
NJ(M)
a
b
a
b
a
b
a
b
66 K
45 K
35 K
24 K
18.4 K
14.3 K

unfractionated wheat embryo tRNA in the presence of [^{35}S]Met (Stanley, 1972) and subsequently extracted with hot phenol as described in methods. The specificity of the aminoacylation is due to the fact that only wheat embryo $tRNA_1^{Met}$ and not $tRNA_2^{Met}$ is recognized by E coli methionyl-tRNA synthetase (Ghosh et al, 1974).

The amino group of [^{35}S]methionine in [^{35}S]Met-$tRNA_1^{Met}$ was chemically formylated by employing the formyl ester of N-hydroxysuccinimide (Gillam et al, 1968). This was done to prevent internal incorporation of [^{35}S]methionine in translation. The extent of formylation was determined by ribonuclease A digestion of the sample followed by separation and quantitation of the f[^{35}S]Met oligonucleotide and the [^{35}S]Met oligonucleotide, as described in methods. The extent of formylation of f[^{35}S]Met-$tRNA_1^{Met}$ varied from 89-99% in different preparations. Cell free translation was carried out in the presence of 10^3 fold molar excess of non radioactive methionine to methionine introduced as label, to further avoid internal incorporation of unformylated [^{35}S]Met.

6.5.1 Cell Free Translation

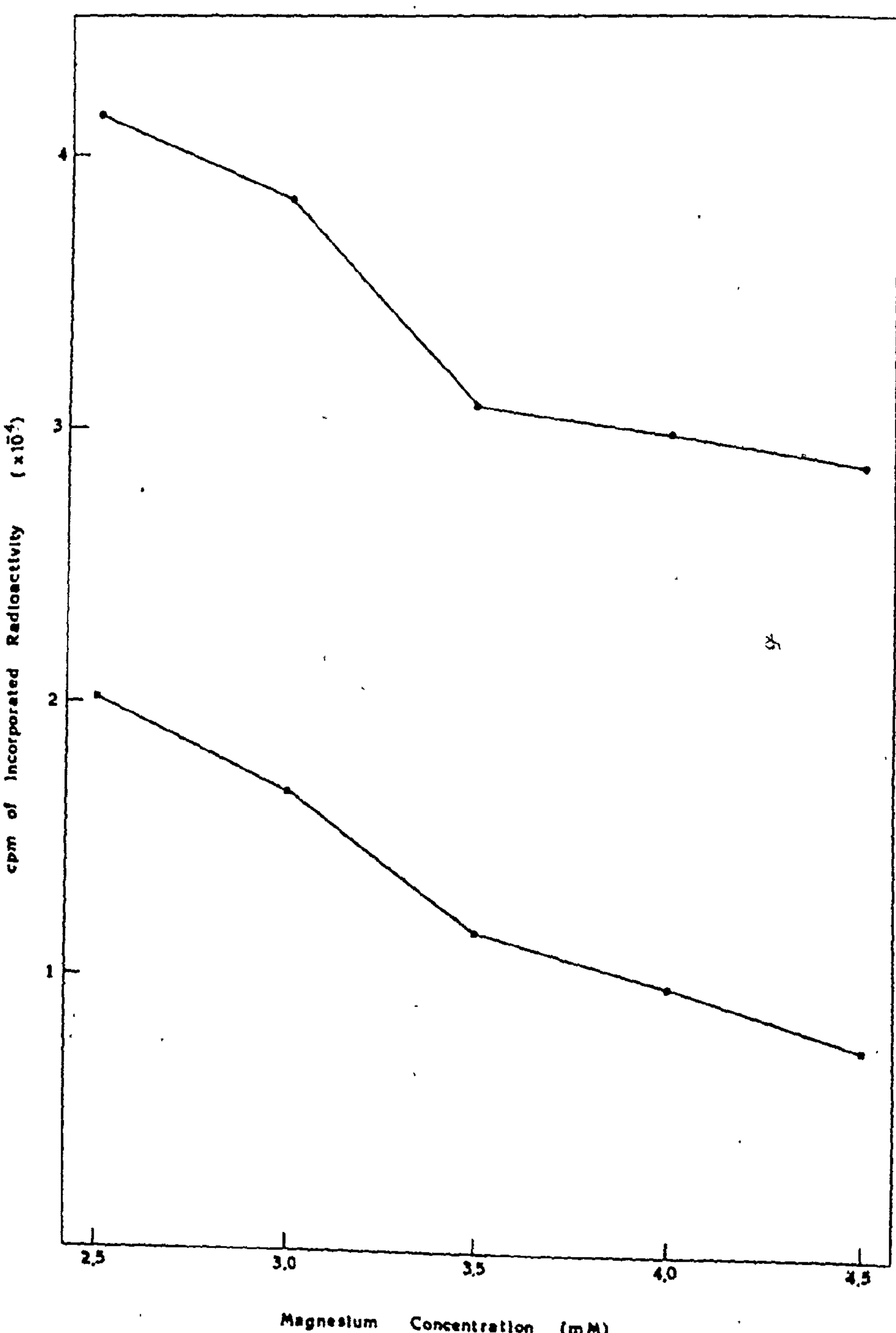
Cytoplasmic extracts of L cells were initially employed to synthesize f[^{35}S]Met-labeled proteins but were found to be deficient in initiation of protein synthesis. This translation system was effective at synthesis of virus specific proteins, incorporating [^{35}S]Met but did not incorporate significant quantities of f[^{35}S]Met. This was evidenced in a VS Cocal infected L cell extract which incorporated 0.4 mole % as much f[^{35}S]Met as [^{35}S]Met. Low incorporation was not due to limiting

quantities of substrate as only 0.2% of the input f[^{35}S]Met-tRNA$_i$^{Met} radioactivity was incorporated relative to 23% of the input [^{35}S]Met. The specific activity of the [^{35}S]Met and f[^{35}S]Met were the same and radioactivity was determined as 0.5 M KOH resistant TCA precipitable radioactivity. It was clear that infected L cell extracts were not efficient at initiation of protein synthesis and would not be useful for preparation of amino terminally labeled proteins. These extracts incorporated radioactivity internally as a result of translation on preexisting polysomes present in the extract.

It is known that cell free translation employing HeLa cell or rabbits reticulocyte extracts contain a hemin dependent inhibitor of initiation (Weber et al, 1975; Hunt et al, 1972). To test the possibility that the lack of initiation in infected L cell extracts was due to a hemin dependent inhibitor of translation. VS Cocal infected L cell extracts were prepared with (+H) and without (-H) 50 uM hemin.

The magnesium ion optima of Cocal infected L cell extracts prepared with hemin (+H) or without hemin (-H) were determined by assaying the hot TCA resistant radioactivity of aliquots after translation in the presence of [^{35}S]Met for 1 hr. Translation was as described in methods for the -H extract with a final concentration of 50 uM hemin in the +H translation mixture. The magnesium ion dependence of translation of +H and -H extracts is shown in Figure 23 and demonstrates a two fold enhancement of [^{35}S]Met radioactivity incorporation in the +H extract relative to the -H extract at 2.5 mM magnesium acetate. The enhancement of [^{35}S]Met incorporation could be due either to

Figure 23. Incorporation of [^{35}S]Met on cell free translation employing VS Cocal infected L cell extracts prepared with and without Hemin: Dependence on Magnesium concentration. Translation mixtures containing 10 uCi [^{35}S]Met (1200 Ci/ mmole) in a 50 ul volume were incubated at 32^{0}C. Aliquots (2 ul) were withdrawn at times, 0, 15, 30, 45, and 60 min. and the NaOH resistant TCA precipitable radioactivity was quantitated by LSC as described in methods. The radioactivity values were subtracted by the 0 time value and plotted on a graph from which plateau values were obtained, since reaction was seen to be essentially complete after 30 min. The maximum [^{35}S]Met incorporation per 2 ul of extract prepared with hemin ● and without hemin ■ are shown.



cpm of Incorporated Radioactivity (x10⁻⁴)
4
3
2
1
2,5
3,0
3,5
4,0
4,5
Magnesium Concentration (mM)

reinitiation of polypeptide chains or to an increase in the ribosomal density of polysomes present in the infected L cell extract.

The finding that the +H extracts were not more efficient in initiation than the -H extracts came from the result of translation in the presence of f[^{35}S]Met-tRNA$_1$^{Met} and subsequent fractionation on SDS-PAGE as seen in Figure 24. It can be seen by comparison with the proteins of purified Cocal virus (lane 1) that [^{35}S]Met-labeled Cocal virus proteins were synthesized in the +H and -H extracts (lanes 2 and 3), but there was barely detectable labeling with f[^{35}S]Met (lanes 4 and 5). There was a very slight enhancement of f[^{35}S]Met incorporation in the +H sample but the quantities were of no use for preparative purposes.

Rabbit reticulocyle extract is efficient at translation of exogenous mRNA (Pelham and Jackson, 1976), and is commercially available. When a rabbit-reticulocyte translation mixture was augmented with an additional 10% (v/v) of Cocal infected L cell extract in the presence of f[^{35}S]Met-tRNA$_1$^{Met} and incubated for 1 hr at 32°C, virus specific proteins were labeled, as can be seen in Figure 24, lane 6. The labeling represented 2% of the input radioactivity. In the mixed translation system, translation of endogenous reticulocyte mRNA was not detectable, as seen in Figure 24, although a prominant band was observable in the reticulocyte extract without added mRNA (lane 7).

Different amounts of infected L cell cytoplasmic extracts were added to the standard reticulocyte extract. When 10, 20 and 30% (v/v) amounts of VS Cocal infected L cell extract were added to a standard

Figure 24. Fluorogram of SDS-PAGE analysis of cell free translation products labeled with [^{35}S]Met or f[^{35}S]Met. Protein samples containing 10^4 cpm of [^{35}S] radioactivity were analyzed on a gel containing 10% acrylamide and 0.13% NN'-methylenebisacrylamide. Electrophoresis was at 150 V and detection was by fluorography. Samples were labeled with [^{35}S]Met (m) of f[^{35}S]Met-tRNA$_1$Met(fm).

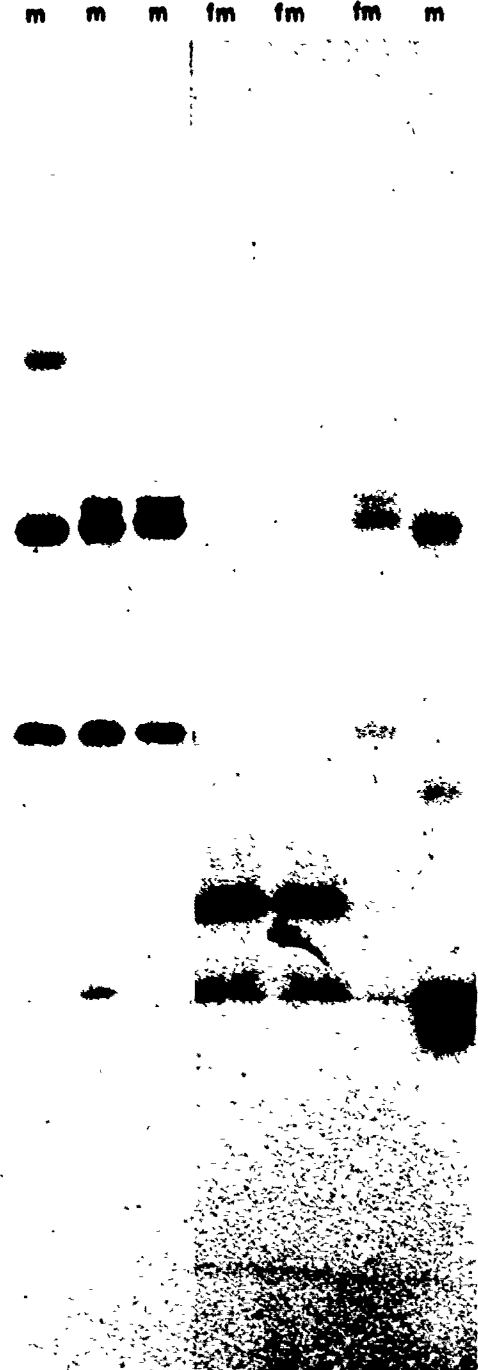
Lane 1 - Purified VS Cocal virus.

Lane 2 and 4 - Translation of +H VS Cocal infected L cell extract.

Lane 3 and 5 - Translation of -H VS Cocal inflected L cell extract.

Lane 6 - Cell free translation of nuclease treated rabbit reticulocyte extract augmented with 15% (v/v) +H VS Cocal infected L cell extract.

Lane 7 - Control cell free translation of nuclease treated rabbit reticulocyto extract, without added mRNA.


1 2 3 4 5 6 7
m m m fm fm fm m

reticulocyte extract without added magnesium, the alkali resistant TCA precipitable radioactivity represented 2.6, 4.2, and 2.6% of the input f[^{35}S]Met-tRNA$_i^{Met}$ radioactivity respectively. Since 20% (v/v) of infected L cell extract resulted in the greatest incorporation of f[^{35}S]Met, this amount was employed routinely. Cell free translation mixtures containing f[^{35}S]Met-tRNA$_i^{Met}$ and 10^3 fold molar excess Met were incubated at 32°C for 1 hr.

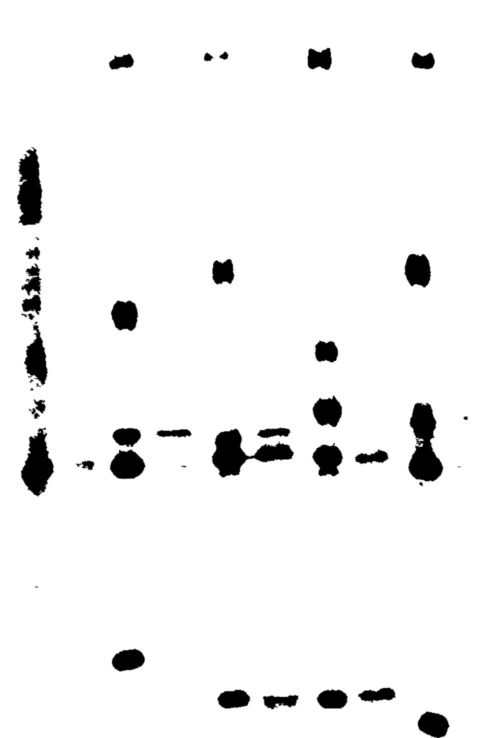
6.5.2 Synthesis of f[^{35}S]Met-Labeled Protein

Cytoplasmic extracts of L cells infected with VS Ind(T), VS Cocal, VS NJ(M) D1R4, and Piry viruses were prepared as described in methods. The VS NJ(M) D1R4, N protein is representative of the VS NJ(M) wild type protein because it results in an identical NCS cleavage pattern as seen in Figure 38. Viral proteins were labeled uniformly with [^{35}S]Met in infected L cells and at their amino terminus with f[^{35}S]Met in reticulocyte extracts augmented with infected L cell extracts. Figure 25 shows the SDS-PAGE profiles of the [^{35}S]Met and f[^{35}S]Met-labeled proteins. Three proteins were synthesized in abundance in the cell free extracts labeled with f[^{35}S]Met: NS, N, and M. These proteins had the same electrophoretic mobilities as the corresponding proteins in infected cells. The same samples analyzed in Figure 25 were fractionated by preparative scale SDS-PAGE and the M and N proteins from each preparation were extracted by electroelution.

6.5.3 Linear Mapping of Nucleocapsid Proteins

Proteins labeled uniformly with [^{35}S]Met and N-terminally with f[^{35}S]Met were reacted with NCS and fractionated in parallel by SDS-

Figure 25. Fluorogram of SDS-PAGE analysis of viral proteins labeled with [^{35}S]Met or f[^{35}S]Met. Samples indicated (m) contained 10 K cpm of [^{35}S]Met and those indicated (fm) contained 4-7 K cpm of f[^{35}S]Met. The gel was composed of 10% acrylamide and 0.13% NN'-methylenebisacrylamide and electrophoresis was at 150 V. Bands were detected by fluorograph following impregnation with PPO. Cells infected with [^{35}S]Met: VS Indiana Toronto (IND), VS Cocal (COC), VS New Jersey Missouri [NJ(M)], and Piry (PIR). An aliquot of uninfected L cell is included (L cell). Rabbit reticulocyte lysates were augmented with cytoplasmic extracts of L cells which had been infected with the indicated serotypes. VS NJ(M) D_1lR4 infected L cell extract was used to prepare f[^{35}S]Met protein instead of the wild type. Rabbit reticulocyte extract translated in the presence of [^{35}S]Met was included as a control (RETIC).


L CELL
RETIC
IND
COC
NJ(M)
PIR
m m m fm m fm m fm m fm

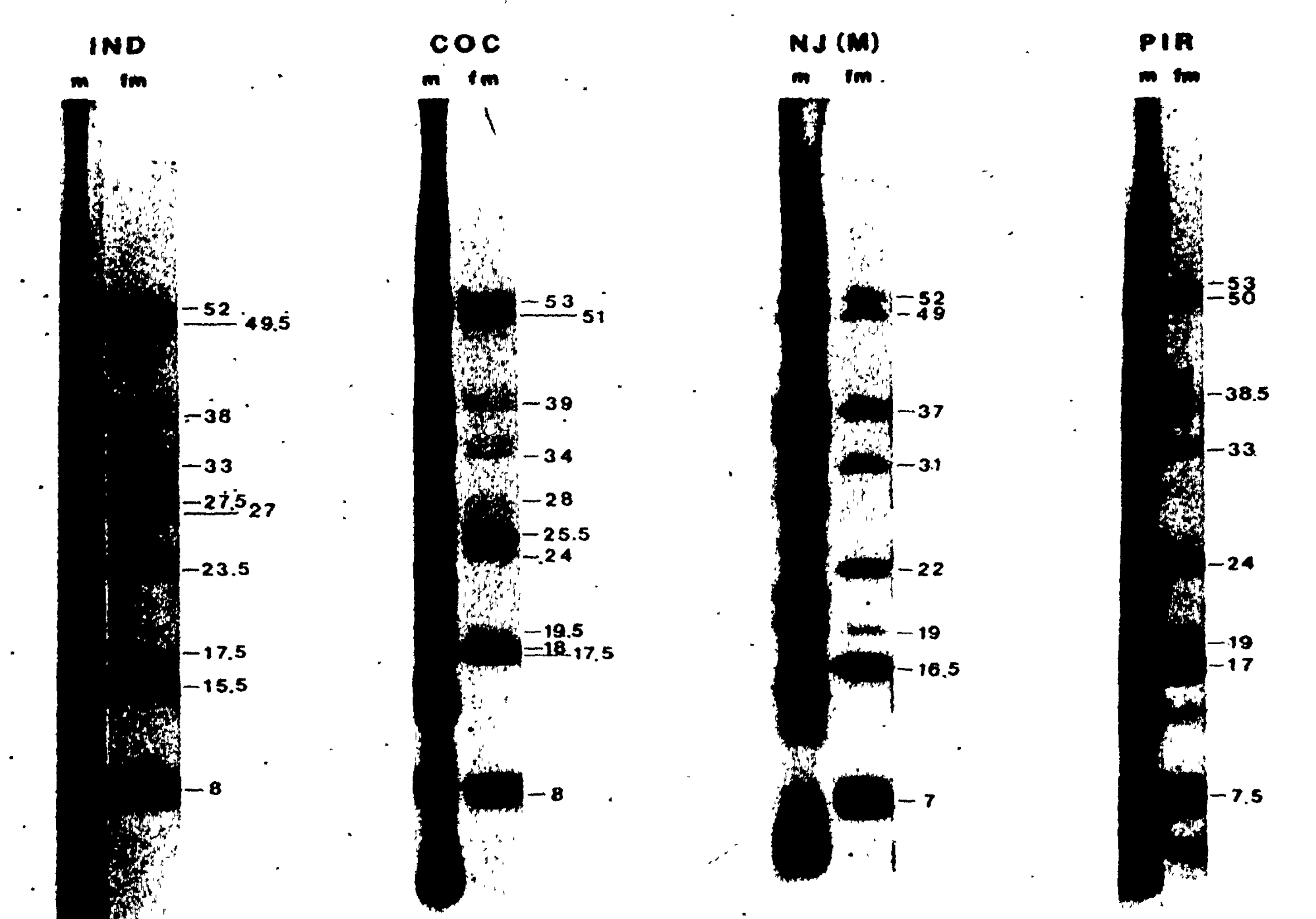
PAGE. The reaction conditions employed 0.5 M acetic acid and 15 mM NCS as described in methods.

The apparent MW of the f[^{35}S]Met-labeled peptides was obtained from interpolation on graphs of relative electrophoretic mobility and log molecular weight according to the procedure of Weber and Osborn (1969). The molecular weight standards and their corresponding molecular weight values are BSA 66K, ovalbumin 45K, carboxypeptidase A 35K, trypsinogen (PMSF) 24K, β-lactoglobulin 18.4K and lysozyme 14.3K. Figure 26 represents the NCS cleavage patterns of N proteins labeled with [^{35}S]Met and f[^{35}S]Met. The terminally labeled protein produced NCS cleavage patterns which were subsets of the corresponding uniformly labeled NCS cleavage pattern as would be predicted. Incorporation of [^{35}S]Met in the f[^{35}S]Met-labeled preparations was very low as evidenced by the lack of bands in map positions corresponding to high intensity bands in the uniformly [^{35}S]Met-labeled samples.

The f[^{35}S]Met labeled NCS cleavage patterns of the four N proteins were very similar. The VS Ind(T) and VS Cocal NCS cleavage pattern of end labeled protein were more complex than either VS NJ(M) DlR4 or Piry virus, possessing two or three extra components. Some of the higher molecular weight end labeled fragments in VS Ind(T) and VS Cocal were very faint but examination of 3 different f[^{35}S]Met-labeled preparations has consistently provided the same peptide patterns.

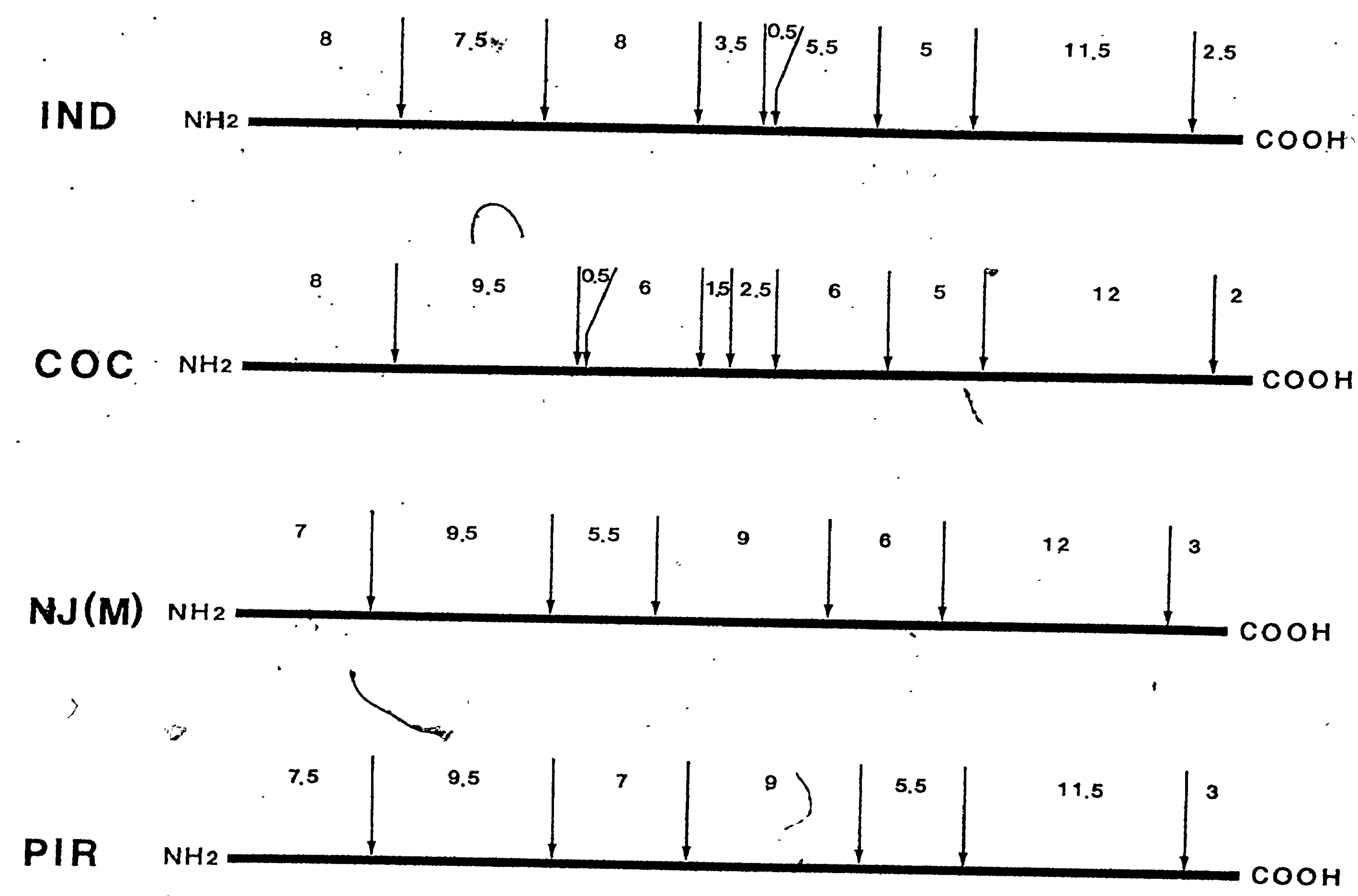
Linear maps of tryptophan positions were composed by plotting the apparent molecular weights of the end labeled fragments on a line representing the polypeptide chain and are presented in Figure 27. The

Figure 26. Fluorogram of SDS-PAGE analysis of peptides resulting from NCS cleavage of different N proteins labeled with [^{35}S]Met and f[^{35}S]Met. Proteins were extracted after preparative SDS-PAGE electroelution and subsequently treated with 15 mM NCS by as described in methods. Samples containing 40 K cpm of [^{35}S]Met radioactivity (m) or 5-7K cpm of f[^{35}S]Met radioactivity were employed. The gel contained 15% acrylamide and 0.2% NN'-methylenebisacrylamide and electrophoresis was at 125 V. Peptides were detected by fluorography after PPO impregnation. The molecular weight (x 10^{-3}) of f[^{35}S] Met labeled peptides are indicated and were calculated using the observed mobilities of MW standards: BSA 66K MW, ovalbumin 45K MW, CPA 35K MW, PMSF trypsinogen, 24K MW, β lactoglobulin 18.4K MW and lysozyme 14.3K MW. The N proteins represent: VS Indiana Toronto (IND), VS Cocal (COC), VS New Jersey Missouri (m) and VS New Jersey Missouri $D_1$1R4 (fm) [NJ(M)], and Piry (PIR).



IND
m fm
52
49.5
38
33
27.5
27
23.5
17.5
15.5
8
COC
m fm
53
51
39
34
28
25.5
24
19.5
18
17.5
8
NJ (M)
m fm
52
49
37
31
22
19
16.5
7
PIR
m fm
53
50
38.5
33
24
19
17
7.5

Figure 27. Linear oriented physical maps of NCS cleavage sites of N proteins of four serotypes of the Vesiculoviruses. The lines represent the primary structure of the N proteins oriented with respect to amino (NH_2) and carboxyl (COOH) termini. Length and sizes (x 10^{-3} MW) are proportionate to apparent molecular weight. The distance between cleavage sites was calculated by subtracting the apparent MW values of f[^{35}S]Met labeled NCS peptides. N protein maps are representative of VS Indiana Toronto (IND), VS Cocal (COC), VS New Jersey Missouri [NJ(M)], and Piry virus (PIR).



IND
NH2
8
7.5
8
3.5
0.5
5.5
5
11.5
2.5
COOH
COC
NH2
8
9.5
0.5
6
1.5
2.5
6
5
12
2
COOH
NJ(M)
NH2
7
9.5
5.5
9
6
12
3
COOH
PIR
NH2
7.5
9.5
7
9
5.5
11.5
3
COOH

four N protein tryptophan maps are compositionally and spatially similar. There may be six tryptophan residues in similar positions in the different proteins. The Piry virus and VS NJ(M) DlR4 tryptophan maps are strikingly similar in pattern with some differences in position. The tryptophan site indicated by the third smallest end labeled peptide in each pattern was not placed on the linear maps since its authenticity is questionable due to its small quantity.

6.5.4 Calculation of the Extent of NCS Cleavage

Densitometer tracings of each f[^{35}S]Met labeled band in the fluorographic image was made to quantitate the extent of cleavage at the different tryptophan positions (Laskey and Mills, 1975). X-ray film was preexposed to produce image densities proportional to radioactivity, and all image density values were within the linear range. The extent of cleavage at any one Trp residue was determined by dividing the quantity of radioactivity in the end labeled band which results from its cleavage, by the total quantity of radioactivity in the labeled bands which contain the Trp site in question (Appendix II). This calculation is only possible with terminally labeled molecules.

The susceptibilities to cleavage of the detected tryptophan sites by NCS under the conditions employed were listed in Table 6. Tryptophanyl peptide bonds of model proteins have been shown to have susceptibilities to cleavage by NCS ranging from 19-58% (Schechter et al, 1976). In R4 N protein, all cleavage sites had cleavage susceptibilities which fell approximately within this range. Cleavage site 7, which is near to the C-terminus, is predicted to be two

TABLE 6

SUSCEPTIBILITY OF NCS CLEAVAGE SITES IN VS NEW JERSEY(M) N PROTEIN

Site (from NH2 terminus)	Yield (% cleavage)
1	23.4
2	50.5
3	2.4
4	23.8
5	17.1
6	50.2
7[a]	65.5

The N protein of VS New Jersey(M) D_1R4 labeled with $[^{35}S]$ Met was treated with 15 mM NCS and fractionated by SDS-PAGE. Values were calculated from the relative abundance of $[^{35}S]$ Met labeled NCS fragments as determined from densitometry tracings of fluorographic images.
(a) Represents 2 tryptophan residues (McGeoch *et al*, 1980).

tryptophan residues according to nucleotide sequence analysis of McGeoch et al, (1980) and thus the average susceptibility is 32.8%. Cleavage site 3 (counting from the amino terminus) is of very low susceptibility and may not have represented a Trp residue, but was considered here since it was a constant feature of all R4 N protein f[^{35}S]Met labeled NCS cleavage patterns.

6.5.5 Linear Mapping of Tryptophan in Matrix Proteins

The matrix proteins are smaller and their NCS cleavage patterns less complex than the nucleocapsid proteins. NCS cleavage of f[^{35}S]Met and [^{35}S]Met-labeled M proteins was carried out as described in methods and the apparent molecular weights of the end labeled fragments were determined as before for N proteins.

The fluorograph of cleavage products of uniformly and terminally labeled NCS M protein after SDS-PAGE fractionation are presented in Figure 28. Again the f[^{35}S]Met labeled patterns consisted of a subset of the uniformly labeled patterns because only the amino terminus was labeled. One f[^{35}S]Met labeled NCS fragment of Piry M protein was not considered to be authentic since it corresponded with the most intensely labeled peptide band of the uniformly labeled pattern and may have represented internal incorporation of [^{35}S]Met since 11% of the f[^{35}S]Met-tRNA$_i^{Met}$ used in translation was nonformylated. VS Ind(T) and Piry M proteins contained 3 NCS cleavage sites whereas, VS NJ(M) D1R4 and VS Cocal M proteins contained 4.

Linear maps were composed from the apparent MW values of the terminally labeled peptides and are shown in Figure 29. There were

Figure 28. Fluorogram of SDS-PAGE analysis of peptides resulting from NCS cleavage of different M proteins labeled with [^{35}S]Met and f[^{35}S]Met. Proteins were extracted from preparative polyacrylamide gels by electroelution and subsequently treated with 15 mN NCS as described in methods. Samples containing 10 K cpm of [^{35}S]Met radioactivity (m) or 3-4 K cpm of f[^{35}S]Met were employed. The gel contained 15% acrylamide and 0.2% NN'-methylenebisacrylamide and electrophoresis was at 125 V. Peptides were detected by fluorography after PPO impregnation. The molecular weights (x 10^{-3}) of f[^{35}S]Met labeled peptides are indicated and were calculated from reference to the observed mobilities of MW standards: BSA 66K MW, ovalbumin 45K MW, CPA 35K MW, PMSF trypsinogen 24K MW, β lactoglobulin 18.4K MW and lysozyme 14.3K MW. The M proteins represent VS Indiana Toronto (IND), VS Cocal (COC), VS New Jersey Missouri [NJ(M)], and Piry (PIR).

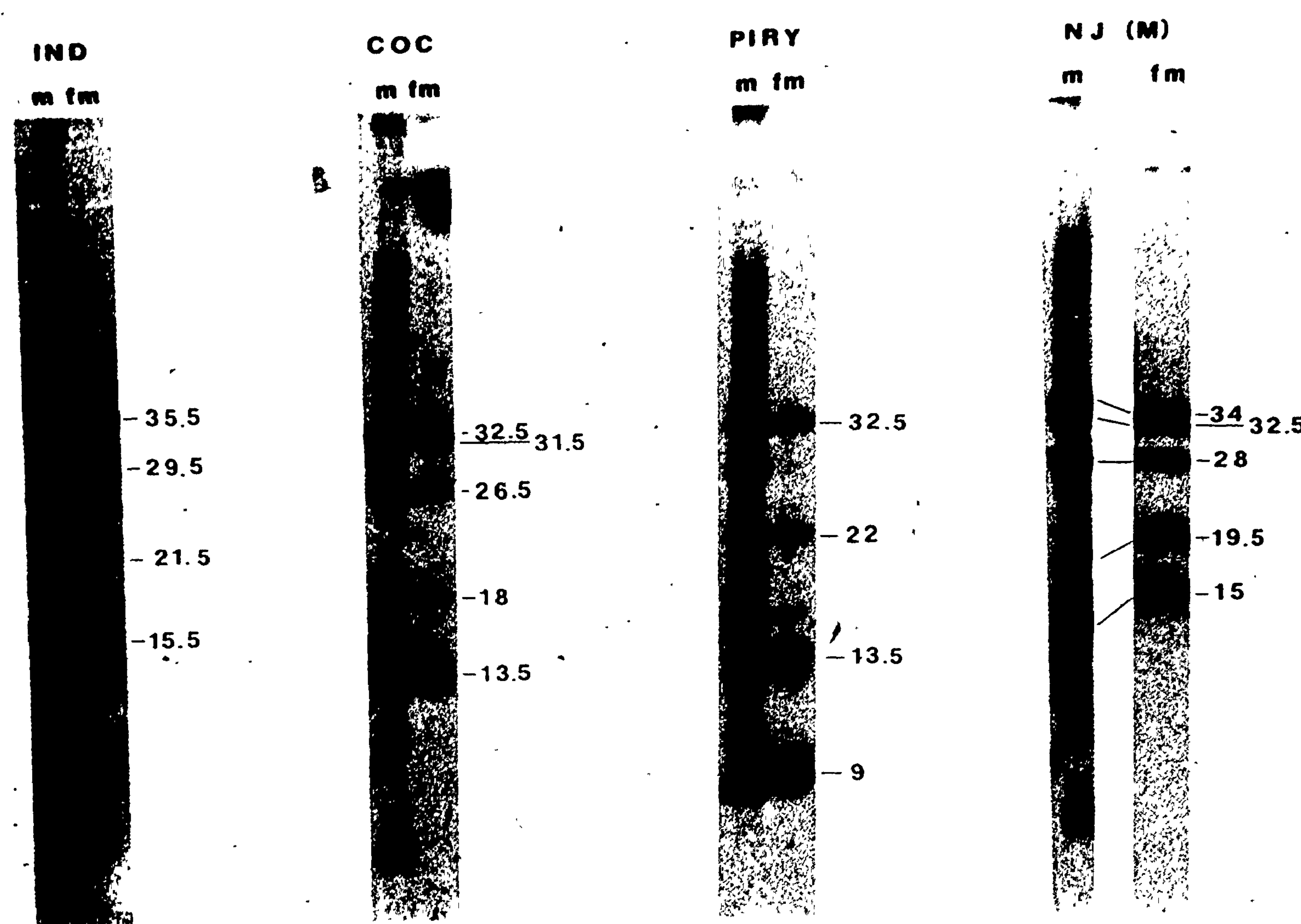
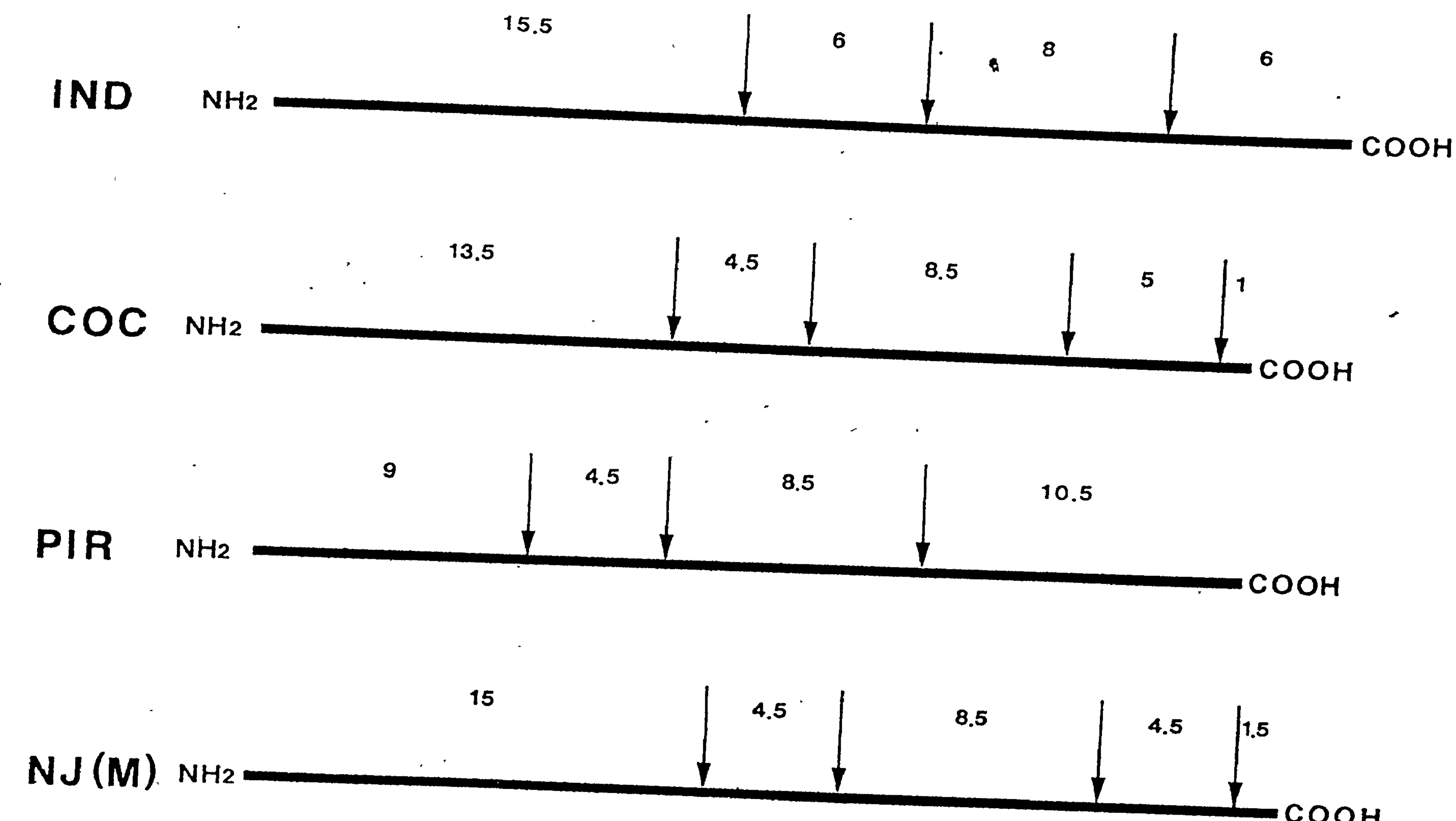

IND
m fm
35.5
29.5
21.5
15.5
COC
m fm
32.5
31.5
26.5
18
13.5
PIRY
m fm
32.5
22
13.5
9
NJ (M)
m
fm
34
32.5
28
19.5
15

Figure 29. Linear oriented physical maps of NCS cleavage sites of M proteins of four serotypes of the Vesiculoviruses. The lines represent the primary structure of the M proteins oriented with respect to amino (NH_2) and carboxyl (COOH) termini. Lengths and indicated sizes are proportionate to apparent molecular weight. The distance between cleavage sites - indicated here as MW x 10^{-3} were calculated by subtracting the apparent MW values of f[^{35}S]Met-labeled NCS peptides. N protein maps are representative of VS Indiana (IND), VS Cocal (COC), VS New Jersey Missouri [NJ(M)] and Piry (PIR).


IND
NH2
15.5
6
8
6
COOH
COC
NH2
13.5
4.5
8.5
5
1
COOH
PIR
NH2
9
4.5
8.5
10.5
COOH
NJ (M)
NH2
15
4.5
8.5
4.5
1.5
COOH

three tryptophan residues in similar positions among VS Ind(T), VS Cocal and VS NJ(M) D1R4. Piry M protein may have had one or three tryptophan residues in common positions with the other Vesiculovirus M proteins depending on the register of comparison. If termini are aligned, only one tryptophan site is coincident among the serotypes, but all three tryptophans can be aligned if the termini are taken out of register. This situation requires further sequence analysis to resolve but could suggest an intragenic translocation event. VS Cocal and VS NJ(M) D1R4 protein share the feature of a tryptophan residue near their carboxyl termini.

J. Rose predicts three tryptophan residues in VS Ind(SJ) M protein from nucleotide sequencing of the M gene (personal communication) in agreement with my finding employing NCS cleavage of f[^{35}S]Met-labeled protein.

6.5.6 Confirmation of Tryptophan Positions: 2 Dimensional Partial Cleavage Mapping

Verification of the Trp site arrangement employed partial proteolysis of SDS-PAGE separated NCS cleavage products and separation in a second dimension by SDS-PAGE. The sequence of sites which were cleaved to result in the peptide fragments separated in the first dimension can be determined by comparison of the fractionated partial proteolysis products generated from them.

Sequencing by this approach rests on the detection of peptides which contain one or other of the proteins termini. Terminus containing fragments can be identified by comparisons of the second dimensional

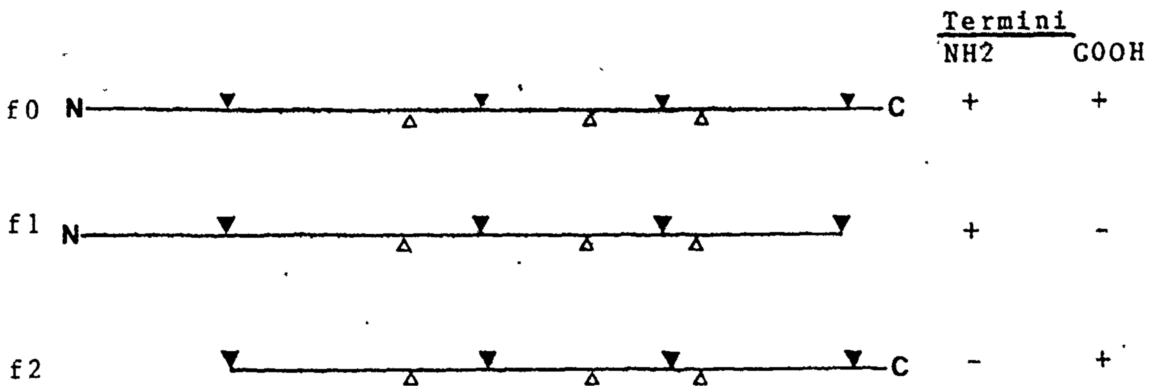
peptides of the intact protein (f0), relative to a fragment containing one of the termini (f1), and a fragment containing the other terminus (f2). In most cases the two largest NCS fragments will differ from f0 by lacking the N-terminus in one and the C-terminus in the other. In some cases, both f1 and f2 will contain the same terminus but this can be recognized by the fact that the f2 second dimensional peptides, which are common with f0, are also a subset of f1.

To explain diagrammatically I have presented in Figure 30 a sample protein which possesses NCS cleavage sites indicated by the closed triangles and V8 protease cleavage sites indicated by open triangles. In the diagram the two NCS cleavage fragments, f1 and f2, were aligned with f0, and the terminus specific V8 generated peptides can be identified by their pattern of occurrence, f0+, f1+, f2- or f0+, f1-, f2+. The NCS fragment in the example is identified as containing the terminus which f1 possesses.

Figure 31, panel A shows a two dimensional SDS-PAGE peptide map of uniformly [^{35}S]Met labeled VS Ind(T) N protein fragments generated by NCS cleavage in one dimension and V8 protease cleavage in the other. Cleavage during electrophoresis in the second dimension was carried out as described in methods. The gel strip was excised after separation in the first dimension and equilibrated for 15 min by shaking in 125 mM Tris HCl (pH 6.8) containing 0.1% SDS. The equilibrated gel strip was affixed to the top of a stacking gel previously overlaid with 0.2 ml of sample gel buffer containing 1% agarose and 20 ug V8 protease/ml before electrophoresis was begun.

Figure 30. Diagramatic representation of the rationale employed to detect peptides possessing common termini after two dimensional partial proteolysis mapping. Molecules are represented as lines oriented with respect to amino (N) and carboxyl (C) termini. Cleavage sites are indicated: NCS sites (▼), V8 protease site (▲). In this presentation NCS cleavage is followed by SDS-PAGE fractionation in one dimension and is followed by V8 protease mediated proteolysis and subsequent fractionation in another dimension by SDS-PAGE. Three fragments separated in the first dimension representing uncleaved protein (f0), and protein bands subjacent to it (f1, f2) are indicated. Two of several possible end specific peptides detected in the second dimension are given and an example peptide is presented.

First Dimension: NCS Peptides



Second Dimension: V8 Peptides

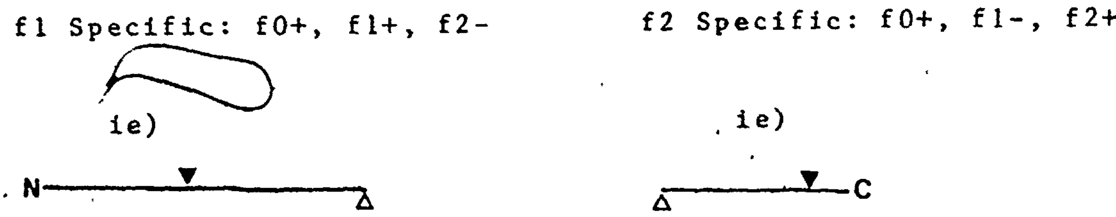
End specific peptides

f1 Specific: f0+, f1+, f2-

ie)

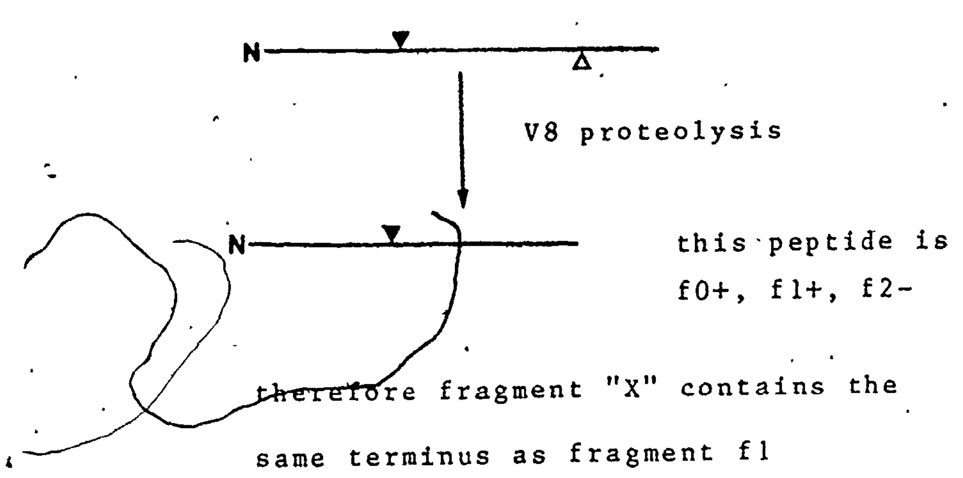
f2 Specific: f0+, f1-, f2+

ie)



EXAMPLE

NCS fragment "X"



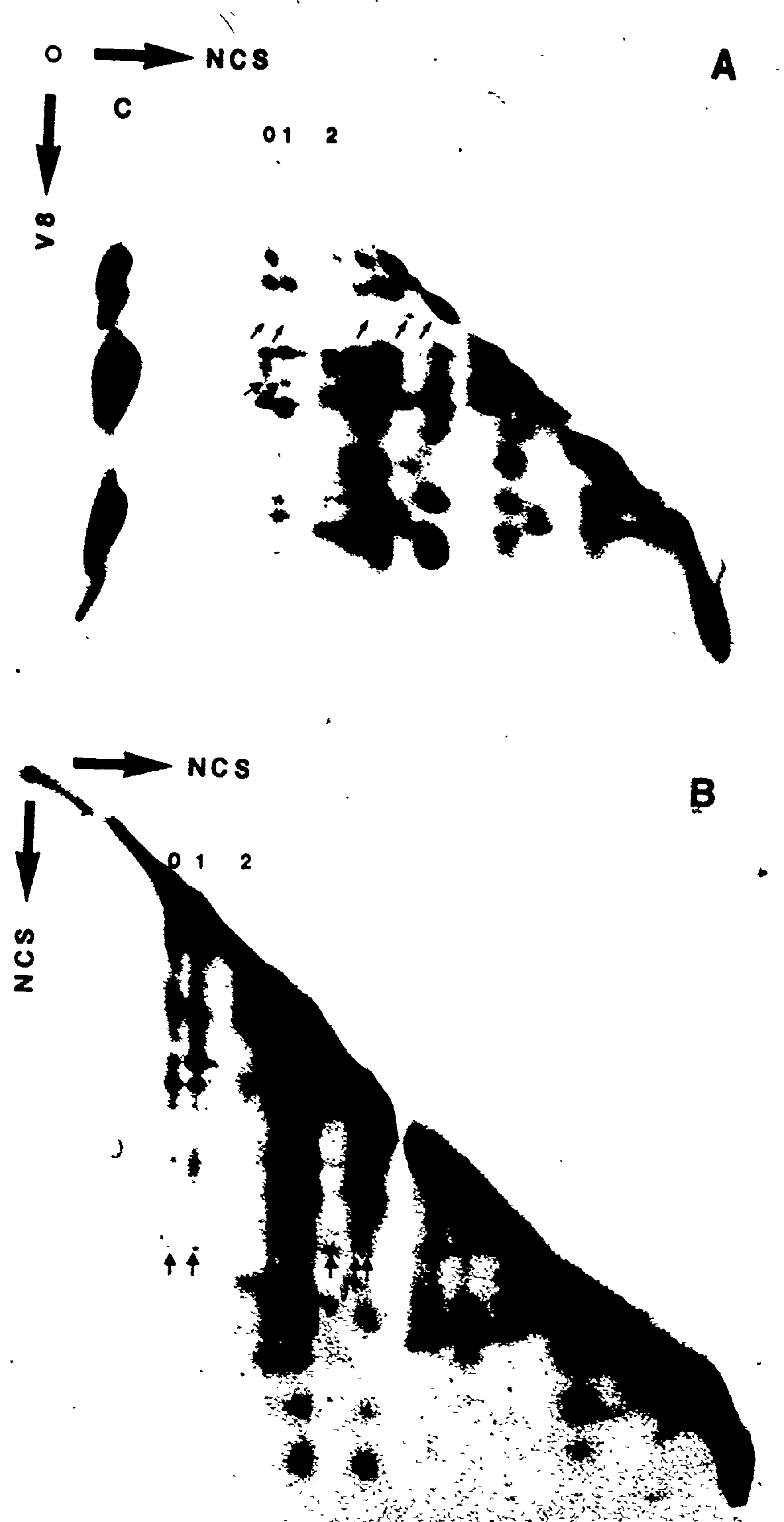
this peptide is f0+, f1+, f2-

therefore fragment "X" contains the same terminus as fragment f1

Figure 31. Fluorogram of two dimensional partial proteolysis maps of VS Indiana (T) N protein cleaved with NCS and V8 protease or NCS cleavage in both dimensions. VS Ind(T) N protein labeled with [^{35}S]Met was isolated from infected cells and reacted with 15 mM NCS as described in methods. The NCS peptides were fractionated by SDS-PAGE employing a 15% polyacrylamide 0.2% NN'-methylenebisacrylamide gel. Strips were cut from this gel and subsequently subjected to partial proteolysis and analyzed by SDS-PAGE in another dimension. Detection was by fluorography subsequent to impregnation with PPO.

Panel A - Fractionated NCS peptides in a gel strip were applied to a second identical polyacrylamide gel along with 4 ug of S. aureus V8 protease and subsequently subjected to electrophoresis. A portion of untreated VS Ind N protein was included in the second dimension (C). The origin is indicated by an open circle and the three NCS peptides, f0(0), f1(1), and f2(2) are indicated. Large arrows indicate the dimension of separation of peptides generated after NCS and V8 cleavage. Small arrows mark peptides containing the terminus in common with f1.

Panel B - A gel strip containing fractionated NCS generated peptides was recleaved with NCS in a solution containing 15 mM NCS as was routinely employed for proteins in solution. Cleavage was allowed to proceed for 20 min with shaking and then the gel strip was equilibrated in a solution of 125 mM Tris HCl (pH 6.7) containing 0.1% SDS. The resulting peptides were then fractionated in a second dimension. The origin (0) and NCS peptide f0(0), f1(1), and f2(2) are indicated. Peptides fractionated in the second dimension containing the same terminus as f1 are marked by small arrows.



O
NCS
A
C
0 1 2
V8
NCS
B
0 1 2
NCS

A portion of VS Ind(T) N protein without NCS treatment was introduced in the second dimension and was labeled C in Figure 31, panel A and 3 NCS fragments were identified as 0, 1, and 2. Two V8 peptides marked with arrows were terminus specific peptides of f0 and f1 since they arise from fragments 0 and 1 but not 2. From the V8 products of the NCS fragments, 5 other NCS fragments could be identified as containing this terminus as well. These 5 fragments and f1 were identified as amino-terminus containing fragments by f[^{35}S]Met labeling, thus these data represented independent support for a portion of the tryptophan map.

Panel B of Figure 31 shows a two dimensional partial proteolysis profile of [^{35}S]Met labeled VS Ind(T) N protein after cleavage with NCS in both dimensions. Recleavage of the NCS fragments after separation in the first dimension by SDS-PAGE was as described in Figure 31. After cleavage the gel strip was equilibrated in a solution containing 125 mM Tris HCl (pH 6.8) and 0.1% SDS for 15 min before application to a second polyacrylamide gel. The gel strip was affixed with sample buffer containing 1% agarose and then electrophoresis was carried out.

In Figure 31, panel B, it was apparent that the second cleavage with NCS (in the second dimension) was not extensive since most of the radioactivity is in uncleaved fragments lying on a diagonal line. However, detectable quantities of fragments were generated and a peptide is identified with arrows as containing the terminus of f1 as evidenced by its presence in f0 and f1 but not f2 derived peptides. Five other NCS peptides possessed this specific peptide which indicated that they

all contained a terminus in common with f1. This group of six peptides had been identified by NCS cleavage of f[^{35}S]Met-labeled protein in previous experiments.

Two dimensional partial proteolysis mapping can serve as a means of deriving linear maps of cleavage sites but such maps are unoriented with respect to N and C termini.

6.5.7 Tryptic Peptide Mapping of NCS Peptides

The f0 and f1 NCS peptides of [^{35}S]Met-labeled VS Ind(T) N protein were tryptic peptide mapped by two dimensional fractionation on cellulose thin layer sheets (Figure 32). The f1 tryptic peptide map was a subset of the f0 map and lacked 2 peptides. These two peptides could be identified as 11 and 12 by reference to Figure 5. This information supports the contention that f1 arose from cleavage and not another type of chemical modification, which could have resulted in increased electrophoretic mobility. The nucleotide sequence analysis of McGeoch *et al* (1980) predicts that two methionine containing tryptic peptides would be lost concomitant with removal of a C-terminus containing peptide by cleavage at the ultimate Trp residue. This is consistent with the tryptic peptide analysis.

Section II: Characterization of the N protein Alteration in VS NJ(M) D_1

The N and G proteins of the VS NJ(M) D_1 ts mutant have altered electrophoretic mobilities on SDS-PAGE corresponding to MW decreases of 1,000 and 3,500 respectively (Wunner and Pringle, 1974). This original report of the N and G abnormality in the VS NJ(M) D_1 ts mutant also noted a direct relationship between the N protein and the ts phenotype;

Figure 32. Fluorogram of tryptic peptide map comparison of [^{35}S]Met labeled peptides f0 and f1 of N protein of VS Ind(T). N proteins isolated from infected cells was cleaved with 15 mM NCS and the peptides were subsequently extracted by diffusion after preparative scale SDS-PAGE. TLE employed (pH 3.5) and 450 V for 2-2.5 hr. Samples containing 10^4 cpm of [^{35}S] radioactivity were applied to MN400 cellulose thin layer sheets in combination with 10 nmoles each of Met (▲) and Arg (●). Amino acids were detected by ninhydrin staining. Peptides were detected by fluorography after impregnation with PPO in naphthalene.
Panel A - N protein of VS Ind(T), untreated control.
Panel B - NCS treated protein f0 of VS Ind(T) N protein.
Panel C - NCS peptide f1 of VS Ind(T) N protein.

A

KODAK SAFETY FILM

B

KODAK SAFETY FILM

C

that is, on reversion, there was a transformation of N protein mobility to wild type.

7. PRODUCTION OF INDEPENDENT REVERTANTS AND DETERMINATION OF TS^+ PHENOTYPE

I obtained a stock of NJ(M) D_1 from Dr. C.R. Pringle (Glasgow) and isolated subclones from it by plaquing at permissive temperature, 32°C. Independent revertants were subsequently isolated from each D_1 subclone by plaquing at non permissive temperature, 39.5°C. The ts subclones and their revertants were titrated at permissive and non permissive temperature to calculate the efficiency of plating (eop) which is the fraction of plaque forming ability of a virus preparation at non permissive temperature relative to permissive temperature. Table 7 lists the eop values of 4 mutant subclones and their revertants. The eop for revertant clones covered a range from 0.1 to 0.6 which included the eop of 0.4 for VS NJ(M) wild type, whereas the eop values of the mutant subclones ranged from $3x10^{-6}$ to $1x10^{-4}$. It appeared that the plaques isolated at nonpermissive temperature had become ts^+ and thus represented true revertants.

8. ANALYSIS OF MUTANT AND REVERTANT PROTEINS BY SDS-PAGE

To compare the electrophoretic mobilities of the virus specific proteins of the mutant and revertants, L cells were infected with the four D_1 subclones and their independent revertants and then labeled with [^{35}S]Met and the proteins analyzed by SDS-PAGE, as described in methods. It was clear from the results (Figure 33), that the ts phenotype was not tightly linked with the altered mobility phenotype of the N protein.

TABLE 7 EFFICIENCY OF PLATING OF D_1 SUBCLONES AND THEIR REVERTANTS

D_1 Subclones	Efficiency of Plating (pfu/ml@39.5 C/pfu/ml@ 32 C)
$D_1 1$	$1x10^{-4}$
$D_1 2$	$1x10^{-4}$
$D_1 5$	$4x10^{-5}$
$D_1 6$	$3x10^{-6}$
ts^+ Revertants	
$D_1 1R4$	0.4
$D_1 2R3$	0.6
$D_1 5R8$	0.2
$D_1 6R1$	0.1
VS NJ(M) wild type	0.4

The titre of virus suspensions were determined by plaque assay on monolayers of L cells incubated at 39.5 C and 32 C. The efficiency of plating ia presented as the fraction of infectivity at 39.5 C relative to 32 C.

Figure 33. Autoradiogram of SDS-PAGE analysis of [^{35}S]Met-labeled cells infected with NJ(M) D_1 ts subclones and independent ts+ revertants. Cells were infected with VS NJ(M) wild type (wt), VS NJ(M) D_1 subclones 1, 2, 5, and 6 ($D_1$1, $D_1$2, $D_1$5, and $D_1$6) and independent revertants isolated from these subclones 1, 3, 4, and 8 ($D_1$6R1, $D_1$2R3, $D_1$1R4 and $D_1$5R8). An uninfected L cell (L cell) sample was included as a control. Infection was at an moi of ~10 pfu/cell for wild type $D_1$1 and $D_1$1R4 and ~100 pfu/cell for the others; labeling was as described in methods. The gel was 10% acrylamide and 0.13% NN'-methylenebisacrylamide and electrophoresis was at 150 V. Each sample contained 10^5 cells.

wt
D1 1
D1 1 R4
D12
D12 R3
D16
D16 R 1
D1 5
D 5 R8
L CELL

Temperature-sensitive mutant subclones had either altered or normal mobility N proteins and, as well, the ts+ revertants possessed both forms of N protein. The G protein mobility was seen to be restored to normal in two independent revertants, D_1 6R1 and D_1 5R8. It should be noted that the four ts subclones described here are a random sampling and have not been selected after SDS-PAGE analysis from a larger group of plaque isolates.

The mutant subclone $D_1$1, designated hereafter as D1, and its revertant $D_1$1R4, designated hereafter as R4, were analyzed further to characterize the structural basis for the observed difference in migration of N protein. This pair of viruses was chosen since they differ from each other and wild type by SDS-PAGE analysis thus providing a means of discrimination when checking continuity or verifying virus type.

The proteins of D1 and R4 were analyzed by two other SDS polyacrylamide gel systems to see if the N protein mobility difference was observable under different conditions. [^{35}S]Met-labeled proteins produced in infected cells were fractionated in SDS-continuous phosphate (pH 7.4) polyacrylamide gels and discontinous SDS-PAGE containing 7 M urea. Both electrophoresis systems demonstrated the same mobility difference as detected in discontinuous SDS-PAGE of [^{35}S]Met-labeled purified virus (Figure 34). The difference in mobility did not appear to be the artifact of one gel electrophoresis system and could not be removed by high concentrations of urea.

Figure 34. <u>Demonstration of the anomalous mobility of D1 N protein under different conditions of SDS-PAGE analysis.</u>

Discontinuous SDS-PAGE - Panel A - Purified [^{35}S]Met-labeled virions of NJ(M) wt, D1 and R4, as indicated, were fractionated by <u>dis</u>continuous SDS-PAGE. The gel was 10% acrylamide and 0.13% NN'-methylenebisacrylamide and electrophoresis was carried out at 150 V. Samples containing 10K cpm of [^{35}S]Met radioactivity were detected by fluorography.

Discontinuous SDS-PAGE in the presence of 7 M urea - Panel B - Proteins labeled with [^{35}S]Met were from pure virus in the case of VS NJ(M) wild type and from infected cells in the case of D1 and R4. The gel was 10% acrylamide, 0.13% NN'-methylenebisacrylamide and contained 7 M urea. Detection of samples containing 10^5 cells was by autoradiography.

Continuous SDS-PAGE - Panel C - Proteins labeled with [^{35}S]Met were from infected or noninfected cells. The gel was 7.5% acrylamide, 0.1% NN'-methylenebisacrylamide prepared as described in methods. Samples containing 10^5 cells were detected by autoradiography. Electrophoresis was carried out at 60 V.

A

NJ (M) wt
NJ (M) D11
NJ (M) D11 R4

L

G =
NS
N =

M

B

NJ (M) wt
D11
D11 R4

N =

C

L CELL
D11
D11 R4

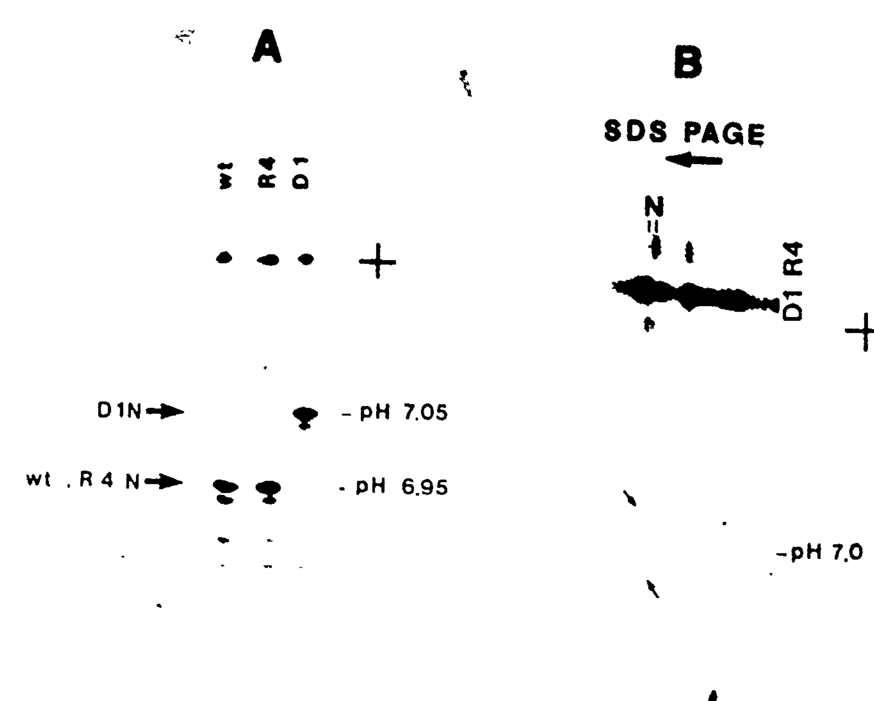
N =

9. DETECTION OF ALTERED ISOELECTRIC POINT

I wanted to determine whether the D1 N protein had an altered isoelectric point relative to R4 or wild type. Purified virus labeled with [^{35}S]Met was disrupted in urea and NP40, and the viral proteins were separated according to their isoelectric point in prerun slab gels containing 2% ampholines. Figure 35, panel A represents an autoradiogram of VS NJ(M) wild type, R4 and D1 proteins fractionated according to isoelectric point. It was observable that a major intensity protein band which was present in the wild type and R4 channels was absent in the D1 channel, whereas the D1 channel possessed a protein band of similar prominence at a more basic position on the pH gradient. That the two bands in question were in fact nucleocapsid proteins was determined by subjecting isoelecric focusing gels to SDS-PAGE separation in another dimension in the presence of viral protein markers (Figure 35 panel B). The N proteins were identified after two dimensional separation of the [^{35}S]Met labeled proteins from a mixture of D1 and R4 infected cells by reference to D1 and R4 proteins included in the second dimension.

From pH determinations in the isoelectric slab gel as described in methods the wild type and R4 N proteins were found to have a Pi of 6.95 and the D1 N protein a value of 7.05. An isoelectric point increase of 0.1 pH unit may represent the acquisition of one positively charged group or the loss of one negatively charged group (Harley et al, 1980). This finding was consistent with a base substitution resulting in a missense mutation or a nonsense mutation.

Figure 35. Isoelectric focusing of D1, R4 and wild type viral proteins. Panel A - Isoelectric focusing of [^{35}S]Met-labeled virus was carried out as described in methods. Samples containing 20K cpm of purified virus were applied to each slot. The pH of the gel at which the N proteins were detected is indicated. The polarity of the applied electric field is indicated. Panel B - Two dimensional separation of viral proteins by isoelectric focusing in one dimension and SDS-PAGE in the other. The gel for SDS-PAGE was 10% acrylamide and 0.13% NN'-methylenebisacrylamide. Radioactively labeled samples of infected cells were combined and subjected to fractionation in both dimensions as described in methods. Aliquots of [^{35}S]Met-labeled D1 and R4 infected cells were included in the second dimension in order to locate the corresponding N proteins. The mutant and revertant N proteins are indicated by arrows.


A
B
SDS PAGE
wt
R4
D1
N
D1 R4
D1N
- pH 7.05
wt . R 4 N
- pH 6.95
-pH 7.0
-pH 6.5

10. INCORPORATION OF PHOSPHATE INTO N PROTEINS

To test the possibility that the D1 N protein alteration was due to a change in phosphorylation, N proteins synthesized in the presence of [^{32}P]orthophosphate were examined by SDS-PAGE fractionation.

Wild type and D1 infected L cells were labeled with [^{35}S]Met and [^{32}P]phosphoric acid as described in methods except that 333 uCi of [^{32}P] was employed for labeling each monolayer. Labeled cells were dissolved in lysis buffer and centrifuged at 5K rpm for 5 min in a Sorvall GSA rotor to remove particulate components. The antigen containing supernatant was mixed with hyperimmune anti-VS NJ(M) serum and protein A sepharose CL 4B as described in methods. The sepharose beads were washed in washing buffer before the bound immune complexes were eluted with SDS-PAGE sample buffer and analysed by SDS-PAGE.

Figure 36 represents the immunoprecipitated proteins and the infected cell mixtures they were derived from after separation on SDS-PAGE. The G and N protein shifts were detectable in the channels containing [^{35}S]Met-labeled D1 and wild type infected cell proteins and their respective immunoprecipitates, but, the only [^{32}P]-labeled viral protein was NS. A 3 fold longer exposure of this autoradiogram still did not indicate [^{32}P] incorporation into wild type or D1 N proteins.

If a phosphorylation change were the basis for the D1 N mobility shift, then the mutant was expected to be nonphosphorylated since it had a more basic isoelectric point than wild type or revertant. Since the D1 and wild type N proteins were not phosphorylated, it

Figure 36. Autoradiogram of SDS-PAGE analysis of VS NJ(M) wild type and DI infected cells labeled with [^{35}S]Met and [^{32}P]-orthophosphate. Samples of infected or noninfected cells were labeled with [^{35}S]Met (^{35}S met) or [^{32}P] orthophosphate ($^{32}PO_4$) as described in methods. Samples were subjected to SDS-PAGE after immunoprecipitation with hyperimmune anti-VS NJ(M) serum (immunoprecipitated) or without prior immunoprecipitation (unfractionated). The position of the NS protein is indicated. Samples of infected cells without prior immunoprecipitation containing 10^5 cells were employed, whereas, an unknown quantity of material was present in the samples of immunoprecipitated material.

UNFRACTIONATED | IMMUNO-PRECIPITATED

$^{32}PO_4$ | ^{35}S met | $^{32}PO_4$ | ^{35}S met

L CELL | wt INF | D1 INF | L CELL | wt INF | D1 INF | L CELL | wt INF | D1 INF | L CELL | wt INF | D1 INF

NS →

appeared that phosphorylation was not the basis for the electrophoretic mobility alteration.

11. LINEAR MAPPING OF R4 N PROTEIN WITH CNBr AND NCS

Linear oriented maps of N protein were produced by subjecting formyl-[^{35}S]methionine labeled protein to limited cleavage with chemical agents and separation of the resulting fragments according to size by SDS-PAGE, as outlined in section I. Protein labeled at its amino terminus was synthesized in a rabbit reticulocyte lysate translation system utilizing f[^{35}S]Met-tRNA$_i^{Met}$ and infected L cell cytoplasmic extracts as a source of mRNA, as described in methods. Although only wheat embryo tRNA$_i^{Met}$ is recognized by *E coli* methionyl-tRNA synthetase, formylation was essential, due to internal incorporation of methionine by Met-tRNA$_i^{Met}$ in the heterologous rabbit translation system (Ghosh *et al*, 1974; personal observation).

Proteins were cleaved either with cyanogen bromide or N-chlorosuccinimide which cleave after methionyl and tryptophanyl peptide bonds, respectively. CNBr specially cleaves peptide bonds involving the carboxyl group of methionine. The agent has been used extensively by others for complete cleavage of methionyl bonds (Kasper, 1975). The fact that CNBr only cleaves reduced methionine residues allowed limited digestion under conditions which cleave susceptible bonds extensively, since the protein substrates were partially oxidized during the manipulations involved in their preparation. It was possible that the presence of SDS in the reaction played a role in partially protecting methionine residues, but prior reduction of samples by incubation with

1% (v/v) thioglycollic acid for 24 hours (Omenn et al, 1970) rendered them much more susceptible to CNBr cleavage (data not shown). The fact that only reduced methionine residues will be lost due to cleavage, made it possible to employ the label f[^{35}S]Met when cleaving with CNBr.

R4 N protein labeled with f[^{35}S]Met was synthesized in vitro and was then reacted with NCS (15mM) or with CNBr, (19mM) in 70% formic acid for 4 hr, as described in methods.

Figure 37 presents the SDS-PAGE analysis of end labeled peptides of R4 N protein after cleavage with CNBr and NCS. It can be seen that the same pattern of 7 fragments, as described in section I, were obtained after NCS cleavage, but only 4 CNBr generated peptides are observed.

12. LOCATION OF THE SITE OF THE D1 N PROTEIN ALTERATION ON THE LINEAR MAP

It was possible to locate the D1 N protein alteration on the linear map by generating partial cleavage maps and referencing the amino terminal f[^{35}S]Met-labeled fragments to those peptides which contain the site of alteration. Both NCS and CNBr cleavage site maps were employed so that the location could be more narrowly defined.

12.1 Cleavage with NCS

Nucleocapsid proteins labeled with a mixture of 16 [^{3}H]amino acids or [^{35}S]Met were isolated from purified virus, whereas R4 N protein labeled with f[^{35}S]Met was prepared by cell free translation. The proteins were cleaved with NCS (15 mM) as described in methods and the peptides were separated by electrophoresis on 15% SDS polyacrylamide

Figure 37. Fluorogram of SDS-PAGE analysis of f[^{35}S]Met-labeled NCS and CNBr peptides of VS NJ(M) D_1lR4 N protein. Proteins synthesized in vitro using the precursor f[^{35}S]Met-tRNA$_1$ Met were reacted in 20 mM NCS or 19 MM CNBr as described in methods. Samples containing 10K cpm of f[^{35}S]Met radioactivity and resulting from the indicated cleavage were fractionated by SDS-PAGE at 125 V. The gel was 15% acrylamide and 0.2% NN'-methylenebisacrylamide. The peptide fragments resulting from each cleavage are labeled f1-f7 for NCS cleavage and f1-f4 for CNBr cleavage. FO is the uncleaved molecule in each pattern. The positions of molecular weight standards are indicated by arrows and corresponding MW values x 10^{-3}. The MW standards indicated were analyzed on the slab gel in which the CNBr peptides were fractionated: BSA 66K MW, ovalbumin 45K MW, CPA 35K MW, PMSF trypsinogen 24K MW, and β lactoglobulin 18.4K MW.

^{35}S fMET

NCS

CNBr

f1 f0

f0 f1

66 K

45 K

f2

f3

f2

f3

35 K

f4

24 K

f4

f5

f6

18.4 K

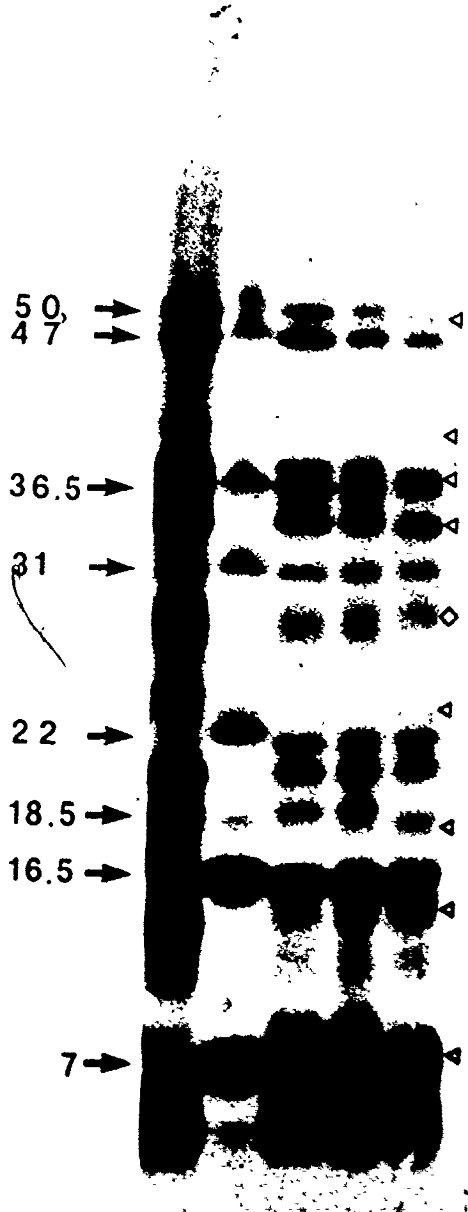
f7

gels in the presence of MW standards. In Figure 38 it could be seen that the pattern of cleavage fragments generated from uniformly [^{3}H]-labeled wild type N protein was identical to the pattern generated from similarly labeled R4 N protein. Comparison of the [^{3}H]-labeled NCS cleaved N protein of D1 virus with these patterns demonstrated that 7 fragments marked with triangles had an increased electrophoretic mobility corresponding to the difference observed between intact mutant and revertant N proteins. The same patterns were generated by cleavage with BNPS-skatole, which cleaves after tryptophan residues (data not shown). One peptide of the D1 N protein marked with a diamond had a decreased electrophoretic mobility relative to the wild type pattern. The significance of this is not known.

Calculation of the apparent MW of those fragments labeled with f[^{35}S]Met provided a linear oriented map of tryptophan residue positions. The site of the D1 N protein alteration was located on the linear map by referencing amino terminus containing fragments to altered mobility fragments. The point of comparison, beginning from the full size molecule, at which the mutant amino terminus containing fragment has the same mobility as the revertant indicates that the site of alteration exists to the carboxyl side of the cleavage site which generated the fragment.

It is seen in Figure 38 that the largest amino terminal fragment of the mutant N protein is indistinguishable by electrophoretic mobility from wild type or revertant. This indicated that the increased electrophoretic mobility in D1 N protein required a 3,000 dalton region

Figure 38. Fluorogram of SDS-PAGE fractionation of NCS peptides of D1, R4 and wild type N proteins labeled with [^{3}H] amino acids, [^{35}S]Met or f[^{35}S]Met. Proteins labeled with a mixture of 16[^{3}H] amino acids or [^{35}S]Met were isolated from purified virus. Protein labeled with f[^{35}S]Met was synthesized *in vitro* in the presence of the precursor f[^{35}S]Met-$tRNA_1^{Met}$. Cleavage with 15 mM NCS was as described in methods. The gel was 15% acrylamide and 0.2% NN'-methylenebisacrylamide and electrophoresis was at 125 V. Detection was by fluorography following impregnation with PPO. The molecular weight values (x 10^{-3}) are indicated for NCS peptides labeled with f[^{35}S]Met (MW*). Apparent MW values were derived by reference to the mobility of molecular weight standards: BSA 66K MW, ovalbumin 45K MW, CPA 35K MW, PMSF trypsinogen 24K MW, β lactoglobulin 18.4K MW and lysozyme 14.3K MW. Peptides of D1 N protein which possessed a faster electrophoretic mobility than corresponding wild type peptides are indicated (Δ). A slower peptide is marked (◇).



MW*
wt met
R4 f met
R4 3H
wt 3H
D1 3H
50
47
36.5
31
22
18.5
16.5
7

of polypeptide defined in the wild type linear map between the carboxyl terminus and the carboxyl-most tryptophan residue.

Also included in Figure 38 is the [^{35}S]Met-labeled wild type N protein after NCS cleavage. The pattern was an overlapping subset of the uniformly [^{3}H]-labeled N protein. By comparison with the f[^{35}S]Met-labeled pattern, it was apparent that the fragment corresponding to the 22K MW amino terminal fragment was not labeled, indicating that this region did not contain methionine. This was corroborated by the f[^{35}S]Met labeled CNBr map of R4 N protein, seen in Figure 37, which showed that no methionine residues existed in the amino terminal half of the protein. The finding that the 22K NCS fragment was not labeled in [^{35}S]Met-labeled N protein from purified virus also indicated that a post-translational processing event removed the initiating methionine residue.

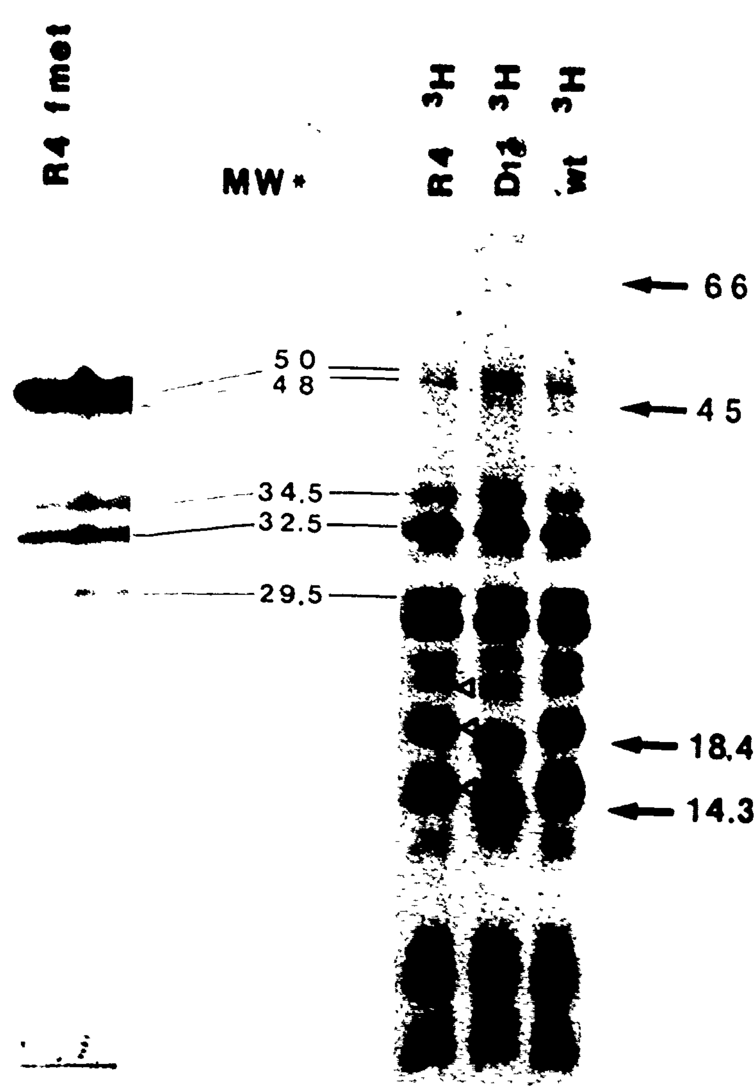
12.2. CNBr Cleavage

The same [^{3}H] and f[^{35}S]Met-labeled proteins employed for NCS cleavage were subjected to CNBr cleavage and analyzed by SDS-PAGE in 15% gels and are represented in Figure 39.

The relationship among the CNBr cleavage patterns of D1, R4 and wild type N proteins is consistent with the NCS derived patterns; R4 and wild type were indistinguishable whereas the D1 N protein derived pattern had three fragments of higher mobility than 3 wild type fragments marked with triangles.

The smallest amino terminal f[^{35}S]Met-labeled peptide of R4 N protein is 29.5K MW. This meant that a 60% portion of the molecule

Figure 39. Fluororgram of SDS-PAGE fractionation of CNBr peptides of D1, R4 and VS NJ(M) wild type N proteins labeled with a mixture of 16[^{3}H] amino acids or f[^{35}S]Met. The same labeled protein preparations used in Figure 38 were employed for this experiment. All apparent molecular weight values indicated are x 10^{-3} (MW*) and were derived by comparison with the mobilities of MW standards: BSA 66K MW, ovalbumin 45K MW, β lactoglobulin 18.4K MW and lysozyme 14.3K MW. CNBr peptides present in the R4 pattern which are altered in the D1 pattern are indicated (Δ), the difference in intact molecules is shifted but was not indicated by this symbol. The gel was 15% acrylamide and 0.2% NN'-methylenebisacrylamide and electrophoresis was at 125 V. Detection was by fluorography after impregnation with PPO.



R4 fmet
MW*
R4 ^{3}H
wt ^{3}H
66
50
48
45
34.5
32.5
29.5
18.4
14.3

Figure 40. Amino Acid sequence of the C-terminal region of the N protein of VS NJ(M). This represents the predicted amino acid sequence of 53 amino acids including the C-terminus published by McGeoch et al (1980). Three sites which have been located by procedures in this thesis are indicated by open arrows and trypsin susceptible peptide bond positions are marked by filled arrows. The peptide fragments are numbered beginning with the C-terminal peptide and are prefixed by C to denote this.

C8

--- LYS GLY ARG ASN VAL VAL ASP TRP LEU GLY TRP TYR ASP

ASP ASN GLY GLY LYS PRO THR PRO ASP MET LEU ASN PHE

C7 C6 C5 C4

ALA ARG ARG ALA VAL ASN SER LEU GLN SER LEU ARG GLU LYS

C3 C2 C1

THR ILE GLY LYS TYR ALA LYS ALA GLU PHE ASN LYS-**COOH**

beginning at the amino terminal was free of methionine and that the 4 methionine residues were clustered in the distal 40% of the molecule.

Mapping of the site of alteration by cross referencing f[^{35}S]Met-labeled R4 and tritium labeled D1, R4, and wild type N protein patterns, located the alteration in the fragment removed to generate f1. Approximately 2,000 daltons of protein material mapped in this region bounded by the carboxyl terminus and the ultimate methionine residue.

The D1 N protein alteration had been mapped to the carboxyl region of 3K MW bounded by tryptophan and more narrowly by a 2K MW carboxyl region bounded by methionine using NCS and CNBr cleavage.

Sequencing of a portion of the coding region at the 3' end of the VS NJ(M) N mRNA molecule by McGeoch et al (1980) has resulted in the prediction of the carboxyl amino acid sequence of 52 amino acids. The predicted sequence is presented in Figure 40 and supports the NCS and CNBr maps since it predicts the same position of the ultimate methionine and tryptophan residues.

12.3 Mapping of the D1 N Alteration by Carboxypeptidase Treatment

The predicted carboxyl amino acid sequence of VS NJ(M) N proteins contained a proline residue at the 32nd amino acid position from the C-terminus. The combined activities of carboxypeptidase A (CPA) and carboxypeptidase B (CPB) can remove all carboxyl amino acid residues except proline therefore providing a means of confirmation of the location of the D1 N protein alteration. If the alteration existed in the C-terminal region bounded by the last methionine residue, it was

expected that removal of this region with exopeptidases would leave a protein fragment which was the same for mutant or wild type.

Purified N proteins were incubated in the presence of DFP treated CPA and CPB and subsequently analyzed on 10% polyacrylamide gels by SDS-PAGE. Two levels of carboxypeptidase treatment were employed. Protein samples containing 25K cpm of [^{3}H] were reacted with 10 ug each of CPA and CPB for 5 hr at 37°C or 0.2 ug each of CPA and CPB for 1 hr at 37°C in 100 ul of 0.2 M N-ethylmorpholine acetate (pH 8.0). The reactions were stopped by lyophilization. The protein substrates were the pooled products of reduced and alkylated proteins obtained from virus particles and infected cells grown in the presence of a mixture of 16 [^{3}H]amino acids. The difference in apparent size of N proteins of D1, R4 and NJ(M) wild type is clearly discerned on SDS-PAGE analysis, Figure 41, tracks 4-6. In aliquots of these samples treated with carboxypeptidase A and B for an hour, the apparent sizes decrease to a uniform level, where D1 N protein was not perceptibly different from wild type or R4 N proteins (Figure 41, tracks 7-9). A more extensive exposure to carboxypeptidase A+B, as seen tracks 1-3 results in more discreet bands of indistinguishable apparent molecular weight between samples. Some endopeptidase activity was evident in the more extensive enzyme treatment as several lower molecular weight bands were observed. It appeared that enzymatic removal of 2K daltons of amino acid residues from the COOH-end of D1 N protein produced a residual peptide which was indistinguishable in electrophoretic mobility from the corresponding R4 or wild type peptide.

Figure 41. Fluorogram of SDS-PAGE analysis of uniformly [^{3}H]-labeled, reduced and carboxymethylated D1, R4, and VS NJ(M) wild type N proteins before and after treatment with the combined carboxypeptidases A and B. Proteins labeled with a mixture of 16[^{3}H] amino acids were the pooled isolates from preparations of viruses and infected cells. Proteins were reduced and subsequently aklylated using iodoacetic acid prior to preparative SDS-PAGE, all as described in methods. The protein samples in 0.2 M N-ethylmorpholine acetate (pH 8.0) were reacted without exopeptidases (CONTROL), with 0.2 ug each of DFP CPA, and DFP CPB for 1 hr or with 10 ug each of DFP CPA and DFP CPB for 5 hr. Samples of the preparations containing 7K cpm [^{3}H] were analyzed by SDS-PAGE at 150 V. The apparent MW value is x 10^{-3} (a) and was derived from comparison with mobility of D1 N protein 49K MW, R4 N protein 50K MW, and R4 M protein 32K MW.

CPA + B
10 ug ea
x 5 hr
CONTROL
CPA + B
0.2 ug ea
x 1 hr
wt D1 R4 wt D1 R4 wt D1 R4
48 K[a]

13. C-TERMINAL AMINO ACID DETERMINATION

The evidence now suggested that the D1 N protein alteration involved, or was contained in, a short carboxyl region of 29 amino acids with a subtractive apparent MW of 2K. If the D1 N protein differed in apparent size on SDS gels because it was physically smaller, it could have been the product of premature termination. If so then the C-terminal amino acid of mutant and revertant N proteins would be different.

I used two methods of C-terminal determination: selective tritiation and carboxypeptidase treatment. In the chemical tritiation procedure, the carboxyl-terminal amino acid residue of a protein is selectively tritiated through racemization by way of the oxozolone intermediate formed by the action of acetic anhydride in a reaction containing T_2O and pyridine (Matsuo and Narita, 1975). The C-terminal determination data of N proteins done by this procedure were inconclusive but are included in Appendix III.

Carboxypeptidase A and B remove amino acids sequentially from the C-terminus but each enzyme has a different substrate specificity. Carboxypeptidase A releases C-terminal residues with aromatic or large aliphatic groups the most rapidly, the other amino acids at various rates and Arg and Pro, not at all. Carboxypeptidase B releases Lys and Arg rapidly, neutral amino acids very slowly and other amino acids not at all. The combined enzymatic activities of CPA and CPB remove all C-terminal amino acids except Pro.

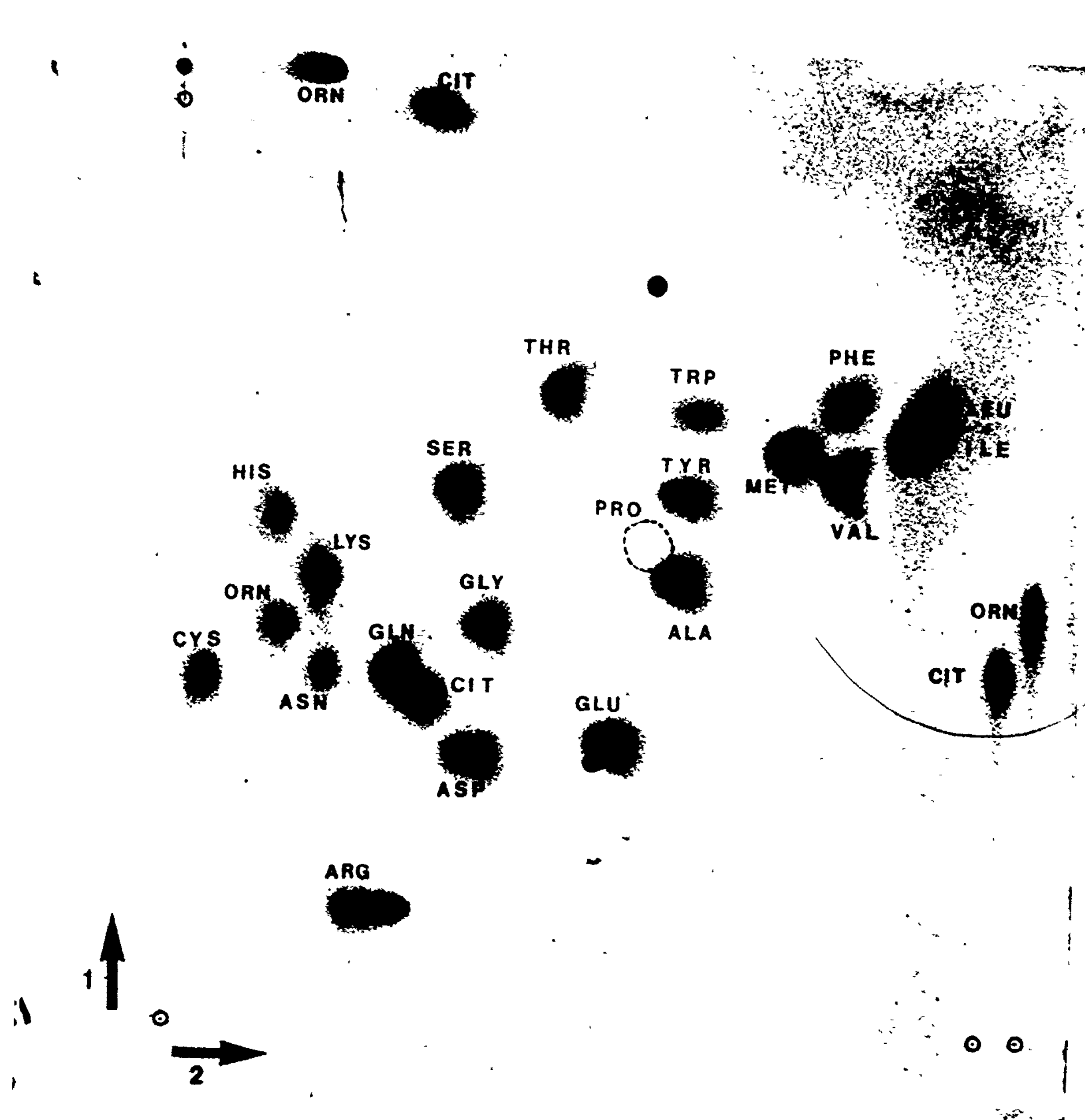
It was seen in the amino acid sequence in Figure 40 that McGeoch et al (1980) had predicted a carboxyl-terminal lysine residue in VS NJ(M) N protein. Such a residue was expected to be susceptible to removal by CPB with the technical advantage that only one amino acid would be removed making a time course of analysis unnecessary.

In order to calculate the moles of the amino acid residues release by CPB treatment the proportion of radioactivity per amino acid residue in the protein preparations was determined. The relative proportion of [^{3}H] radioactivity per amino acid species in each protein preparation was determined by quantitating the radioactivity of amino acids after acid hydrolysis and chromatographic separation.

Amino acid mixtures were separated on MN300 cellulose thin layers by two dimensional chromatography. The chromatography solvents were n-butanol:acetone:diethylamine:water (10:10:2:5) (pH 12) employed in the first dimension, followed by chromatographic separation in isopropanol:formic acid:water (40:2:10) (pH 2.5). Figure 42 showed a mixture of 22 amino acids after fractionation by two dimensional chromatography and ninhydrin detection. All amino acids were resolved except the pair, leucine and isoleucine.

The amino acid content of NJ(M) nucleocapsid protein was approximated by that of VS Indiana(T) N protein (Brown and Prevec, 1978) with corrections for those amino acid substitutions predicted from nucleotide sequence comparisons (McGeoch et al, 1980), see Table 8. The radioactivity per amino acid residue was then calculated by dividing the proportional quantity of each amino acid species by its corresponding

Figure 42. Fractionation of amino acids by two dimensional TLC on MN300 cellulose thin layer sheets. A mixture containing 10 nmoles each of the 22 amino acids indicated was applied to a MN300 cellulose thin layer sheet along with two additional applications of Orn and Cit at the positions indicated by open circles. Separation in the first dimension indicated by a numbered arrow (1) employed the solvent n-butanol: acetone:diethylamine:water (pH 12) (10:10:2:5:). Development in the second dimension employed the solvent isopropanol:formic acid:water (pH 2.5) (40:2:10). Detection was by ninhydrin staining. The proline spot stained yellow and was not detectable after photography but is circled.



ORN
CIT
THR
TRP
PHE
SER
HIS
TYR
MET
VAL
PRO
LYS
GLY
ALA
ORN
ORN
CYS
GLN
CIT
CIT
ASN
GLU
ASP
ARG
1
2

TABLE 8

RADIOACTIVITY OF INDIVIDUAL AMINO ACID RESIDUES IN TRITIUM LABELED PROTEINS

Amino Acid		Radioactivity/Residue[a]			
Species	no./N Protein[b]	D_1 N	R_4 N	wt N	R_4+D_1 M[c]
ARG	31.9	2150	1900	2150	5200
ASP+ASN	50	20	30	30	30
GLT+GLN	28.8	170	160	280	300
GLY	34.1	100	110	130	200
LYS	38	980	1080	1270	2740
HIS	8.2	920	980	570	810
SER	24.3	420	370	520	520
ALA	31.2	120	290	250	240
PRO	25.2	2550	2840	2580	6600
THR	21	990	1430	1400	2350
TYR	21.3	1120	870	1050	2310
MET	5	1580	3000	1130	1860
VAL	27.9	1200	1100	950	2450
PHE	18.1	7450	6900	6600	13490
LEU+ILE	55.9	1320	1280	1260	470
CYS	-	Not Labeled			
TRP	6.8	Not Labeled			

(a) Protein samples containing 100K cpm tritiated amino acids were hydrolyzed with HCl, fractionated by two dimensional TLC, eluted, and quantified by LSC; values presented are radioactivity/residue/500K cpm.
(b) The amino acid content is that of VS Ind(T) N protein adjusted by the changes predicted by McGeoch *et al* (1980).
(c) Calculated using the amino acid content data of Kendal and Cohen (1976).

molar representation per mole of protein. The radioactivity per amino acid residue for the [^{3}H] nucleocapsid proteins of D1, R4 and wild type, as well as for the pooled M protein from D1 and R4 are presented in Table 8. The incorporation of radioactive aspartic acid and asparagine was so low as to prevent accurate quantitation of released residues. The labeling of all other residues was several fold greater except for Cys and Trp which were not labeled.

Uniformly [^{3}H]-labeled N proteins from D1, R4, and wild type, as well as M protein pooled from D1 and R4, were incubated with CPB. The same reduced and alkylated protein substrates used in Figure 41 were used in this experiment. Reaction was achieved by adding 5 ug of DFP treated CPB to [^{3}H]-labeled protein (3-7x10^5 cpm) in 1.5 ml of 0.2 M N-ethylmorpholine acetate (pH 8.0) and incubating for 20 min at 37^{0}C. An aliquot of [^{3}H]-proline was added to each sample as an internal standard and a mixture composed of the 20 common amino acids was added (10 nmoles each) for ninhydrin detection.

The amino acid residues removed by CPB treatment are listed in Table 9. Values of 0.6, 0.8, and 0.7 moles of lysine were removed per mole of wild type, D1 and R4 N proteins respectively. Lysine was removed from the M protein sample as well, which was unexpected since this sample was included as a blank control. No amino acid sequence data was available for NJ(M) matrix protein. An untreated control sample of wild type N protein did not yield significant quantities of any amino acid. Some radioactivity was also detected in the histidine and alanine spots which could be explained by the partial overlap of the

TABLE 9

AMINO ACIDS RELEASED BY CPB TREATMENT OF TRITIUM LABELLED N AND M PROTEINS

Amino acid	Moles released/Mole of protein				
	wt N	D_1 N	R_4 N	wt N Control[a]	D_1+R_4 M
ARG	0.0	0.0	0.0	0.0	0.0
ASP[b]	0	0	0	20	0
GLT	0.0	0.2	0.1	0.1	0.0
ASN[b]	3	3	30	10	60
GLN	0.0	0.0	0.0	0.0	0.0
GLY	0.0	0.2	0.0	0.1	0.1
LYS	0.6	0.8	0.7	0.0	0.5
HIS	0.2	0.1	0.0	0.0	0.2
SER	0.0	0.0	0.0	0.0	0.0
ALA	0.1	0.1	0.1	0.1	0.2
PRO	INTERNAL STANDARD				
THR	0.0	0.0	0.0	0.0	0.0
TYR	0.0	0.0	0.0	0.1	0.0
MET	0.0	0.0	0.0	0.0	0.0
VAL	0.0	0.0	0.0	0.0	0.0
PHE	0.0	0.1	0.0	0.0	0.0
LEU+ILE	0.0	0.0	0.0	0.0	0.0
CYS	NOT LABELLED				
TRP	NOT LABELLED				
Recovery (%)	85	83	81	41	78
Sample size (cpm)	501K	341K	683K	900K	611K

Samples of reduced and alkylated protein labelled with a mixture of 16 tritiated amino acids were treated with DFP treated CPB. The liberated amino acids were fractionated by 2 dimensional TLC, eluted and quantitated by LSC.
(a) Without CPB treatment.
(b) Values given as cpm normallized to a sample size of 500K cpm.

lysine spot and the internal standard proline spot respectively. The data suggested that the C-terminus was identical in the N proteins of mutant, revertant and wild type.

The data represented a paradox. Linear mapping on SDS gels suggested that a carboxyl-terminal region of 29 amino acids in the wild type and revertant N proteins was smaller in the mutant. The only known revertible mutation which results in a smaller protein is a nonsense mutation leading to premature termination. Premature termination should produce a new C-terminus, but carboxypeptidase data indicated that the mutant and wild type proteins both terminated with lysine. Thus, although the data suggested a truncated protein, it had not resulted in a change in the C-terminal amino acid.

The predicted amino acid sequence in Figure 40, shows a tyrosine at position 8 from the C-terminus, coded for by UAU (McGeoch *et al*, 1980). If this codon mutated to either of two termination codons, the new C-terminus would be the lysine at position 9, making the C-terminus identical to wild type. Thus it was possible that the D1 N protein was shorter but maintained an identical C-terminal residue.

To distinguish between these possibilities I attempted to determine some fine structural features of the carboxyl region bounded by the ultimate proline residue. To this end, [^{3}H]-labeled N proteins were tryptic peptide mapped after CPB treatment, CPA plus CPB treatment, and no treatment, to identify and compare specific tryptic peptides. I also attempted to determine the amino acids released by CPA plus CPB

treatment to see if fewer amino acids were removed from D1 than from wild type N protein.

13.1 AMINO ACIDS REMOVED FROM N PROTEINS BY CPA PLUS CPB TREATMENT

The same uniformly [^{3}H]-labeled, reduced, and alkylated proteins subjected to CPB treatment were employed for CPA plus CPB treatment. The [^{3}H]-proline used as internal standard was repurified before use by two dimensional chromatography on thin layers of cellulose, eluted with 10 mM HCl and stored as a solution in 2 mM HCl. This was done to reduce the background level of other contaminating [^{3}H]-labeled amino acids. The protein samples (~5x10^5 cpm of [^{3}H] radioactivity) were reacted by incubation at 37^{0}C for 2 hr in 1.5 ml of N-ethylmorpholine acetate (pH 8.0) containing 10 ug DFP treated CPA, 10 ug of DFP treated CPB, 6.1K cpm [^{3}H]-proline internal standard and a mixture of 10 nmoles each of the 20 amino acids normally constituting proteins. The carboxypeptidase reaction was stopped by heating in boiling water for 5 min, and the released amino acids were bound to cationic exchange resin and the protein fraction saved for further use. The amino acids were fractionated by two dimensional chromatography, detected with ninhydrin, eluted, and quantified by LSC in triton X114:xylene based scintillation cocktail. The relative radioactivity values in Table 8 were employed to calculate the quantities of amino acids released. Table 10 lists the amino acids released from R4, D1, and wild type N proteins and from R4 plus D1 M protein. The recoveries calculated from the yield of internal standard ranged from 41-51%. The asparatic acid and asparagine values

TABLE 10

AMINO ACIDS RELEASED BY CPA+CPB TREATMENT OF TRITIUM LABELLED N AND M PROTEINS

Amino acid[a]	Moles released/Mole of protein			
	wt N	D_1 N	R_4 N	D_1+R_4 M
ARG	2.5	1.8	2.4	0.2
ASP[b]	190	160	90	30
GLT	2.3	2.2	2.7	0.5
ASN[b]	190	2030	290	250
GLN	o.2	0.3	0.4	0.4
GLY	2.3	2.3	3.1	1.1
LYS	4.8	5.5	5.1	1.3
HIS	1.4	1.2	1.1	0.9
SER	1.7	1.4	1.7	0.5
ALA	2.0	4.0	1.8	0.7
PRO[c]	INTERNAL STANDARD			
THR	1.0	1.1	1.2	0.1
TYR	1.3	1.2	1.4	0.2
MET	0.7	0.9	0.2	0.3
VAL	2.1	1.7	1.9	0.4
PHE	1.7	1.4	2.2	0.9
LEU+ILE	5.2	4.7	5.3	0.6.
TOTAL (Moles/Mole)	29.2	29.7	30.5	8.1
Sample size (cpm)	668K	341K	456K	339K
Recovery[d] (%)	51	46	50	45

(a) All amino acids except Cys and Trp were labelled.
(b) Values given as cpm normallized to a sample size of 500K cpm.
(c) Tritiated proline (6100 cpm) was added to each reaction.
(d) Calculated from the recovery of the internal standard.

were given as cpm since the radioactivity incorporated per residue was very low.

The total number of amino acids released from the different N proteins was very similar with 29.2, 29.7, and 30.5 for wild type, D1 and R4 N protein respectively. Only 8.1 amino acid residues were released from M protein. The amount of the individual amino acids was comparable among the N proteins, but there was a much greater quantity of label associated with the D1 N asparagine spot than with wild type or R4. This radioactivity was probably not due to asparagine since this would represent a 10 fold increase over wild type and R4. The fact that two post-translational modifications of arginine result in the formation of the compounds, ornithine and citrulline (Uy and Wold, 1977), which are found in positions near to asparagine after fractionation by the two dimensional chromatography system employed (von Arx and Neher, 1963), suggested that a modified form of arginine could have comigrated with asparagine. The quantity of radioactivity detected in the asparagine spot was the amount expected in one arginine residue. These facts, in combination with the observation in Table 10, that the D1 N protein yielded less arginine than R4 or wild type N protein, suggested the existence of a modified arginine group in D1. To determine if D1 N protein contained a modified arginine residue the chromatographic behavior of [^{3}H]-labeled amino acid components of D1, R4, and wild type N proteins which had been labeled with [^{3}H]-arginine was determined. Infected cells were labeled for 30 min with [^{3}H]-arginine as described in methods. Proteins were fractionated by SDS-PAGE, eluted

electrophoretically, precipitated with 20% TCA, washed with acetone and hydrolyzed in 6 M HCl as described in methods. When acid hydrolyzates of these proteins and uninfected cellular proteins were subjected to two dimensional chromatography and fluorography, only arginine contained [^{3}H] radioactivity (data not shown). Exposure times were employed which would detect 3% of the total label in a second spot in order to accurately assess the presence or absence of a second form of arginine present as 1 residue in 32. The result of this experiment indicated that Dl N protein did not contain an acid stable, modified arginine residue.

To be able to determine that the Dl N protein did not contain a more labile modified arginine, a Dl N protein sample labeled with [^{3}H]-arginine was hydrolyzed enzymatically with trypsin and chymotrypsin followed by carboxypeptidase A and carboxypeptidase B. Dl N protein labeled with [^{3}H]Arg digested at 37^{0}C with two aliquots of 10 ug TPCK trypsin in 50 mM ammonium bicarbonate (pH 8.5), the second portion being added 2 hr after the first. After 4 hr of trypsinization 10 ug of alpha chymotrypsin was added and digestion continued for 2 hr at 37^{0}C. The protein digest was then diluted into 10 volumes of 0.2 M N-ethyl-morpholine acetate (pH 8.0) and 10 ug each of CPA and CPB were added. Incubation was continued for 4 hr at 37^{0}C to complete hydrolysis. The resulting hydrolyzate was bound to H^+ form Amberlite IR 120, eluted, fractionated by 2 dimensional chromatography in combination with 10 nmoles each of 20 amino acids, stained with ninhydrin, impregnated with PPO in naphthalene and fluorographed. The resulting fluorogram had only

one spot at the arginine position and indicated that a post translationally modified form of arginine was not present in D1 N protein (data not shown). It was concluded therefore that the radioactivity detected in the Asparagine fraction of the D1 N protein was probably the result of contamination during sample analysis.

14. FINE STRUCTURAL MAPPING OF THE CARBOXYL REGION OF N PROTEIN

Treatment of wild type, D1 and R4 N proteins with CPA plus CPB released similar types and quantities of amino acids, suggesting that the region from the C-terminus to the first proline residue were of similar sizes. However, the protein change appeared to be located within this region of the physical map.

An attempt was made to locate the site of alteration within the terminal sequence of 31 amino acids. There was no positive data identifying the protein alteration in the carboxyl region, since the mapping data was of a substractive nature. That is; the alteration had been mapped to a specific location by the fact that its removal resulted in the loss of detectable alteration. I wanted to identify and locate altered tryptic peptide fragments of D1 N protein. To do this, I prepared tryptic peptide maps of untreated, CPB treated, and CPA plus CPB treated [^{3}H]-labeled N proteins of D1, R4, and wild type. Comparison of the tryptic peptide map of untreated protein with the map of CPB treated protein was expected to identify the C-terminal peptide of each protein. From the predicted sequence of amino acids (Figure 40) it could be seen that 7 tryptic peptides would be expected to be affected by CPA plus CPB treatment and thus identified in tryptic

peptide maps of CPA plus CPB treated protein. Comparison among the tryptic maps of D1, R4, and wild type N proteins was used to determine if the previously identified C-terminal peptide or other carboxyl region peptides were altered.

The trypsin susceptible cleavage sites are indicated in Figure 40 and examination of the 7 CPA plus CPB susceptible peptides indicated that 4 peptides contained lysine, 2 peptides contained arginine and one contained both arginine and lysine. This latter peptide, C7, had a C-terminal arginine and an internal lysine which was expected to be trypsin resistant since its carboxyl group was bound to proline. C7 also contained a methionine residue. One of the arginine-containing tryptic fragments was free arginine.

Tryptic mapping of [^{35}S]Met and [^{3}H]Lys-labeled protein preparations were employed to classify tryptic peptides. One of the expected tryptic peptides from the C-terminal region, C4, was the dipeptide Glu-Lys. Authentic α-L-Glu-α-L-Lys was obtained commercially to compare its map position with tryptic fragments.

Proteins labeled with a mixture of 16 [^{3}H]amino acids were portions of the previously employed reduced and alkylated preparation characterized in Table 7. N proteins treated with CPB, and CPB plus CPA, as well as aliquots of untreated N protein, were digested with trypsin. In preparation for tryptic digestions the proteins were lyophilized, dissolved in water, precipitated overnight with 5 volumes of acetone, collected by centrifugation at 10K rpm for 20 min in a Sorvall GSA rotor, dissolved in 70% formic acid and lyophilized.

Trypsinization was as described in methods, employing 20 ug of TPCK trypsin. The 2 dimensional maps were prepared as described in methods except that Silica G thin layers were employed. After 2 dimensional separation, the thin layer sheets were impregnated with 8% PPO (w/v) in naphthalene and fluorographed.

The protein subtrates employed for tryptic mapping analysis were subjected to SDS-PAGE fractionation (Figure 43). Carboxypeptidase B treatment did not change the electrophoretic mobility of the proteins, whereas CPA plus CPB treatment reduced the apparent molecular weights as was shown previously.

14.1 Identification of the C-terminal Tryptic Peptide

Figure 44, panels A and B represent fluorograms of tryptic peptide maps of untreated and CPB treated wild type N protein. On comparison, the tryptic peptide map of CPB treated wild type N protein was qualitatively identical to the untreated wild type N protein, but one peptide was present in greatly diminished relative quantity suggesting that it was the C-terminal peptide, C1. A shorter exposure of the fluorograms, allowed detection of a novel spot in the peptide map of CPB treated protein and was indicated in Figure 44, panel B by a heavy arrow. This new peptide spot had a similar chromotographic mobility to C1 but differed in that it did not move appreciably on electrophoresis.

14.2 Identification of CPA plus CPB Susceptible Tryptic Peptides

On tryptic peptide mapping of CPA plus CPB treated wild type N protein, and observation of the resulting fluorogram, it could be seen

Figure 43. Fluorogram of SDS-PAGE analysis of VS NJ(M) wild type, D1 and R4 N proteins before and after treatment with CPB or CPA plus CPB. Proteins samples were the reduced and alkylated pooled isolates of preparations of viruses and infected cells labeled with a mixture of 16 [^{3}H] amino acids. N protein samples of VS NJ(M) wild type (wt), D1 and R4 were treated with CPB, CPA plus CPB or left untreated (CONT) before analysis by SDS-PAGE. Aliquots of protein containing 5K cpm of [^{3}H] radioactivity were applied to a gel composed of 10% acrylamide and 0.13% NN"-methylenebisacrylamide. Electrophoresis was at 150 V and detection was by fluorography following impregnation with PPO.

wt D1 R4

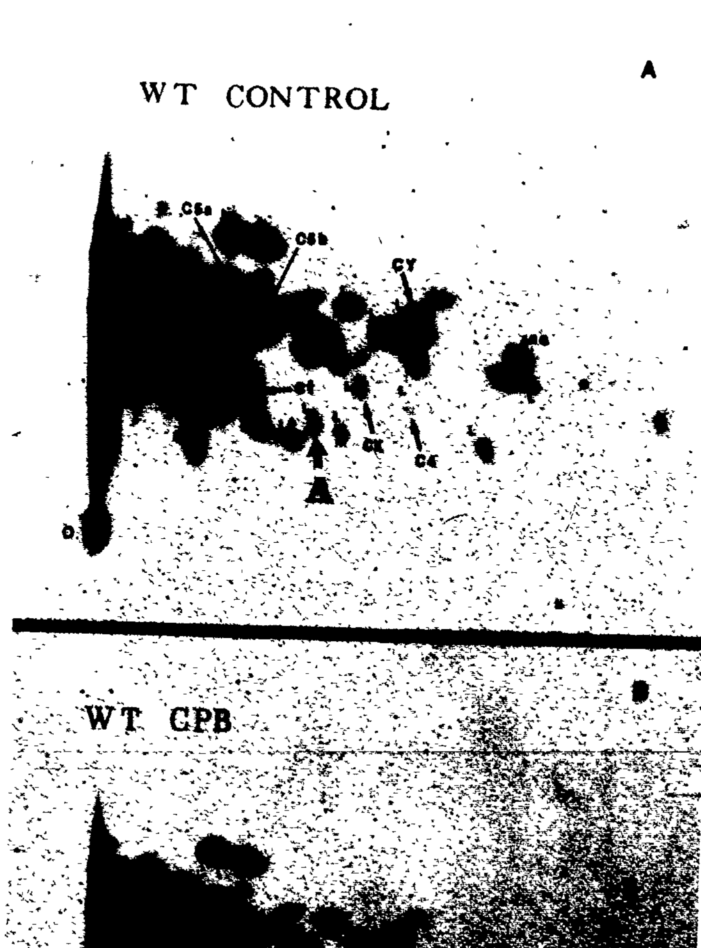
CPB CONT CPB+A CPB CONT CPB+A CPB CONT CPB+A

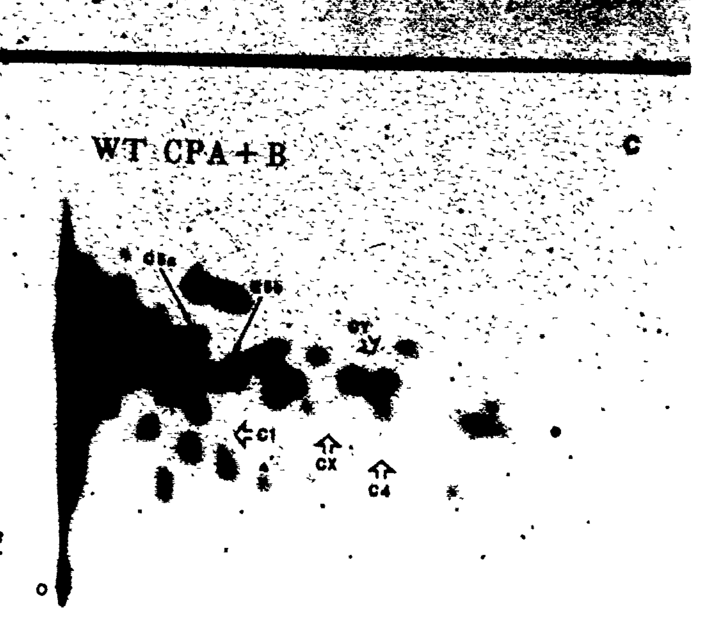
Figure 44. Fluorogram of tryptic peptide maps of VS NJ(M) wild type N Protein labeled with a mixture of 16[^{3}H] amino acids, before and after treatment with CPB or CPA plus CPB. The protein samples were the reduced and alkylated pooled isolates of preparations of viruses and infected cells labeled with a mixture of 16[^{3}H] amino acids. Protein samples which had been reacted with carboxypeptidases were freed of the released amino acids by treatment with H+ form sulfonated polystyrene beads. The protein samples were lyophilized and subsequently dissolved in 0.1 ml water and precipitated with acidified acetone (2% v/v acetic acid) along with 100 ug of BSA added as carrier. The samples were dissolved in 70% formic acid, lyophilized and then trypsinized as described in methods. Samples containing 7-10x10^4 cpm of [^{3}H] radioactivity were applied to Silica G thin layer sheets along with 10 nmoles each of Lys, Arg, and Asp-Lys. Electrophoresis was at 450 V until a phenol red marker had migrated 9 cm. TLC employed the solvent n-butanol: Pyridine:water: acetic acid (204:143:143:50). The peptide maps were stained with ninhydrin to detect Arg, Lys ( ) and Asp-Lys (Δ). Radioactive components were detected by fluorography after impregnation with PPO in naphthalene.

Panel A - VS NJ(M) wild type N protein. Peptides referred to in the text are labeled A, C1, C4, CY, CX C5a, and C5b. Peptides which contained methionine are labeled (M) as are those which contained lysine (L).

Panel B - DFP CPB treated VS NJ(M) wild type N protein. The position of C1 is noted by an open arrow. Short exposure allowed detection of an additional peptide marked by the filled arrow.

Panel C - DFP CPA plus DFP CPB treated VS NJ(M) wild type N protein. The position of peptides affected by exopeptidase treatment are indicated.


A
WT CONTROL
WT CPB


WT CPA + B
C

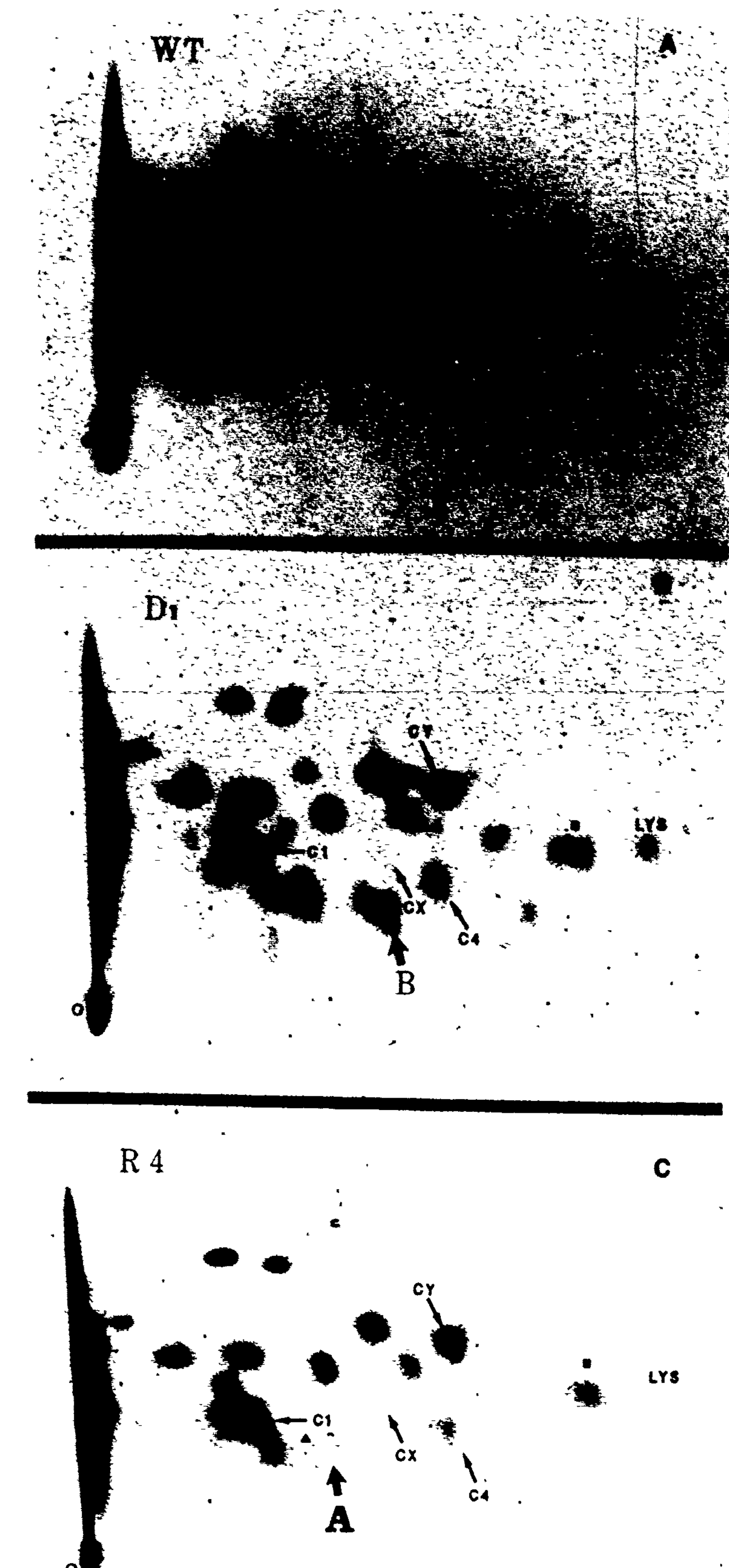
that 6 peptides were affected (Figure 44), four peptides were totally absent and 2 were reduced in quantity relative to the untreated control. The C1 peptide which represents the C-terminal peptide was removed by CPA plus CPB treatment, as was expected.

In order to identify the lysine containing peptide tryptic maps of reduced and alkylated [^{3}H]Lys-labeled N proteins of D1, R4, and wild type proteins were generated from proteins isolated from infected cells. Figure 45, panel A represented the fluorogram of the tryptic peptide map of [^{3}H]Lys-labeled wild type N protein. Four of the peptides previously shown to be affected by CPA plus CPB treatment were lysine labeled and are indicated.

To identify methionine containing peptides a tryptic digest of nonoxidized [^{35}S]Met-labeled wild type N protein was mapped in combination with uniformly [^{3}H]-labeled untreated N protein. The 2 dimensional tryptic peptide map was subjected to autoradiography to detect [^{35}S]Met-labeled peptides and subsequently fluorographed to detect [^{3}H]-labeled peptides. Two [^{35}S]Met-labeled peptides are indicated in Figure 44, panel A but most of the peptides labeled with [^{35}S]Met appeared as an indistinct array of spots which only migrated in the chromatographic dimension. None of the 6 tryptic peptides identified by CPA plus CPB susceptibility were observed to possess methionine.

Peptide C4 was predicted to have the sequence Glu-Lys (Figure 40). Authentic α-L-Glu-α-L-Lys was obtained commercially and peptide mapped in mixture with uniformly [^{3}H]-labeled wild type N protein to

Figure 45. Fluorogram of tryptic peptide maps of VS NJ(M) wild type, D1 and R4 N proteins labeled with [^{3}H] Lys. Proteins from [^{3}H] Lys-labeled infected cells were reduced and alkylated and then precipitated with acidified acetone (2% v/v acetic acid), dissolved in 70% formic acid and lyophilized before tryptic digestion as described in methods. Samples containing ~1x10^{5} cpm of [^{3}H] radioactivity were applied to Silica G thin layer sheets and subjected to TLE at 450 V until a phenol red marker had migrated 9 cm followed by TLC with the solvent n-butanol:pyridine:water:acetic acid (204:143:143:50). Detection was by fluorography after impregnation with PPO in naphthalene. The panels contain N proteins from VS NJ(M) wild type (A), D1, (B) and R4 (C). The location of Arg (●) and Asp-Lys (▲) are indicated.


WT
A
D1
CY
LYS
C1
CX
C4
B
O
R 4
C
CY
LYS
C1
CX
C4
A
O

locate peptide C4. The peptide C4 was also located on the [^{3}H]-labeled peptide map (Figure 45, panel A) confirming the fact that it contained lysine.

Most of the CPA plus CPB susceptible tryptic peptides were provisionally identified. Peptide C1 was identified by CPB susceptibility, as well as lysine content. Peptide C4 was identified by comigration on peptide mapping with α-L-Glu-α-L-Lys, and lysine content. Fragment C6 was expected to be free arginine, the position of which was located by comigration with unlabeled arginine detected by ninhydrin staining. The susceptibility of C6 to CPA plus CPB could not be detected due to the presence of free arginine removed from elsewhere in the protein, as predicted by McGeoch _et al_ (1980).

Peptides C5a and C5b were identified as the only fragments which did not contain lysine nor methinine but were affected by carboxypeptidases A plus B. It was assumed by default that they possessed C-terminal arginine. Peptide C5 probably exists in two forms as a function of incomplete cleavage of its C-terminal Arg bond. This may have been due to the considerably slower rate of hydrolyis reported for repetitive sequences of Lys and Arg (Kasper, 1975). Peptide C5b was absent in some tryptic peptide map preparations (Figure 43, panel B and Figure 44, panel B). Also, a completely independent preparation of tryptic peptide maps of D1 and R4 N proteins possessed C5a but not C5b suggesting that C5a is the authentic end product peptide (data not shown).

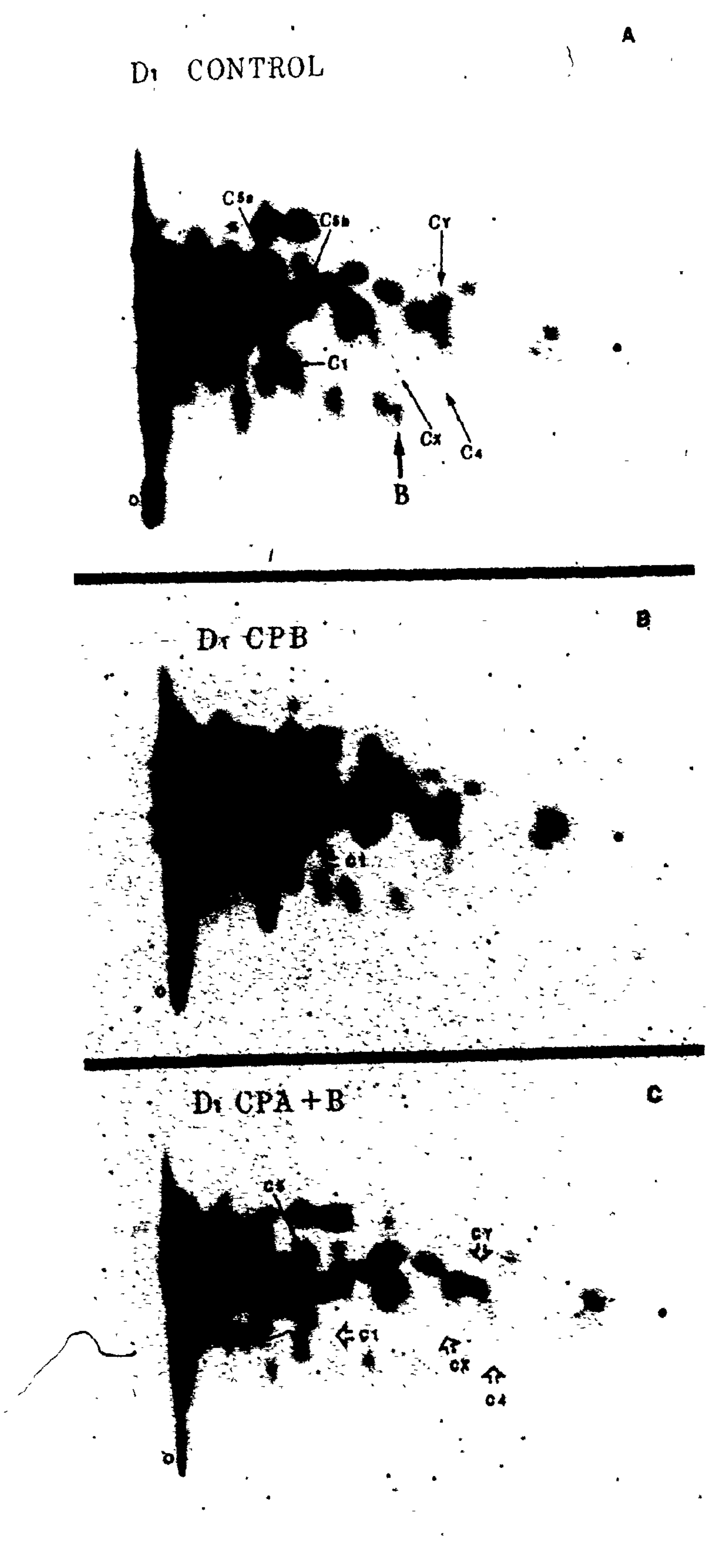
In addition to the two lysine peptides C1 and C4, three other Lys-containing peptides were predicted to be carboxypeptidase susceptible. Two unassigned peptides were affected by CPA plus CPB treatment which are also observed on lysine-labeled peptide maps (Figure 45, panel A) and were marked as Cx and Cy. The other tryptic peptide which was expected to be Lys-labeled was C7 which also contained Met, however only one peptide was detected which contained Lys and Met, yet it was not detectably affected by CPA plus CPB treatment (Figure 44 and 45). Peptide C7 may not have been resolved in the tryptic peptide map or not significantly reduced in quantity by CPA plus CPB treatment.

Comparison of the tryptic peptide maps of untreated D1 and R4 N proteins with the wild type map was done to allow detection of peptide differences. Comparison among the untreated peptide maps in panel A of Figures 44, 46 and 47 shows one peptide spot present in the wild type and R4 N protein patterns, labeled A, which was absent in the D1 N protein pattern, whereas a novel peptide spot designed B was present instead.

The electrophoretic behavior of peptide B indicates that it was more positively charged than peptide A since it migrated to a position nearer to the cathode. This peptide may have contained the structural alteration responsible for the increased isoelectric point of D1 N protein.

Peptide C1 was present in tryptic peptide patterns of D1 and R4 N proteins and was removed by CPB treatment (Figures 46, and 47, panel B). This finding confirmed the fact that the termini of D1, R4, and

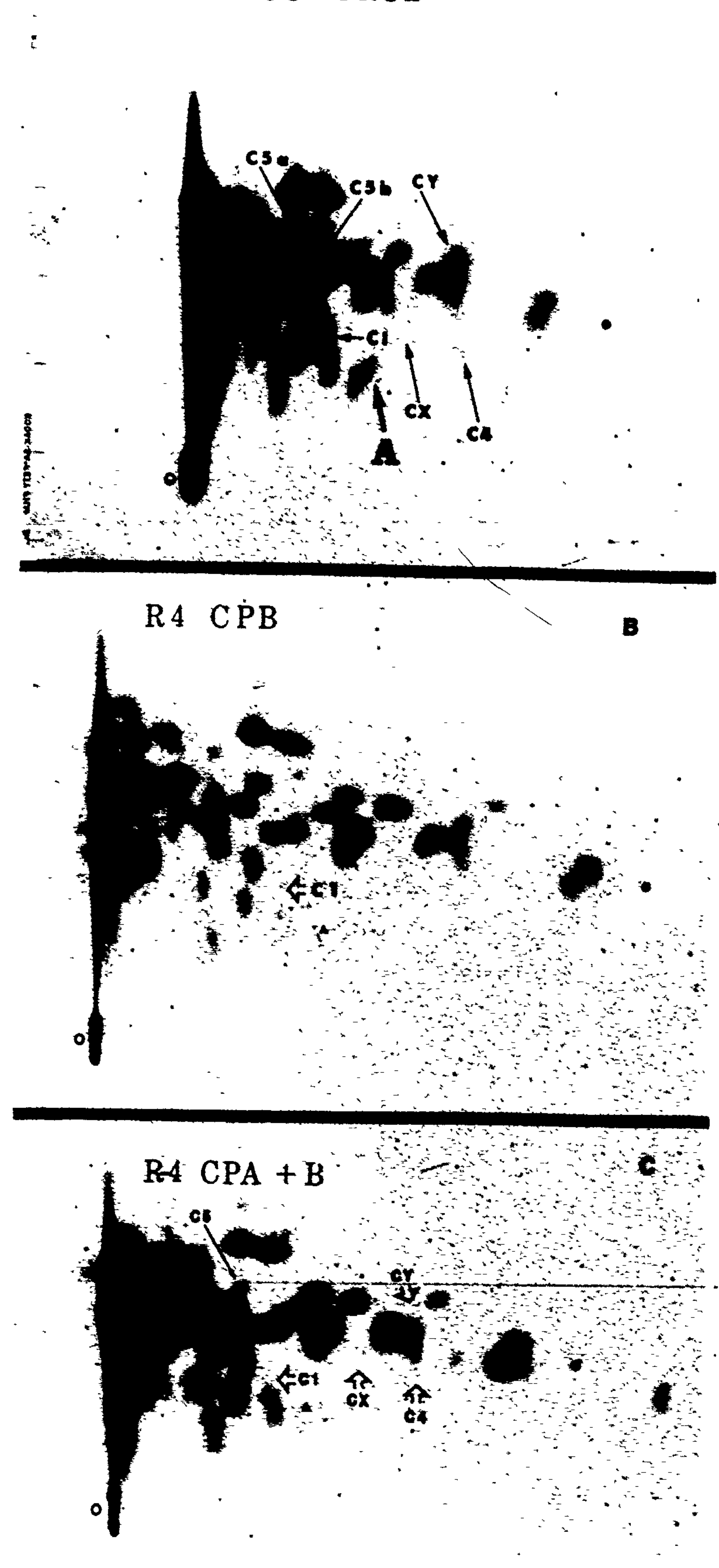
Figure 46. Fluorogram of tryptic peptide maps of D1 N protein labeled with a mixture of 16[^{3}H] amino acids, before and after treatment with CPB or CPA plus CPB. The protein samples were the reduced and alkylated pooled isolates of preparations of viruses and infected cells labeled with a mixture of 16[^{3}H] amino acids. Protein samples which had been reacted with carboxypeptidases were freed of the released amino acids by treatment with H+ sulfonated polystyrene beads. The protein samples were lyophilized and subsequently dissolved (0.1 ml of water) and precipitated with acidified acetone (2% v/v acetic acid) along with 100 ug of BSA added as carrier. The samples were dissolved in 70% formic acid, lyophilized and then trypsinized as described in methods. Samples containing 7-10x10^4 cpm of [^{3}H] radioactivity were applied to Silica G thin layer sheets. Electrophoresis was at 450 V until a phenol red marker had migrated 9 cm. TLC employed the solvent N-butanol:pyridine:wter:acetic acid (204:143:143:50). Peptide maps were stained with ninhydrin to detect Arg, Lys (●) and Asp-Lys (▲). Radioactive spots were detected by fluorography after impregnation with PPO in napthalene. The location of specific peptides are indicated.
Panel A - D1 N protein control
Panel B - D1 N protein after treatment with DFP CPB
Panel C - D1 N protein after treatment with DFP CPA and DFP CPB.



D1 CONTROL
A
C5a
C5b
CY
C1
CX
C4
B
O
D1 CPB
B
C1
O
D1 CPA + B
C
C5
CY
C1
CX
C4
O

Figure 47. Fluorogram of tryptic peptide maps of R4 N protein labeled with a mixture of 16[^{3}H] amino acids, before and after treatment with CPB or CPA plus CBP. The protein samples were the reduced and alkylated isolates pooled from preparations of viruses and infected cells labeled with a mixture of 16[^{3}H] labeled amino acids. Protein samples which had been reacted with carboxypeptidases were freed of the released amino acids by treatment with H+ form sulfonated polystyrene beads. The protein samples were lyophilized and subsequently dissolved in 0.1 ml of water and precipitated with acidified acetone (2% v/v acetic acid) along with 100 ug of BSA added as carrier. The samples were dissolved in 70% formic acid, lyophilized and then trypsinized as described in methods. Samples containing 6-7x10^4 cpm of [^{3}H] radioactivity were applied to Silica G thin layer sheets. Electrophoresis was at 450 V until a phenol red marker had migrated 9 cm. TLC employed the solvent n-butanol;pyridine:water:acetic acid (204:143:143:50). Peptide maps were detected by fluorography after impregnation with PPO in naphthalene. The location of specific peptides are marked by arrows and the position of Arg, Lys (●) and Asp-Lys (▲) are noted.

Panel A - N protein of R4, control.
Panel B - N protein of R4 after treatment with DFP CPB.
Panel C - N protein of R4 after treatment with DFP CPA and DFP CPB.


R4 CONTROL
A
C5a
C5b
CY
C1
CX
C4
A
O
R4 CPB
B
C1
O
R4 CPA + B
C
C5
CY
C1
CX
C4
O

wild type N proteins were identical and refuted the hypothesis that the D1 N protein alteration was a function of premature termination.

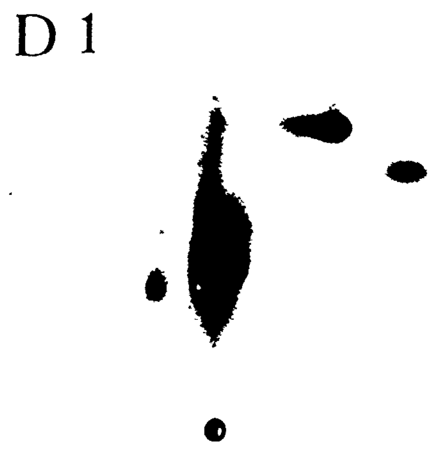
All the other identified CPA plus CPB susceptible peptides C1, C4, C5, Cx, and Cy, were present among D1, R4, and wild type N proteins (Panel A of Figures 44, 46 and 47). Treatment of D1 and R4 N proteins with CPA plus CPB was seen to result in decreased yields of this group of peptides.

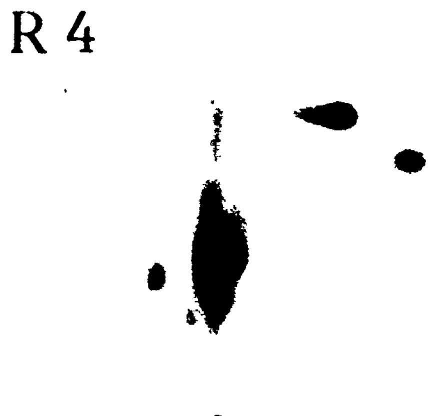
It appeared that the revertible mutation underlying the D1 N protein alteration was associated with a protein component which was not contained in the terminal sequence of 31 amino acids or in the 56 amino acids contained in tryptic peptides affected by CPA plus CPB treatment. However the largest tryptic peptide, C7, consisting of 25 amino acids including one Lys and Met had not been located. To circumvent this problem, comparison of N protein tryptic peptide maps was done employing the two labels [^{35}S]Met and [^{3}H]Lys.

The [^{3}H]Lys-labeled tryptic peptide maps of D1, R4, and wild type N protein are presented in Figure 45. The maps were identical except for the presence of peptide A in R4 and wild type and the presence of peptide B in D1, thus both these peptides contained lysine.

D1 and R4 N proteins labeled with [^{35}S]Met were isolated from infected cells and tryptic mapped after oxidation as described in methods. Figure 48 represents the [^{35}S]Met-labeled D1 and R4 N protein tryptic peptide maps prepared on Silica G thin layer plates and it appeared that the patterns were identical. The same digests were also mapped on MN400 cellulose thin layer sheets, to produce patterns

Figure 48. Autoradiogram of tryptic peptide maps of [^{35}S]Met-labeled N proteins of DI and R4. Proteins labeled with [^{35}S]Met were isolated from infected cells and were oxidized with performic acid prior to trypsinization. Samples containing ~5×10^4 cpm of [^{35}S] radioactivity were applied to Silica G thin layer sheets and subsequently fractionated by TLE at 450 V until a phenol red marker had migrated 9 cm. TLC employed the solvent n-butanol:pyridine:water:acetic acid (204:143:143:50). Detection was by autoradiography.


D 1


R 4

identical to the wild type pattern shown in Figure 7 (data not shown). N proteins labeled with [^{35}S]Met and isolated from pure virus of other D1 mutant subclones and a revertant from one of them ($D_1 2$, $D_1 6$, and $D_1 6R1$) were tryptic mapped on MN400 cellulose, producing indistinguishable patterns again (data not shown). Although the identity of C7 was not determined by these tryptic map comparisons, it followed logically from the invariance of the total [^{35}S]Met-labeled patterns and the [^{3}H]Lys-labeled patterns-excepting peptides A and B-that peptide C7 was invariant among wild type, D1 and R4 N proteins.

DISCUSSION

SECTION I: STRUCTURAL COMPARISONS OF PROTEINS

Structural comparisons of proteins are of value both for taxonomic purposes and for the identification of evolutionarily conserved amino acid sequences. Viral proteins were compared by tryptic peptide mapping and by 2 modifications of the partial proteolysis peptide mapping technique of Cleveland *et al* (1977).

The different mapping procedures suggested different extents of homology. The possible reasons for this will be discussed. As well, I will discuss the interpretation of partial proteolysis mapping with specific reference to the relationship between peptide map complexity and the number of susceptible cleavage sites. The ancestral relationship among the *Vesiculoviruses*, VS Ind, VS Cocal, VS NJ, Piry, and Chandipura, as evidenced by peptide structure comparisons will be placed in perspective with the existing information on their classification. Table 11 shows a summary of the *Vesiculovirus* relationships to VS Ind(T) detected by tryptic and NCS peptide mapping.

1. ANCESTRAL RELATIONSHIPS DEMONSTRATED BY TRYPTIC MAPPING

Ancestral relationships can be detected by demonstrating sequence homology in proteins. Functionally similar proteins which are no more structurally homologous than would be predicted by chance are termed analogous proteins and are assumed to have resulted from convergent evolution within ancestrally distinct organisms (Fitch, 1973). Since tryptic peptides are submolecular sequences of amino

TABLE 11

SUMMARY OF RELATIONSHIPS OF VESICULOVIRUSES TO THE PROTOTYPE VS INDIANA AS SHOWN BY COMPARISON OF PROTEIN STRUCTURE

VIRUS	Tryptic Peptide Mapping			Limited Proteolysis Mapping			
				N-Chlorosuccinimide			V8 protease
	G protein	M protein	N protein	G protein	M protein	N protein	N protein
VS Cocal	+	+	+	+	+	+	+
VS New Jersey	–	+	+	+	+	+	+
Piry	–	–	+	–	+	+	+
Chandipura	ND	ND	ND	ND	+	+	ND

(ND) not done.

acids, their conservation among proteins of different organisms is evidence of sequence homology and thus common genealogy.

The ancestral relationships demonstrated by tryptic peptide mapping of the N, M, and G proteins of VS Ind, VS Cocal, VS NJ, and Piry are consistent with previously published serological cross relationships (Cartwright and Brown, 1972b). It is evident from the results that functionally corresponding proteins in different Vesiculovirus serotypes can be readily distinguished by the pattern of methionine-containing tryptic peptides. Despite this general dissimilarity there were common features observed between the prototype VS Ind(T) and some serotypes.

The virus with the closest relationship to VS Ind was VS Cocal. Structural features in the N and G proteins were conserved in the two viruses. The G protein homology indicates a close relationship, since this protein is the type specific antigen and has been observed to be the most variable protein (Cartwright and Brown, 1972b). VS Cocal has previously been shown to possess homologous structures to VS Ind by interserotypic complementation of ts mutants, immunological cross-reactivity, heterologous interference, RNA hybridization and interserotypic reconstitution of in vitro transcription.

The Missouri and Concan strains of VS NJ serotype both showed the same level of tryptic peptide homology to VS Ind. VS NJ was less closely related to VS Ind than was VS Cocal. Tryptic peptide sequences in N and M proteins were conserved. No G protein homology was detected which is consistent with the lack of cross-neutralization with VS Ind antiserum (Cartwright and Brown, 1972b), VS NJ has been related to VS

Ind by a limited number of biological tests which include *in vivo* rescue of the transcription defect in the VS Ind ts mutant G114 and by heterologous interference. Low levels of RNA homology have been detected by RNA-RNA hybridization between VS NJ and VS Ind (Repik *et al*, 1974), but, nucleotide sequence data of McGeoch *et al* (1980) have demonstrated 71% homology. RNA-RNA hybridization data and my tryptic mapping data underestimate homology probably due to the fact that both techniques require conservation of sequence of subunits rather than of individual nucleotide bases.

Tryptic peptide mapping data of Piry virus proteins showed conservation of only one tryptic peptide with VS Ind. This peptide, number 3, of the N proteins may be significant since it is the only peptide which is common among the four serotypes tested. There is one report of cross-neutralization by antisera prepared against VS Ind, and there is some detectable RNA homology with the other *Vesiculoviruses* (Bishop and Smith, 1971).

2. Tryptic Peptide Homology Between VS NJ Strains Missouri and Concan

The Missouri strain is a member of the VS NJ Hazelhurst subtype, and the Concan strain is a member of the VS NJ Concan subtype (Reichmann *et al*, 1978). Both viruses react with anti-VS NJ(C) antiserum but they can be distinguished by the quantitative reaction indicating some structural differences in the G protein. The strains can also be distinguished by differences in electrophoretic mobility of their constituent polypeptides on SDS-PAGE. It has also been demonstrated that the RNA homology by hybridization is limited to ~20% and the T_1

oligonucleotide patterns are distinct (Clewley et al, 1977; Reichmann et al, 1978).

In apparent contrast to this degree of dissimilarity in the two strains are the tryptic peptide maps presented in this thesis. With the exception of a few peptides, there is almost complete homology of methionine containing peptides of M, N, and NS proteins. The G protein patterns were the most varied, sharing half of the constituent peptides in common. My data is not consistent with the findings of Metzel et al (1977) who have reported that tryptic peptides of the G, M, and N proteins of the two subtypes migrate identically in the electrophoretic dimension but separate in the chromatographic dimension.

A partial nucleotide sequence of the N mRNA of VS NJ Ogden strain of the Concan subtype (Rhodes and Banderjee, 1980) shows 82% homology compared to the VS NJ(M) sequence of McGeoch et al(1980). This is consistent with the high degree of conservation of protein structure detected by tryptic peptide mapping. It may be a general phenomenon that nucleic acid homology, as detected by hybridization of nuclease resistant acid precipitable material, can grossly underestimate true homology. Homology as detected by hybridization between the Papova viruses BK and SV40 is 11-20% whereas direct sequence comparison showed 70% homology (Yang et al, 1980). Presumably the lower level of homology by hybridization analysis as compared to direct sequence comparison is due to the fact that the majority of base paired sequences go undetected because they are small and thus are acid soluble.

3. Evolutionary Rate of Change of Protein Structure

Conserved amino acid sites can be operationally defined as serving specific functions. That is; conservation signifies functional necessity. Evolutionary rate of change of polypeptide structure has been observed to be dependent on the functional density of the polypeptide (Zuckerkandl, 1976), where functional density is the ratio of amino acids serving specific functions relative to those serving general functions. Specific functions are served by one invariant amino acid such as the proximal histidine in hemoglobin, or a specific constellation of amino acids such as the active site of enzymes. General functions are responsible for maintaining physical properties such as charge density, isoelectric point and solubility. Functionality implies a certain measure of invariance and this measure is insured by natural selection. Specific structural components of Vesiculovirus virions must be responsible for the gamut of intra- and intermolecular reactions which occur in infection and reproduction. Those proteins serving more specific functions will have a higher functional density and thus be more highly conserved. Such proteins will be more lasting documents of natural history than the less functionally dense proteins according to the evolutionary clock hypothesis (Wilson et al, 1977).

Tryptic mapping data demonstrated that the N protein was the most highly conserved with peptide number 3 being common among all serotypes tested: VS Ind, VS NJ, VS Cocal, and Piry, and peptides 4 and 1 being common among all except Piry virus. The fact that N protein appears to evolve more slowly suggests that it has a higher functional

density than the M or G proteins. This is not unexpected when it is considered that N protein interacts not only with RNA and other N protein molecules in the RNP complex but must also interact with proteins L and NS to yield functional transcriptive and replicative complexes (Emerson and Yu, 1975) and probably with protein M during assembly to yield a structurally stable virion (Dubovi and Wagner, 1977; Schnitzer and Lodish, 1979). The N protein may also serve specific functions in one or the other of the events which occur during in vitro transcription, such as polyadenylation, methylation, capping, or regulation of these events (Banerjee et al, 1977). Serological data of Cartwright and Brown (1972b) showed that the N protein was the most highly conserved antigen in support of the tryptic peptide finding.

The identification of specific conserved peptide sequences such as VS Ind N protein peptides 1, 3 and 4 may serve as a useful reference for further studies relating structure and function. Location of these peptide regions in the native protein may aid in location of sites responsible for specific functions or interactions.

The M protein was not seen to be conserved to the same extent as N protein but was more conserved than the G protein. The different proteins accept mutations at different rates making them useful for examining different extents of relatedness.

4. Partial Proteolysis Mapping on SDS-PAGE

A protein molecule containing n cleavage sites is expected to generate $(n+1)(n+2)/2$ peptide fragments after limited cleavage.

It appears from observation of NCS cleavage maps of proteins labeled N-terminally or uniformly with [^{35}S]Met, that the observed complexity of the uniformly labeled peptide pattern underestimates the complexity which would be predicted from the number of susceptible sites indicated by end labeling. For example, in the case of VS NJ(M) N protein, 16 component peptides were observed in the [^{35}S]Met-labeled NCS pattern, whereas 28 would be predicted from the 6 Trp sites observed in the f[^{35}S]Met labeled NCS cleavage pattern. There are several possible explanations for this situation:

i) There may be an uneven distribution of methionine resulting in peptide fragments devoid of [^{35}S]methionine.

ii) The peptide fragments may be present in different abundances with the result that only a subset of the fragments are detected.

iii) The f[^{35}S]Met-labeled NCS cleavage maps may not be a true representation of the number of cleavage sites and may result in overestimates of n.

The possibility that a large number of peptides are not labeled with [^{35}S]methionine is not an adequate explanation of the complexity problem because even in the case of NJ(M) N protein, which has its Met residues clustered to the carboxyl end, only 3 extra peptides were detected in the uniformly [^{3}H]-labeled N protein NCS pattern (Fig. 38). Ten peptides would be predicted to lack [^{35}S]Met but all of these would be labeled with [^{3}H]amino acids. This suggests that fragments which are not detected in the [^{35}S]Met-labeled NCS patterns, presumably because

they lack label, are still undetected in the NCS patterns of [^{3}H]-labeled protein even though they are labeled.

This situation makes the second possibility more plausible; that the different abundances of peptides result in some fragments going undetected. To examine this possibility, I will present a hypothetical situation in order to predict the yield of all possible fragments resulting from partial cleavage of a protein containing 10 cleavage sites. It will also be assumed that all sites are cleaved to 20% yield, since this value falls within the range of cleavage yields of Trp bonds when employing NCS. The abundance or yield of any peptide will be a function of the efficiency of cleavage at its termini and also the number of sites contained within it, which are susceptible to cleavage. Thus the amount of a fragment bounded by cleavage sites 1 and 5 and containing sites 2, 3, and 4, with k1-k5 representing the extent of cleavage at these sites, is represented by the relationship:

amount of fragment (1,5) = C (k1xk5)(1-k2)(1-k3)(1-k4) where C is the amount of protein substrate.

The yield of all possible fragments is given in Table 12. All fragments can be classed as resulting from one or two cleavages, it is clear that fragments resulting from single cleavage are more abundant. The average abundance of the fragments generated by single cleavage is 0.09, and those generated by double cleavage 0.02, relative to the quantity of protein C. This represents a fourfold difference in abundance; however, the peptide products are detected by fluorography and the content of labeled atoms per fragment must be taken into

TABLE 12

YIELDS OF CLEAVAGE PRODUCTS RESULTING FROM PARTIAL PROTEOLYSIS OF A HYPOTHETICAL PROTEIN

Class (no. of internal sites)	no./Class	Abundance (C)	Size (relative)	I
Fragments Produced by Single Cleavage				
0	2	0.20	1	0.20
1	2	0.16	2	0.32
2	2	0.13	3	0.39
3	2	0.10	4	0.40
4	2	0.08	5	0.40
5	2	0.06	6	0.36
6	2	0.05	7	0.35
7	2	0.04	8	0.32
8	2	0.03	9	0.27
9	2	0.03	10	0.30
TOTAL:	20	AVERAGE: 0.09	2.75	0.33
Fragments Produced by Double Cleavage				
0	9	0.04	1	0.04
1	8	0.03	2	0.06
2	7	0.03	3	0.08
3	6	0.03	4	0.08
4	5	0.02	5	0.08
5	4	0.02	6	0.08
6	3	0.01	7	0.07
7	2	0.01	8	0.06
8	1	0.01	9	0.06
TOTAL:	45	AVERAGE: 0.02	3.69	0.07
Uncleaved Protein				
10	1	0.09	11	0.95

consideration with the abundance. Equal amounts of large and small fragments would contain different amounts of label depending on the distribution of isotopically labeled sites. To correct the values in Table 12 for their different contents of radioisotope the further provision will be made that the cleavage sites are equally spaced along the molecule and that the labeling is constant per unit length. It is assumed that the protein contains 11 units of radioactivity. Given these assumptions each yield value is multipled by a number corresponding to its size and hence its amount of label, indicated in Table 12. The numbers in column I are the products of isotope content and abundance, and represent the quantity of radioactivity in the fragment species in relative units and become relevant to autoradiographic or fluorographic intensities. When the average values are compared, it becomes clear that the fluorographic image of this partial proteolysis pattern would be made up of two intensity classes since the 20 fragments resulting from one cleavage event have an average radioactivity of 0.33 compared to 45 fragments resulting from 2 cleavage events with an average radioactivity of 0.07. The same two average values were calculated for the hypothetical model but with cleavage increased to 30%. In this instance the average values were predicted to be 0.29 for single cleavage fragments versus 0.11 for double cleavage fragments.

A fluorogram of a partial proteolysis pattern will show those fragments with the most radioactivity due to abundance or distribution of labeled isotope. Longer exposures can be used to detect low

intensity fragments in simple peptide maps but in the case of complex patterns longer exposure usually results in a total loss of resolution due to overexposure; such was the case in NCS cleavage patterns of [^{35}S]Met-labeled G and N proteins.

The fact that cleavage site efficiencies vary, and also that the distribution of labeled sites vary, will also serve to generate fragments of unequal fluorographic intensity and thus detectability. Pattern complexity is further underestimated when peptides are obscured by overlapping fragments. It would appear that partial proteolysis map complexity, in practice, is better approximated by 2n+1 rather than (n+1)(n+2)/2. That is; partial proteolysis maps usually present a subset of the total fragments, made up of those fragments which are the most abundant or most densely labeled. This explanation of why detectable complexity of partial proteolysis maps underestimates the true complexity is compelling but consideration must still be given to the possibility that the number and location of cleavage sites may not be correctly derived-within the limits of SDS-PAGE separation-by limited cleavage of f[^{35}S]Met-labeled protein.

An independent method of identification of terminus containing NCS fragments was made by partial proteolysis in a second dimension employing V8 protease. The position of fragments which were identified by this procedure as terminus containing fragments were in agreement with the findings when f[^{35}S]Met labeled protein was employed for NCS cleavage.

Further evidence that a specific fragment (f_1) identified by f[^{35}S]Met labeling was a genuine N-terminal fragment was suggested by tryptic mapping of the corresponding [^{35}S]Met-labeled NCS fragment of VS Ind N protein. The largest NCS fragment of 49.5K MW generated a tryptic map differing from the map of the full size molecule by lacking peptides 11 and 12. This demonstrated that this fragment was the result of cleavage and not other chemical modifications resulting in altered electrophoretic mobility on SDS-PAGE analysis. From the amino acid sequence predicted by McGeoch et al (1980), this fragment was expected to be lacking 2 methionine containing peptides. As an aside, both of these fragments, 11 and 12, are conserved between the N proteins of VS Cocal and VS Indiana.

Tryptic peptide mapping of other [^{35}S]Met-labeled proteins produced peptide maps with identical patterns to the intact molecule due to the fact that some fragments overlap and cannot be isolated in pure form following SDS-PAGE fractionation. This occurred in tryptic peptide maps of VS Ind N protein NCS peptides of 27+27.5 MW and 23.5 MW (data not shown). Two dimensional partial proteolysis maps of VS Ind N protein also demonstrated overlap of several peptide fragments by the slightly staggered patterns in the second dimension arising from peptide bands in the first dimension (Figure 31).

The coding sequence of VS Ind(SJ) M protein contains 3 tryptophan codons predicted from nucleotide sequence data of J. Rose (personal communication). This offers strong support to my finding that 3 tryptophan residues exist in VS Ind(T) M protein as detected by NCS

cleavage of f[^{35}S]Met-labeled protein. The partial N proteins sequence prediction of McGeogh et al (1980) confirms the tryptophan site near the C-terminus of VS Ind VS NJ(M) N proteins.

5. NCS Cleavage Map Comparisons

Linear oriented maps of cleavage sites derived from NCS cleavage of f[^{35}S]Met-labeled protein were more easily compared than NCS cleavage patterns of [^{35}S]Met-labeled protein. [^{35}S]Met-labeled NCS maps demonstrated structural conservation in most instances but underestimated the conservation of tryptophan residues as detected by linear map analysis. This is probably due to the fact that NCS peptide patterns are affected by the number and distribution of methionine residues which varied more widely than Trp residues, as seen in [^{35}S]Met-labeled tryptic maps of VS Ind and VS NJ N protein.

Extensive Trp sequence conservation was not only observed in the N and M proteins but also to a more limited extent in G proteins. When generating linear maps by NCS cleavage mapping all of the Vesiculoviruses tested were shown to have similar ancestral relationships. This is interesting in light of the relative paucity of data linking Piry and Chandipura with VS Ind. However, an ancestral relationship would be expected from the RNA homology detected by Bishop and Smith (1977) and the serological cross reactivity reported by Dragunova and Zavada (1979) and Murphy and Shope (1971).

Nucleocapsid proteins were peptide mapped employing V8 protease by the standard partial proteolysis mapping technique of Cleveland et al (1977). This method was not as effective as NCS mapping at demonstra-

ting an ancestral relationship of Piry virus N protein, but was able to demonstrate structural conservation among the more closely related VS Ind, VS Cocal, and VS NJ(M). Although Piry and Chandipura viruses are related to VS Ind by conservation of tryptophan residues the question can be raised: "What level of classification does tryptophan residue conservation indicate?" To answer this question, the tryptophan positions of proteins of viruses from outside the Vesiculovirus genus but within the Rhabdoviridae must be compared. The data in this thesis suggested that members of a genus have very smilar tryptophan arrangements in their proteins but it did not indicate whether nongenus members would also show similar conservation of structure.

6. Tryptic Peptide Mapping Versus NCS Peptide Mapping

After employing both tryptic peptide mapping and NCS cleavage mapping for comparison of the same viral proteins, it becomes clear that the techniques can provide different answers to the same question. To place this phenomenon in perspective I will discuss the factors which determine variability as determined by the two approaches.

One difference between the techniques is that peptides are separated as a function of size rather than composition in NCS peptide mapping whereas the detection of peptide similarily in tryptic maps requires exact sequence conservation. Minor differences in peptide composition of tryptic peptides results in a complete loss of detectable similarity.

It has been shown by Dayhoff et al (1972) that different amino acid types are substituted at various rates. While both Met and Trp are

the rarest amino acids on average in proteins, they represent the opposite extremes of amino acid variability. Met has been observed to be the most variable amino acid type being substituted by other amino acids the most frequently, whereas Trp is the most highly conserved amino acid and is rarely substituted for in proteins. This is probably due to the fact that Trp is the largest amino acid with a specific chemistry which cannot be substituted for without disruption or loss of protein function. Methionine, on the other hand, is a hydrophobic amino acid which can be replaced by several other hydrophobic amino acids of similar size.

In analogy with the evolutionary clock hypothesis, methionine variability may measure minutes and tryptophan variability, hours. This has been supported by the findings here. Piry virus was essentially unrelated to other Vesiculoviruses by [^{35}S]Met-labeled tryptic map analysis but closely related by NCS cleavage map comparison.

Section II: Characterization of the N Protein Alteration in VS NJ(M) D1

The VS NJ(M) D1 ts mutant was studied since it possessed an N protein with characteristics which suggested that it was the product of premature termination. The aim of the study was to characterize the N protein alteration and determine if it was the result of premature termination. A conditional lethal mammalian virus which was the result of premature termination, and thus contained a nonsense mutation, would be valuable as a tool in screening mammalian cells for the presence of nonsense suppressor activity.

7. The Location of the D1 N Protein Alteration

Positioning of the N protein alteration on the linear map by partial cleavage of f[^{35}S]Met-labeled protein with NCS and CNBr placed it within a short carboxyl segment of the protein. Figure 49, panels A and B shows the linear oriented maps of Trp and Met positions on NJ(M) N protein and the regions which had been assumed to be responsible for the D1 N protein alteration, by virtue of the fact that removal of these segments resulted in residual peptides which were indistinguishable from the corresponding wild type N protein fragments. The apparent molecular weight of these regions calculated by subtraction of apparent MW values of the amino terminal labeled fragment from the full size molecule were 3K from NCS cleavage and 2K from CNBr cleavage. The regions bounded by the C-terminus and the ultimate Trp and Met residues contain 42 and 29 amino acids respectively (McGeoch et al, 1980).

Treatment of N proteins with a combination of CPA and CPB demonstrated that removal of the carboxyl region of 31 amino acids resulted in a residual protein fragment which was indistinguishable from wild type by SDS-PAGE analysis.

The suggestion from these three map analyses was that the D1 N alteration was contained in a carboxyl region containing 29 amino acids, but all the mapping techniques were of a subtractive nature. That is; the defect was located by removing portions and comparing the remainder to see if it was altered. Thus there was no direct comparison of the carboxyl peptide fragments identified by the mapping procedure. Such small peptides are not resolvable under standard SDS-PAGE conditions.

Figure 49. Linear oriented map of Trp and Met residues in VS NJ(M) N protein and the location of the D_1 N protein defect.

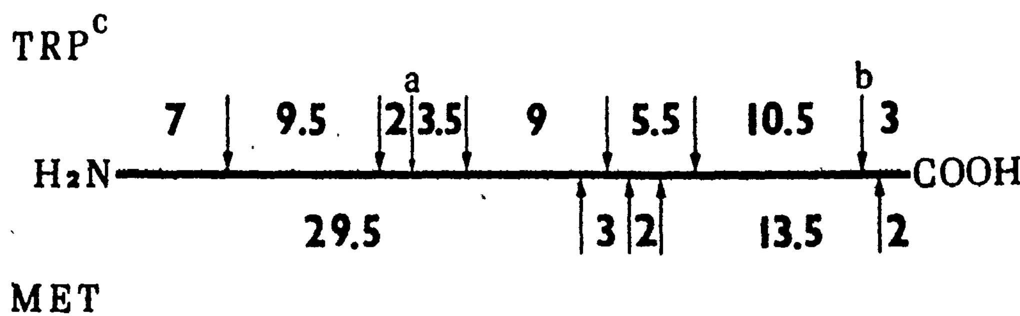
a) This site had a very low susceptibility to NCS cleavage.

b) This site represents two Trp residues McGeoch et al (1980).

c) Values are $MW \times 10^{-3}$ and were determined from the decrease in apparent MW observable when the peptides were removed by cleavage.

d) Regions were identified by the fact that their removal resulted in residual peptides which were indistinguishable among N proteins of D1, R4, and wild type.

A LINEAR ORIENTED MAP OF TRP AND MET RESIDUES IN NUCLEOCAPSID PROTEIN.



B REGIONS IMPLICATED IN D1 MOBILITY SHIFT[d]

H2N CLEAVAGE at TRP 3 COOH

H2N CLEAVAGE at MET 2 COOH

H2N SUSCEPTIBILITY to CPA + CPB 2 COOH

C LOCATION of ALTERATION

H2N 10.5 COOH

The mapping data was consistent with premature termination but C-terminal amino acid determination indicated that the D1, R4, and wild type N proteins all ended in lysine. Further confirmation that the C-termini were conserved came from the identification of an indistinguishable C-terminal tryptic peptide among these N proteins. The C-terminal tryptic fragment was identified by its susceptibility to CPB activity.

Tryptic peptide map comparisons among uniformly [^{3}H]-labeled N proteins of D1, R4 and wild type demonstrated a revertible peptide difference. From the linear peptide mapping data, it was expected that this tryptic peptide would originate from the carboxyl region sequence of 29 amino acids.

Treatment of N proteins with CPA plus CPB was predicted to affect 7 tryptic peptides representing a sequence of 51 amino acids beginning at the C-terminus. All the CPA plus CPB susceptible tryptic peptides were shown to be indistinguishable among D1, R4, and wild type by 2 dimensional fractionation on thin layers, but peptide C7 was not identified. The largest peptide C7, was predicted to contain Lys, Arg and Met among its constituents. Tryptic peptide mapping of [^{3}H]Lys and [^{35}S]Met labeled N proteins, showed complete conservation of structure except for the previously identified altered peptide B in D1 N protein. The only tryptic fragment which was not detected by CPA plus CPB susceptibility was free Arg, since free arginine is predicted to be released by trypsin from elsewhere in the protein (McGeoch et al, 1980). The free arginine residue, C6, is predicted to be between fragment C7

and C5. The existence of fragment C5 as two forms, a and b, in D1, R4, and wild type N proteins, suggests that it is bounded by a sequence of Arg-Arg or Arg-Lys, since repeated trypsin sites are cleaved slowly resulting in fragment heterogeneity.

It is concluded that the D1 N protein alteration manifested by the peptide alteration A to B lies outside the carboxyl region identified by linear mapping. This is interpreted to mean that a portion of the carboyxl end region of the D1 N protein is required to express the alteration in electrophoretic mobility. Removal of 29-42 amino acids from the carboxyl end prevents detection of, rather than removes, the cause of the alteration.

It may still be argued that the tryptic peptide analysis was not exhaustive and that altered peptides had escaped detection after fractionation or that the structural difference in one of the CPA plus CPB susceptible peptides was undetectable. However, nucleotide sequence analysis of the 3' end of the N mRNA of D1 and a ts^+ revertant has shown them to be identical in the region encompassing 52 amino acids from the C terminus (D. McGeoch, personal communication).

If it is accepted that the carboxyl region is required for expression of the mobility shift but that the structural perturbation underlying it exists outside this region, it can then be located on the physical map by detection of the smallest NCS or CNBr fragment which still shows altered electrophoretic mobility. The smallest detectably altered fragment would be assumed to consist of the carboxyl region required for detection of the alteration, as well as the portion of the

protein which contains the structural perturbation in D1 N protein. . In the case of NCS cleavage, a fragment of ~10K MW was present in the wild type pattern but was shifted to lower apparent MW in the D1 N pattern (Figure 38), and a fragment of 15.5K MW was present in the wild type pattern of the CNBr cleavage pattern but was shifted to a lower MW in the D1 N protein (Figure 39). It is assumed that both these fragments contain the C-terminus and arose from cleavage at the penultimate cleavage site. Since the alteration does not exist in the region defined by the ultimate NCS or CNBr cleavage sites and the C terminus, it is concluded that the alteration was contained in the region defined by the penultimate and the ultimate cleavage sites on the physical map. These mappings overlap but the narrowest definition of the location of the D1 N alteration is the 10.5K MW region of the NCS cleavage map adjacent to the short carboxyl region. This region is displayed in Figure 49, panel C.

The D1 N protein alteration is probably due to an amino acid substitution or possibly to a post-translational modification; in either event one amino acid would be involved. Only one peptide is different on tryptic peptide map analysis. The altered peptide, B has a higher mobility towards the cathode on electrophoresis at pH 3.5 and may represent the structural change resulting in the isoelectric point increase of D1 N protein to 7.05 from 6.95 in the wild type and revertant.

Post-translational modification of arginine residues or phosphorylation were not seen to occur in D1 or wild type N protein.

8. Is the D1 N Protein Alteration Responsible for Temperature Sensitivity?

Reversion of ts D1 virus was often concomitant with restoration of wild type electrophoretic mobility to the N protein on analysis in SDS-containing gels. Early results of Wunner and Pringle (1974) suggested that temperature-sensitivity and N protein mobility were tightly linked but more recently they have questioned this relationship (Evans et al, 1979). SDS-PAGE analysis of mutants and revertants presented in this thesis also indicate that the N protein is not necessarily the site of the ts mutation since a revertant exists with an abnormal N protein and a mutant exists with a normal N protein.

The existence of revertants which possess altered N protein could be explained by invoking secondary site changes within the N protein or in another viral protein. Both situations are characteristic of suppression, the first, intragenic, (Schweingruber et al, 1979) and the second, intergenic (Ramig et al, 1977). In suppression, the original mutation remains but a coordinated change in another gene, another mutation, results in restoration of wild type pheotype.

It is more difficult to explain the existence of a D1 ts mutant with a normal N protein. Presumably this would require a primary site change sufficient to restore wild type electrophoretic mobility but insufficient to restore ts+ function. Revertants which contain other than the parental amino acid sequence have been described (Schweingruber et al, 1979), but the situation of a ts D1 subclone with a normal N

protein represents a non selected variant of the original mutant. Since this mutant subclone could not be selected, it is surprising that it was obtained from a plaque after dilution to end point. It would be useful to tryptic peptide map these mutant and revertant N proteins to ascertain whether the peptide A or B or another variant peptide exists.

Characterization of the phenotypic defect of the VS NJ(M) D1 ts mutant may allow identification of the defective protein.

9. Can an Amino Acid Substitution Significantly Alter a Protein's Mobility on SDS-PAGE?

Presently it is a routine assumption that the mobility of a protein on SDS-PAGE is solely a function of its molecular weight (Shapiro et al, 1967; Weber and Osborn, 1969), except for some well characterized exceptions such as glycoproteins and highly basic proteins (Panyim and Chalkley, 1971; Banker and Cotman, 1972).

A linear relationship exists betwen log protein MW and electrophoretic mobility in SDS-containing gels for proteins of ~15-100 K MW. Numerous studies have verified the method from both a theoretical and a practical standpoint for a large number of soluble proteins. The general application of SDS-PAGE to protein molecular weight determination is not in disupte but the extent to which specific proteins may deviate from expected behavior has been questioned (Maddy, 1976).

It has been established that proteins of identical molecular weight but differing in amino composition can have markedly different apparent MW from SDS electrophoresis measurements of hemoglobin alpha

and beta chains (Weber and Osborn, 1975), alpha crystallin A and B chains (van der Ouderaa *et al*, 1974), parvalbumins (Sullivan *et al*, 1975), and cucumber virus coat proteins (Tung and Knight, 1972). It is now known that proteins differing by single amino acid residues show differences in electrophoretic mobility in SDS-containing gels corresponding to apparent MW changes of 1000-2000. This has been described in alpha crystallin proteins (de Jong *et al*, 1978), a Qβ coat protein mutant (Glowacki Strauss and Kaesberg, 1970) and a histidine transport protein of *Salmonella typhimurium* (Noel *et al*, 1979). The Dl N protein shift in electrophoretic mobility on SDS-PAGE analysis may also be an example of a single amino acid change.

In an attempt to explain the basis of this phenomenon reference must be made to the current understanding of SDS protein interaction with respect to electrophoresis in SDS-containing gels. It has been proposed that the binding of large amounts of SDS anions to proteins overshadows any intrinsic charge differences between proteins and allows the relationships between migration in SDS-PAGE and MW (Banker and Cotman, 1972). The protein molecular weight - electrophoretic mobility relationship is based on two assumptions: a) that the binding of SDS is constant and uniform per unit polypeptide chain length and is sufficient to abolish any intrinsic charge differences, and b) that conformation differences are obliterated resulting in complexes whose frictional coefficients are due to variation in molecular weight (Maddy, 1976).

Evidence is available which shows that these two assumptions are not justified in every case. Early studies of SDS binding to proteins

by Reynolds and Tanford (1970) as well as Pitt-Rivers and Impiombato (1968) determined that reduced proteins bind 1.4 g SDS/g protein but several years later a re-evaluation by Takagi et al, (1975) showed tha the amount of SDS bound per gram of protein varied over a range of 1.2-1.5 g. Another instance of abnormal SDS binding was observed in cytochrome b5 which possesses two structural domains, one which binds 2.5 times the normal amount of SDS, and the other which binds 0.5 times the normal amount (Robinson and Tanford, 1975). It is clear that it is not categorically correct to assume uniform and constant SDS-polypeptide interaction.

Less information is available regarding the conformation of SDS-protein complexes but there are currently three models derived from hydrodynamic measurements. These are: the prolate ellipsoid (Reynolds and Tanford, 1970) the deformable prolate ellipsoid (Wright et al, 1975), and the necklace model (Shirahama et al, 1974). The first two models differ primarily in the extent of flexibility attributable to the SDS-protein complex and the third model assumes that the association of SDS with protein occurs through micelle formation. Measurement of the retardation coefficients (Banker and Cotman, 1972) show that the effective size of some protein-SDS complexes deviates from the expected values, indicating some variability in conformation.

The fact that specific single amino acid substitutions can significantly alter electrophoretic mobility suggests that a minor structural change can significantly perturb SDS-protein interaction as a function of altered conformation and/or extent of SDS binding. From

consideration of the previously described instances of this phenomenon, it appears that both SDS binding and the conformation of the resulting SDS-protein complex may be affected by single amino acid substitutions. In alpha crystallin proteins neutral substitution of hydrophilic residues with hydrophobic residues or substitution of proline by both hydrophobic and hydrophilic residues results in increased mobility. There should be no conformational change involved in neutral substitutions of hydrophobic for hydrophilic residues so increased electrophoretic mobility may be attributable to an increased anionic charge to mass ratio. This is consistent with information regarding SDS binding to free amino acids which showed that amino acids with cationic or hydrophobic residues bind strongly with SDS, Ala and Pro to a lesser extent and acidic and neutral hydrophilic bind no SDS at all (Maley and Guarino, 1977).

Substitution of Pro by Thr or Ala in alpha crystallin protein results in increased electrophoretic mobility even though Ala binds to SDS to the same extent as Pro and Thr does not bind SDS. In this instance it would seem reasonable to assume that a conformational change may have resulted from removal of the proline residue which has a restricted peptide bond conformation.

Increased electrophoretic mobility on substitution of cationic Arg for neutral hydrophilic cysteine in the histidine transport protein, is consistent with increased SDS binding. The substitution resulting in increased electrophoretic mobility of Qβ coat protein was Lys to Gln.

The affinities of individual amino acids for SDS cannot serve as an explanation of the electrophoretic behavior of this protein.

If charge differences are responsible, extensive increases in SDS binding may be required since the addition of two negative charged groups to elephant alpha crystallin does not affect electrophoretic mobility (de Jong _et al_, 1978). Presumably the surrounding protein structure is crucial for SDS protein interaction since only specific substitutions result in a change in electrophoretic mobility. The picture emerges, therefore of local SDS-protein interactions due not only to the individual substitution but also to the surrounding residues. Since data is available which suggests a cooperative association of SDS with protein (Takagi _et al_, 1975) single substitutions may alter the interaction of a larger structural unit by enhancing or disrupting a cooperative SDS-protein association.

10. Models for the Increased Mobility of Dl N Protein

I wish to propose two models to explain the increased electrophoretic mobility of Dl N protein. Both models assume a single amino acid substitution and the involvement of the carboxyl region of the protein which is required to manifest the aberration on SDS-PAGE. In the first model (A) the Dl N protein alteration is considered to result in increased SDS binding and the second model (B) invokes a structural change as the result of a point substitution.

Model A - It is assumed in this model that the altered N protein binds more SDS than wild type N to result in an increased anionic charge density. It must also be assumed that increased SDS binding involves

adjacent regions of the protein, since removal of 29 or 42 amino acids from the C-terminus prevents increased SDS binding, even though the protein alteration remains. This is conceivable if the SDS associates with the protein to form micelles. It may be that removal of a piece of the protein normally contained in the SDS micelle prevents its formation and thus increased SDS binding does not occur.

Model B - The increased electrophoretic mobility of Dl N protein may be due to a structural change in the protein-SDS complex. By analogy with the alpha crystallin protein, the substitution of another amino acid for a proline residue may result in increased electrophoretic mobility. Presumably, this would affect the frictional drag of the SDS-protein complex by changing the overall conformation or restricting the flexibility. In this model, the amino acid substitution is near to the carboxyl end of the protein but when a portion of the carboxyl region is removed, the substitution is then situated too near to the end of the protein to exert an effect on the structure of the SDS-protein complex.

The findings of the structural analysis of Dl N protein represent evidence for caution in interpretation of small pertebations in electrophoretic mobility in SDS-containing gels. The Dl N mobility shift is an artefact of SDS-PAGE analysis and does not indicate a change in molecular weight by deletion or premature termination.

APPENDIX I

Figure 50. Autoradiograms of tryptic peptide maps of extra proteins detectable by SDS-PAGE fractionation of [^{35}S]Met-labeled pure viruses, as well as [^{32}P]orthophosphate labeled NS proteins of VS Ind. The bands were minor constituents of SDS-PAGE profiles and possessed various electrophoretic mobilities. Tryptic hydrolysis employed oxidation, as described in methods. Electrophoresis was at pH 3.5 and 450 V for 1.5-2.5 hr on MN400 cellulose thin layer sheets. TLC employed the solvent n-butanol:pyridine: water:acetic acid (204:143:143:50). Spots were detected by autoradiography.

Panel A - Protein subjacent to VS Cocal L protein. (Same patterns as VS Cocal G protein).

Panel B - Protein subjacent to VS G protein (Subset of pattern of VS Cocal G protein).

Panel C - Protein subjacent to VS NJ(M) G protein (Same pattern as VS NJ(M) G protein).

Panel D - Protein subjacent to VS NJ(C) G protein (The pattern of VS NJ(C) G protein is a subset of this pattern).

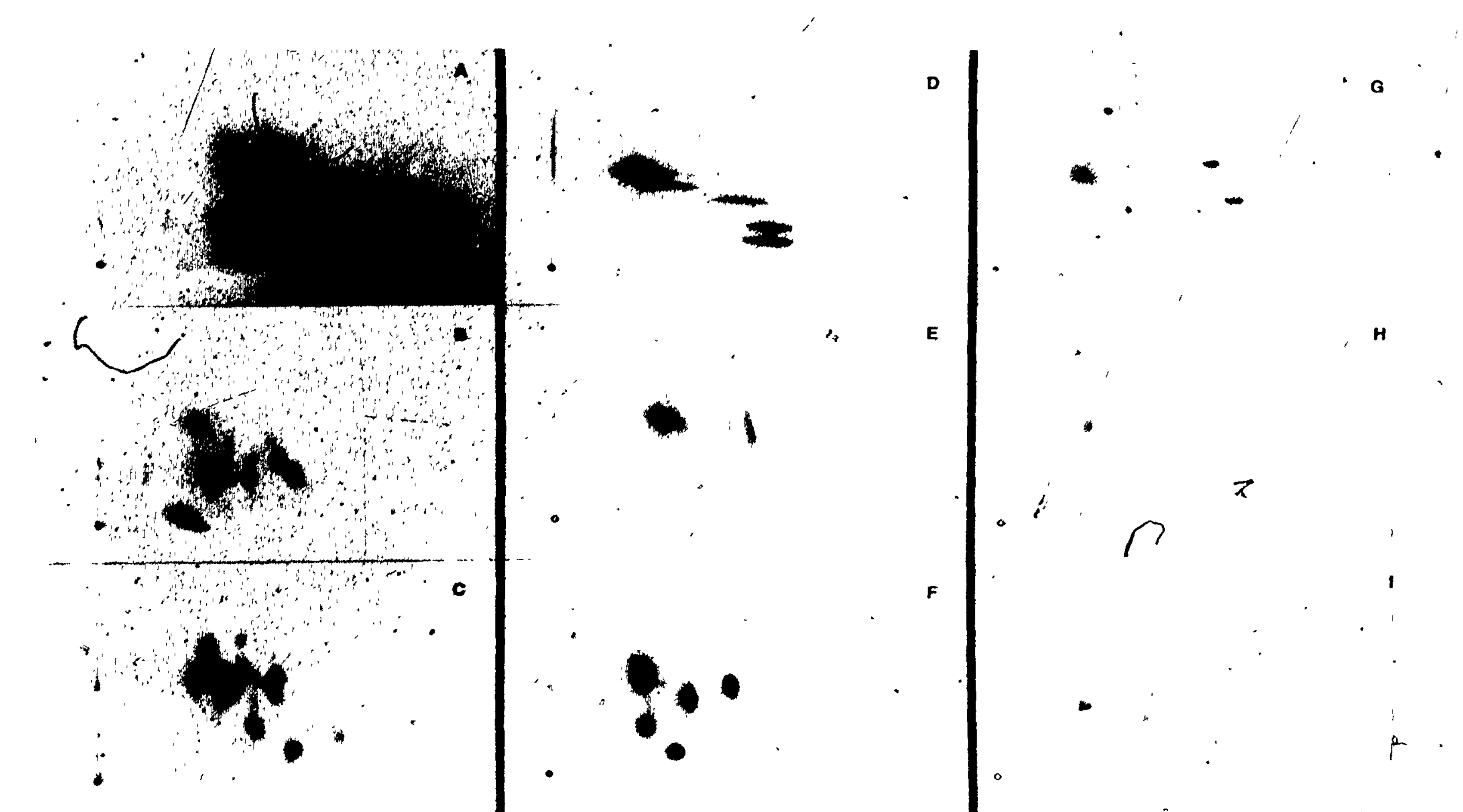
Panel E - Protein subjacent to VS NJ(M) N protein (Pattern cannot be visually correlated with other VS NJ(M) peptide maps).

Panel F - Protein subjacent to VS Cocal N protein (General pattern similarity with NS protein peptide map of VS Cocal).

Panel G - Protein suprajacent to VS NJ(C) M protein (Pattern is identical to VS NJ(C) M protein peptide map).

Panel H - NS protein of VS Ind labeled with [^{32}P] orthophosphate. The protein was isolated from infected cells.

Panel I - NS protein of VS Ind labeled with [^{35}S]Met. The protein was isolated from virions and was analyzed in parallel with the sample in Panel H to serve as a reference.



A
B
C
D
E
F
G
H

APPENDIX II

Determination of Cleavage Yields from the Relative Abundance of End Labeled Peptides

When protein is labeled at its amino terminus with f[^{35}S]Met, the radioactivity of any peptide relative to another indicates its relative molar abundance. After NCS cleavage products of f[^{35}S]Met-labeled protein are fractionated and detected by fluorography, the relative amount of radioactivity in each peptide can be determined by assessement of densitometer tracings. Thus, the relative radioactivity (R) of each peptide is derived. The object of this procedure is to determine the susceptibility to cleavage by NCS at each site.

To provide an example; consider a hypothetical f[^{35}S]Met labeled protein molecule which contains 3 cleavage sites, numbered from the amino-terminus (1, 2, 3). Quantitation of densitometry tracings of the peptides formed by cleavage at these sites has indicated the relative radioactivities R1, R2, and R3, as well as uncleaved protein R4.

The relationships describing the quantity of each peptide are:

$$R1 = RT(K1)$$

$$R2 = RT(K2)(1-K1)$$

$$R3 = RT(K3)(1-K1)(1-K2)$$

where RT is the total radioactivity (ΣR1-4) and K1, K2, and K3 are the cleavage yields at the corresponding sites 1, 2, and 3. In order to determine cleavage yields, the equations must be rearranged and converted to values of R.

$$K1 = R1/RT$$

$$K2 = \frac{R2}{RT(1-K1)}$$

Substituting R1/RT for K1 and solving gives:

$$K2 = \frac{R2}{RT-R1}$$

which in practice is

$$K2 = \frac{R2}{R2+R3+R4}$$

The extent of cleavage at site 3, K3 is equal to:

$$K2 = \frac{R3}{RT(1-K1)(1-K2)}$$

By substituting R2/RT(1-K1) for K2 and R1/RT for K1 and solving:

$$K3 = \frac{R3}{R3+R4}$$

To generalize, the extent of cleavage at site x can be derived by quantitation of end labeled peptides and relating the abundance of the labeled peptide generated by cleavage at x to all the labeled peptides containing site x. (Note: this includes the labeled fragment cleaved at x).

APPENDIX III

Tritium- Labeling of the C-terminus

Nonradioactive protein samples were reacted in a mixture of acetic anhydride, pyridine and T_2O to result in selective incorporation of tritium into the C-terminal amino acid. Protein samples were produced from virus, purified by velocity centrifugation in sucrose gradients and by fractionation employing SDS-PAGE. The proteins were located by staining and eluted electrophoretically. The protein eluates were dialyzed against three changes of 1,000 volumes of water over a 24 hr period at 4^0C and then precipitated with acidified acetone (2% v/v acetic acid). The protein precipitates were collected as a sediment by centrifugation at 10K rpm for 20 min. in a Sorvall GSA rotor, dissolved in 70% formic acid and transferred to heavy walled pyrex reaction tubes before being lyophilized. The lyophilized protein was then ready for reaction with acetic anhydride in the presence of pyridine and T_2O. The acetic anhydride and pyridine were distilled from reagent grade stocks prior to use. The protein samples, N proteins of D1, R4, and wild type, and M protein or R4 were reacted in a mixture containing 100 mCi of tritiated water (5 Ci/ml). The reactions were carried out as described in methods and subsequently vacuum distilled to remove solvents and unincorporated T_2O. Egg white lysozyme which possesses a C-terminal leucine residue was employed as a control. Exchangeable tritium was removed from the samples by repeated additions of 10% acetic acid and subsequent lyophilization. The protein was then acid hydrolyzed with 6

M HCl for 24 hr at 105 $^{\circ}$C as described in methods. The acid was then removed by heating at 80 $^{\circ}$C and flushing with a stream of air. The resulting amino acid residue was dissolved in water and lyophilized before dissolving in water again and application to MN 300 cellulose or EM cellulose thin layer plates along with 10 nmoles each of 20 amino acids commonly found in proteins. The amino acid mixtures were fractionated by 2 dimensional chromatography and then detected by ninhydrin staining before impregnation with PPO:POPOP (40:3) as described in methods. The samples were then fluorographed.

Autoradiograms of the fractionated labeled amino acids are represented in Figure 51, panels A to E. The lysozyme sample had 90% of its radioactivity at the leucine position with the remainder at aspartic acid. Tritiation of internal asparatic acid is a common side reaction of the tritiation procedure. Also present in the autoradiogram of the lysozyme sample is a component which is not one of the twenty amino acids and is labeled X. Component X was present in most hydrolyzates of chemically tritiated samples.

The other four samples, A to D, all contained radioactive glycine. Component X was present in D1 N protein, wild type N protein, and R4 N protein but not in R4 M protein. The M protein in addition contained radioactive aspartic acid and alanine but these were present as very minor components. At face value, these data suggested that mutant, revertant and wild type N proteins, as well as revertant M protein possessed C-terminal glycine. On examination of the experimental regime, it can be noted that the proteins were eluted into

Figure 51. Fluorogram of fractionated amino acids of N and M proteins which were chemically labeled with [^{3}H] in vitro.. Viral proteins were prepared by discontinuous SDS-PAGE and electroelution (A,B,C,D) or continuous SDS-PAGE and extraction by diffusion (G,H,I,J). A commercial preparation of lysozyme (hen egg white) was also employed (E). Protein samples containing ~1 mg of protein were tritiated by reacting with pyridine and acetic anhydride in the presence of T_2O as described in methods. The tritiated protein samples were subjected to acid hydrolysis in 6 M HCl and subsequently fractionated by 2 dimensional TLC on thin layers of cellulose (MN300 cellulose or EM cellulose). The TLC solvents were n-butanol:acetone:diethylamine:water (pH 12)(10:10:2.5) for first dimension, and isoproponal:formic acid:water (pH 2.5)(40:2:10) for the second dimension. Thin layers were stained with ninhydrin in order to locate all 20 amino acids and detection of labeled compounds was by fluorography following impregnation with PPO and POPOP. Amino acids are indicated and two unknown compounds are also indicated (X,Y). The following chemically tritiated samples are presented: VS NJ(M) wild type protein (A,G), R4 N protein (B,H), R4 M protein (C), D1 N protein (D,J) egg white lysozyme control (E), a mixture of 10 nmoles each of the 20 amino acids normally constituting proteins (F), and M protein pooled from D1 and R4 (I).

A

B

GLY

GLY

X

C

D

LYS

X

Y

X

glycine containing buffer. If glycine had remained associated with the protein through preparation, it may have become susceptible to tritiation. The tritiation reaction requires a free carboxyl group and an acyl function, of which glycine possesses the former but not the latter. Amino acids are acetylated in the presence of acetic anhydride and pydridine which are both present in the tritiation reaction (Schechter et al, 1976). Acetyl glycine has an acyl group and is a substrate for tritium incorporation. Since this course of events seemed possible the experiment was repeated with modifications.

Virus purified by differential centrifugation was fractionated in continuous phosphate gels and eluted by diffusion into a buffered SDS solution. Lysozyme was treated in an identical manner to control for effects of preparation. The protein eluates were lyophilized, dissolved and then precipitated with 20% TCA. The precipitates were collected by sedimentation, washed with acetone, dissolved in formic acid, and transferred to reaction tubes. The rest of the procedure was identical to the previous experiment. A sample which contained the 20 amino acids normally encountered in proteins, 250 nmoles each was also included in order to determine if free amino acids could be labeled by the tritiation procedure. The amino acid sample was reacted in 1/4 of the amount of reagents used in the protein samples. Fluorograms of the separated amino acids of the R4, D1 and wild type N proteins, and the R4 plus D1 M proteins, as well as the amino acid mixture after tritiation are presented in Figure 51 panels F-J. The result of the treated

lysozyme control was identical with panel E and is not included in this figure.

With reference to panel F, it can be observed that 13 of the 20 free amino acids became labeled by the tritiation procedure, presumably by first being acetylated. Certain amino acids are not observable in this pattern: cysteine, threonine, serine, proline, glutamine, asparagine, and tryptophan. The latter three would not be expected due to destruction during acid hydrolysis, and proline is the only amino acid not labeled by the Matsuo technique. Most noteworthy, however is the fact that glycine was labeled. This shows that free glycine can be labeled by the tritiation procedure, suggesting that the [^{3}H]-labeled glycine observed in Panels A-D resulted from labeling of free glycine carried over from the buffer used in electroelution. This is further suggested by the lack of significant [3H]-labeled glycine in the repeated attempt at tritiation of N proteins (panels G-I).

Analysis of the amino acids labeled in the N proteins of D1, R4, and wild type, (panels G, H, and I) show several labeled amino acid spots in addition to aspartic acid and component X. All N proteins have labeled lysine, histidine, cysteine, glutamic acid and alanine, with barely detectable label in glycine and serine. Also present in the N protein patterns is a novel component (γ) which migrates to the upper right of each thin layer and is prominent in the D1 N pattern. The R4 N protein also contained labeled methionine.

The M protein sample (Figure 51 panel I) had only one labeled component, lysine, which suggested that Lys was the C-terminal group.

The existence of several labeled amino acids in panel G, H, and J may be due to contaminant proteins in the preparation, although only one extra band of minor relative intensity was observable on SDS-PAGE analysis of the preparation prior to reaction (data not shown). Internal incorporation has been previously reported but only for glutamic and aspartic acid residues (Holcomb _et al_, 1968). The insolubility of N protein during reaction may have prevented C-terminal labeling under conditions which labeled lysozyme and M protein which were both soluble. Matsuo and Narita (1975) state that insolubility makes the tritiation reaction very sluggish but they did not have a reliable solution to the problem.

REFERENCES

Abraham, G., and Banerjee, A.K. (1976). Sequential transcription of the genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. 73:1504-1508.

Abraham, G., Rhodes, D.P., and Banerjee, A.K. (1975). The 5'-terminal structure of the methylated mRNA synthesized *in vitro* by vesicular stomatitis virus. Cell 5:51-58.

Anderson, C.W., Baum, P.R., and Gesteland, R.F. (1973). Processing of adenovirus 2-induced proteins. J. Virol. 12:241-252.

Ambler, R.P. (1975). Enzymatic hydrolysis with carboxypeptidases. In *Methods in Enzymology*, vol. XI, (ed. C.H.W. Hirs), p. 155. New York, Academic Press.

Ambler, R.P., Brown, L.H. (1967). The amino acid sequence of *Pseudomonas fluorescens* azurin. Biochem. J. 104:784-825.

Atkinson, P.H., Moyer, S.A., and Summers, D.F. (1976). Assembly of vesicular stomatitis virus glycoprotein and matrix protein into HeLa cell plasma membranes. J. Mol. Biol. 102:613-631.

Ball, C.A. (1977). Transcriptional mapping of vesicular stomatitis virus *in vivo*. J. Virol. 21:411-414.

Ball, L.A. and White, C.N. (1976). Order of transcription of genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. 73:442-446.

Banerjee, A.K., Abraham, G., and Colonmo, R.J. (1977). Vesicular stomatitis virus: mode of transcription. J. Gen. Virol. 34:1-8.

Banker, G.A. and Cotman, C.W. (1972). Measurement of free electrophoretic mobility and retardation coefficient of protein-sodium dodecyl sulfate complexes by gel electrophoresis. J. Biol. Chem. 247:5856-5861.

Baxt, B., and Bablanian, R. (1976). Mechanisms of vesicular stomatitis virus - induced cytopathic effects. II Inhibition of macromolecular synthesis induced by infectious and defective-interfering particles. Virology 72:383-392.

Bell, J.C., Prevec, L. (1979). Proteins of vesicular stomatititis virus V. Identification of a precursor to the phosphoprotein of Piry virus. J. Virol. 30:56-63.

Bishop, D.H.L., Emerson, S.U., and Flamand, A. (1974). Reconstitution of infectivity and transcriptase activity of homologous and heterologous viruses: VSV Indiana, Chandipura, VSV New Jersey, Cocal. J. Virol. 14:139-144.

Bishop, D.H.L., and Roy, P. (1972). Dissociation of vesicular stomatitis virus and relation of the virion proteins to the viral transcriptase. J. Virol. 10:234-243.

Bishop, D.H.L. and Smith, M.S. (1977). Rhabdoviruses in: The Molecular Biology of Animal Viruses, vol. 1, p. 167 (ed. D.P. Navak) Marcel Dekker, New York.

Blackburn, S. (1968). Amino acid determination. Methods and Techniques p. 16-17. Marcel Dekker, Inc., New York.

Bonner, W.M., and Laskey, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

Bonner, W.M., and Stedman, J.D. (1978). Efficient flourography of ^{3}H and ^{14}C on thin layers. Annal. Biochem. 89:247-256.

Brenner, M., and Niederwieser, A. (1967). Thin-layer chromatography (TLC) of amino acids in: Methods in Enzymology, vol. XI (ed. C.H.W. Hirs) pp. 39-58. Academic Press, New York and London.

Brown, E., and Prevec, L. (1978). Proteins of vesicular stomatitis virus IV. A comparison of tryptic peptides of the vesicular stomatitis group of Rhabdoviruses. Virol. 89:7-21.

Brown, E.G., and Prevec, L. (1979). Comparative analyses of Vesiculovirus proteins utilizing partial cleavage fragments at tryptophan residues. Virol. 95:244-248.

Brown, F., Bishop, D.H.L., Crick, J., Francki, R.I.B., Holland, J.J., Hull, R., Johnson, K., Martelli, G., Murphy, F.A., Obijeski, A.F., Peters, D., Pringle, C.R., Reichmann, M.E., Schneider, L.G., Shope, R.E., Simpson, D.I.H., Summers, D.F., and Wagner, R.R. (1979). Rhabdoviridae. Intervirology 12:1-7.

Buckley, S.M. (1969). Susceptibility of the Aedes albopictus and A. aegypti cell lines to infection with arboviruses. Pro. Soc. Exp. Biol. Med. 131:625-630.

Burge, B.W., and Huang, A.S. (1970). Comparison of membrane protein glycopeptides of Sindbis virus and vesicular stomatitis virus. J. Virol. 6:176-182.

Burge, B.W., and Huang, A.S. (1979). Conserved peptides in the proteins of vesicular stomatitis virus. Virol. 95:445-453.

Capecchi, M.R., Vonder Haar, R.A., Capecchi, N.E., and Sveda, M.M. (1977). The isolation of a suppressible nonsese mutant in mammalian cells. Cell 12:371-381.

Carroll, A.R., and Wagner, R.R. (1979). Role of the membrane (M) protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus. J. Virol. 29:134-142.

Cartwright, B., and Brown, F. (1972a). Glycolipid nature of the complement-fixing host cell antigen of vesicular stomatitis virus. J. Gen. Virol. 15:243-245.

Cartwright, B., and Brown, F. (1972b). Serological relationships between different strains of vesicular stomatitis virus. J. Gen. Virol. 16:391-398.

Cartwright, B., Smale, C.T., and Brown, F. (1970). Dissection of vesicular stomatitis virus into the infective ribonucleoprotein and immunizing components. J. Gen. Virol. 7:19-32.

Cartwright, B., Talbot, P., and Brown, F. (1970). The proteins of biologially active sub-units of vesicular staomtitis virus. J. Gen. Virol. 7:267-272.

Celma, M.L., and Ehrenfeld, E. (1975). Translation of poliovirus RNA *in vitro*: Detection of two different initiation sits. J. Mol. Biol. 98:761-780.

Choppin, P.W., and Compans, R.W. (1970) Phenotypic mixing of envelope proteins of the parainfluenza SV5 and vesicular stomatitis virus. J. Virol. 5:609-616.

Clerx-van Haaster, C.M., Clewley, J.P. and Bishop, D.H.L. (1980). Oligonucleotide sequence analyses indicate that vesicular stomatitis virus large Dl virus particle RNA is made by internal deletion: Evidence for a similar transciprtion polyadenylation signals for the synthesis of all VSV in RNA species. J. Virol. 33:807-817.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W., and Laemmli, U.K. (1977). Peptide mapping by limited proteolysis in sodium dodecylsulfate and analyses by gel electrophoresis. J. Biol. Chem. 257:1102-1106.

Clewley, J.P., Bishop, D.H.L., Kang, C.-Y., Coffin, J., Schnitzlein, W.M., Reichmann, M.E., and Shope, R.E. (1977). Oligonucleotide fingerprints of RNA species obtained from Rhabdoviruses belonging to the vesicular stomatitis virus subgroup. J. Virol. 23:152-166.

Clinton, G.M., Burge, B.W., and Huang, A.S. (1978). Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. J. Virol. 27:340-346.

Clinton, G.M., Burge, B.W., and Huang, A.S. (1979). Phosphoproteins of vesicular stomatitis virus: Identity and interconversion of phosphorylated forms. Virology 99:84-94.

Clinton, G.M., Little, S.P., Hagen, F.S., and Huang, A.S. (1978). The matrix (M) protein of vesicular stomatitis virus regulates transcription. Cell 15:1455-1462.

Cohen, G.H., Atkinson, P.H., and Summers, D.F. (1971). Interactions of vesicular stomatitis virus structural proteins with HeLa plasma membrane. Nat. New Biol. 231:121-123.

Colonno, R.J., and Banerjee, A.K. (1976). A unique RNA species involved in initiation of vesicular stomatitis virus RNA transcription in vitro. Cell 8:197-204.

Cooper, B.D., and Bellet, A.J.D. (1959). A transmissable interfering component of vesicular stomatitis virus preparations. J. Gen. Microbiol. 21:485-497.

Crawford, C.V., and Gesteland, R.F. (1973). Synthesis of polyoma proteins in vitro. J. Mol. Biol. 74:627-634.

Cremer, K.J., Bedemer, M., Summers, W.P., Summers, W.C., and Gesteland, R.F. (1979). In vitro suppression of UAG and UGA mutants in the thymidine kinase gene of herpes simplex virus. Proc. Natl. Acad. Sci. 76:430-434.

Crestfield, A.M., Moore, S., and Stein, W.H. (1963). The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins. J. Biol. Chem. 238:622-627.

Dahlberg, J.E. (1974). Quantitative electron microscopic analysis of the penetration of VSV into L cells. Virol. 58:250-262.

Dayhoff, M.O., (ed.) (1976). Atlas of Protein Sequence and Structure, Vol. 5, supplement 2, p.266. (ed. M.O. Dayhoff). The National Biomedical Research Foundation, Maryland.

Dayhoff, M.O., Eck, R.V., and Park, C.M. (1972). A model of evolutionary change in proteins. in: Atlas of Protein Sequence and Structure, Vol. 5, p.89 (ed. M.O. Dayhoff). The National Biomedical Research Foundation, Maryland.

Degani, Y., and Patchornik, A. (1974). Cyanylation of sulfhydryl groups by 2-nitro-5-thiocyanobenzoic acid. High-yield modification and cleavage of peptides at cysteine residues. Biochem. 13:1-10.

Doel, T.R., and Brown, F. (1978). Tryptic peptide analysis of the structural proteins of vesicular stomatitis virus. J. Gen. Virol. 38:351-361.

Dragunova, J., and Zavada, J. (1979). Cross-neutralization between vesicular stomatitis virus type Indiana and Chandipura virus. Acta. Virol. 23:319-328.

Dubovi, E.J., and Wagner, R.R. (1977). Spatial relationships of the proteins of vesicular stomatitis virus: Induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. 22:500-509.

Dulbecco, R., and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. J. Exptl. Med. 99:167-182.

Emerson, S.U., and Wagner, R.R. (1972). Dissociation and reconstitituion of the transcriptive and template activities of vesicular stomatitis virus B and T virions. J. Virol. 10:297-309.

Emerson, S.U., and Yu, Y.-H. (1975). Both NS and L proteins are required for in vitro RNA synthesis by vesicular stomatitis virus. J. Virol. 15:1348-1356.

Epstein, D.A., Herman, R.C., Chien, I., and Lazzarini, R.A. (1980). Defective interfering particle generated by internal deletion of the vesicular stomatitis virus genome. J. Virol. 33:818-829.

Evans, D., Pringle, C.R., and Szilagyi, J.F. (1979). Temperature-sensitive mutants of complementation groups E of vesicular stomatitis virus New Jersey serotype possess altered NS polypeptides. J. Virol. 31:325-333.

Fan, D.P., and Sefton, B.M. (1978). The entry into host cells of Sindbis virus, vesicular stomatitis virus and Sendai virus. Cell, 15:985-992.

Federer, K.E., Burrows, R., and Brooksby, J.B. (1967). Vesicular stomatitis virus - the relationship between some strains of the Indiana serotype. Res. Vet. Sci. 8:103-117.

Felix, A.M., and Jiminez, M.H. (1974). Usage of fluorescamine as a spray reagent for thin-layer chromatography. J. Chromatography 89:361-364.

Finlayson, A.J. (1969). The performic acid oxidation of egg-white lysozyme. Can. J. Biochem. 47:31-37.

Fitch, W.M. (1973). Aspects of Molecular evolution. Ann. Rev. Genet. 7:343-380.

Flamand, A. (1969). Etude des mutants thermosensibles du virus de la stomatite vésiculaire: Mise au point d'un test de complémentation. C.R. Acad. Sci (Paris) 268:2305.

Flamand, A., and Bishop, D.H.L. (1973). Primary in vivo transcription of vesicular stomatitits virus and temperature-sensitive mutants of five vesicular stomatitis virus complementation groups. J. Virol. 12:1238-1252.

Follett, E.A.C., Pringle, C.R., Wunner, W.H., and Skehel, J.J. (1974). Virus replication in enucleate cells: Vesicular stomatitis virus and influenza virus. J. Virol. 13:394-399.

Garoff, H., and Soderlund, H. (1978). The amphiphilic membrane glycoproteins of Semliki Forest virus are attached to the lipid bilayer by their COOH-terminal ends. J. Mol. Biol. 124:535-549.

Geller, A.I., and Rich, A. (1980). A UGA termination suppression $tRNA^{Trp}$ active in rabbit reticulocytes. Nature 283:41-46.

Genty, N. (1975). Analysis of uridine incorporation in chicken embryo cells infected by vesicular stomatitis virus and its temperature-sensitive mutants: uridine transport. J. Virol. 15:8-15.

Gesteland, R.F. Wills, N., Lewis, J.B., and Grodzicker, T. (1977). Identification of amber and ochre mutants of the human virs Ad2+ND1. Proc. Natl. Acad. Sci. 74:4567-4571.

Ghosh, K., Ghosh, H.P., Simsek, M., and Raj Bhandary, U.L. (1974). Initiation methionine trasnfer ribonucleic acid from wheat embryo. J. Biol. Chem. 249:4720-4729.

Ghosh, H.P., Toneguzzo, F., and Wells, S. (1973). Synthesis in vitro of vesicular stomatitis virus proteins in cytoplasmic extracts of L cells. Biochem. Biophys. Res. Commun. 54:228-233.

Gillam, I., Blew, D., Warrington, R.C., von Tigerstrom, M., and Tener, G.M. (1968). A general procedure for the isolation of specific transfer ribonucleic acids. Biochemistry 7:3459-3468.

Glowacki Strauss, E., and Kaerberg, P. (1970). Acrylamide gel electrophoresis of bacteriophage Qβ:electrophoresis of the intact virions and of the viral proteins. Virology 42:437-452.

Gorini, L. (1970). Informational suppression. Ann. Rev. of Genet. 4:107-134.

Gross, E., and Witkop, B. (1962). Nonenzymatic cleavage of peptide bonds: The methionine residues in Bovine pancreatic ribonuclease. J. Biol. Chem. 237:1856-1860.

Harley, C.B., Pollard, J.W., Chamberlain, J.W., Stanners, C.P., and Goldstein, S. (1980). Protein synthetic errors do not increase during aging of cultured fibroblasts. Proc. Natl. Acad. Sci. 77:1885-1889.

Heinz, J.W., and Schnaitman, C.A. (1971). Entry of vesicular stomatitis virus into L cells. J. Virol. 8:786-795.

Hendrix, R.W., and Casjens, S.R. (1974). Protein fusion: A novel reaction in bacteriophage lambda lead assembly. Proc. Natl. Acad. Sci. 71:1451-1455.

Herman, R.C., Adler, S., Lazzarini, R.A., Colonno, R.J., Banerjee, A.K., and Westphal, H. (1978). Intervening poly A in RNA transcripts of vesicular stomatitis virus. Cell 15:587-596.

Heyward, J.T., Holloway, B.P., Cohen, P., and Obijeski, J.F. (1978). Rhabdovirus nucleocapsid in Rhabdoviruses, chapter 7. CRC Press, Cleveland, Ohio.

Hirs, C.H.W. (1967). Performic acid oxidation. in: Methods in Enzymology, Vol. XI, pp. 197-199 (C.H.W. Hirs Ed.). Academic Press, New York and London.

Holcomb, G.N., James, S.A., and Darrell, N.W. (1968). A critical evaluation of the selective tritiation method of determining C-terminal amino acids and its application to luteinizing hormone. Biochemistry 7:1291-1296.

Holloway, A.F., Wong, P.K.Y., and Cormack, D.V. (1970). Isolation and characterisation of temperature-sensitive mutants of vesicular stomatitis virus. Virology 42:917-926.

Houmard, J., and Drapeau, G.R. (1972). Stophylococcal protease: a proteolytic enzyme specific for glutamoyl bonds. Proc. Natl. Acad. Sci. 69:3506-3509.

Huang, A.S., and Baltimore, D. (1970). Defective viral particles and viral disease processes. Nature (Lond.) 226:325-327.

Huang, A.S., and Wagner, R.R. (1966). Defective T particles of vesicular stomatitis virus II Biologic role in homologous interference. Virology, 30:173-181.

Hunt, D.M., Emerson, S.V., and Wagner, R.R. (1976). RNA^- temperature-sensitive mutants of vesicular stomatitis virus: L protein thermosensitivity accounts for transcriptase restriction of group I mutants. J. Virol. 18:596-603.

Hunt, T. Vanderhoff, G., and London, I.M. (1972). Control of globin synthesis: Role of heme. J. Mol. Biol. 66:471-481.

Hsu, C.-H., Kingsbury, D.W., and Murti, K.G. (1979). Assembly of veicular stomatitis virus nucleocapsids in vivo: a kinetic analysis. J. Virol. 32:304-313.

de Jong, W.W., Zweers, A., and Cohen, L.H. (1978). Influence of single amino acid substitutions on electrophoretic mobility of sodium dodecyl sulfate-protein complexes. Biochem. Biophys. Res. Commun. 82:532-539.

Kang, C.-Y, and Prevec, L. (1970). Proteins of vesicular stomatitis virus. II Immunological comparisons of viral antigents. J. Virol. 6:20-27.

Karabatsos, N. (ed.) (1978). Supplement to International Catalogue of Arboviruses. Am. J. Trop. Med. Hyg. 27:372-440.

Kasper, C.B. (1975). Fragmentation of proteins for sequence studies and separation of peptide mixtures. in Protein Sequence Determination, 2nd edition (ed. S.B. Needleman) p.114-161. Springer-Verlag, N.Y. Heidelberg, Berlin.

Keene, J.D., Schubert, M., and Lozzarini, R.A. (1980). Intervening sequence between the leader region and the nucleocapsid gene of vesicular stomatitis virus RNA. J. Virol. 33:789-794.

Kendal, A.P., and Cohen, P. (1976). Isolation of the matrix (membrane) protein of vesicular stomatitis virus by gel filtration in quanidine hydrochloride. J. Gen. Virol. 33:543-546.

King, A.M., and Newman, J.W.I., (1980). Temperature-sensitive mutants of foot-and-mouth disease virus with altered structural polypeptides. 1: Identification by electrofocusing. J. Virol. 34:59-66.

Kingsford, L. and Emerson, S.U. (1980). Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. J. Virol. 33:1097-1105.

Knipe, D.M., Baltimore, D., and Lodish, H.F. (1977). Maturation of viral proteins in cells infected with temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. 21:1149-1158.

Knipe, D., Lodish, H.F., and Baltimore, D. (1977). Analysis fo the defects of temperature-sensitive mutants of vesicular stomatitis virus: intracellular degradation of specific viral proteins. J. Virol. 21:1140-1148.

Knipe, D., Rose, K.J., and Lodish, H.F. (1975). Translation of individual species of vesicular stomatitis viral mRNA. J. Virol. 15:1004-1011.

Knudson, D.L. (1973). Rhabdoviruses. J. Gen. Virol. 20:105-130.

Kohli, J., Kwong, T., Altruda, F., Soll, D., and Wahl, G. (1979). Characterization of a UGA-suppressing serine tRNA from Schizosaccharomyces pombe with the help of a new in vitro assay system for eukoryotic suppressor tRNAs. J. Biol. Chem. 254:1546-1551.

Kohn, A. (1979). Early interactions of viruses with cellular membranes. Adv. Virus. Res. 24:223-276.

Kostka, V., and Carpenter, F.H. (1963). Inhibition of chymotrypsin activity in crystalline trypsin preparations. J. Biol. Chem. 239:1799-1803.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227:680-685.

Lafay, F. (1974). Envelope proteins of vesicular stomatitis virus; Effect of temperature-sensitive mutations in complementation groups III and V. J. Virol. 14:1220-1228.

Laskey, R.A., and Mills, A.D. (1975). Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biocehm. 56:335-341.

Laurenson, R.A. (1969). An isotope dilution procedure for detection and quantitative estimation of phenylthiohydantoins released in the Edman degradation. Biochem. Biophys. Res. Commun. 37:663-667.

Layne, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. in: Methods in Enzymology, Vol. III, pp.447-454. Academic Press, New York.

Leppert, M., Rittenhouse, L., Perrault, J., Summers, D.F., and Kolakofsky, D. (1979). Plus and minus strand leader RNAs in negative strand virus-infected cells. Cell. 18:735-747.

Lesnaw, J.A., Dickson, L.R., and Curry, R.H. (1979). Proposed replicative role of the NS polypeptide of vesicular stomatitis virus: Structural analysis of an electrophoretic variant. J. Virol. 31:8-16.

Liu, T.-Y., and Chang, Y.H. (1971). Hydrolysis of proteins by p-toluenesulfonic acid. Determination of tryptophan. J. Biol. Chem. 246:2842-2848.

Lodish, H.F., and Porter, M. (1980a). Specific incorporation of host cell surface proteins into budding vesicular stomatitis virus particles. Cell 19:161-169.

Lodish, H.F., and Porter, M. (1980b). Heterogeneity of vesicular stomatitis virus particles: Implications for virion assembly. J. Virol. 33:52-58.

Lodish, H.F., and Rothman, J.E. (1979). The assembly of cell membranes. Sci. Am. 240:48-63.

Maddy, A.H. (1976). A critical evaluation of the analysis of membrane proteins by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate. J. Theor. Biol. 62:315-326.

Maley, F., and Guarino, V. (1977). Differential binding of sodium dodecyl sulfate to amino acids as evidences by elution from Sephadex. Biochem. Biophys. Res. Commun. 77:1425-1430.

Margoliash, E., Smith, E.L., Kreil, G., Tuppy, H. (1961). The complete amino-acid sequence. Nature 192:1125-1127.

Martinet, C., Combard, A., Printz-Anne, C., and Printz, P. (1979). Envelope proteins and replications of vesicular stomatitits virus: <u>in vivo</u> effects of RNA^+ temperature-sensitive mutations on viral RNA synthesis. J. Virol. 29:123-133.

Matsuo, H., and Narita, K. (1975). Improved tritium-labeling for quantitative C-terminal analysis in <u>Protein Sequence Determination, 2nd Ed.</u> (S.B. Needleman, Ed.) pp.104-113. Springer-Verlag, New York, Heidelberg, Berlin.

Maxam, A.M., and Gilbert, W. (1977). A new method for sequencing DNA. Proc. Natl. Acad. Sci. 74:560-564.

McGeoch, D.J. (1979). Structure of the gene N: gene NS intercistronic junction in the genome of vesicular stomatitis virus. Cell, 17:673-681.

McGeoch, D.J., Dolan, A., and Pringle, C.R. (1980). Comparisons of nucleotide sequences in the genomes of the New Jersey and Indiana serotypes of vesicular stomatitis virus. J. Virol. 33:69-77.

McSharry, J.J., and Choppin, P.W. (1978). Biological properties of the vesicular stomatitis virus glycoprotein. 1. Effects of the isolated glycoprotein on host macromolecular synthesis. Virology 84:172-182.

McSharry, J.J., Compans, R.W., and Choppin, P.W. (1971). Proteins of vesicular stomatitis virus and of phenotypically mixed vesicular stomatitits virus - simian virus 5 virions. J. Virol. 8:722-729.

Metzel, P.S., Schnitzlein, W.W., and Reichmann, E. (1977). Characterization of distinct VSV NJ serotype isolates with respect to interference by D.I. particles, nucleic acid homology and protein structures in Negative Strand Viruses. Cambridge, England.

Moore, S., and Stein, W.H. (1963). Chromatographic determination of amino acids by the use of automatic recording equipment. in: Methods in Enzymology, (S.P. Colowick and N.O. Kaplan, eds.) Vol. 6, p.819. Academic Press. New York.

Moyer, S.A., Graubman, M.J., Ehrenfeld, E., and Banderjee, A.K. (1975). Studies on the in vivo and in vitro messenger RNA species of vesicular stomatitis virus. Virology 67:463-473.

Murphy, F.A., and Shope, R.E. (1971). Workshop on bridging groups of viruses. in: Proceedings of the International Congress for Virology, Budapest, p. 261.

Nakai, T., Howatson, A.F. (1968). The fine structure of vesicular stomatitis virus. Virol. 35:268-281.

Narita, K., Matsuo, H., and Nakajima, T. (1975). End group determination in Protein Sequence Determination, 2nd Ed. (S.B. Needleman Ed.), pp.30-103. Springer-Verlag, New York Heidelberg, Berlin.

Nathanson, N.M., and Hall, Z.W. (1979). Subunit structure and peptide mapping of junctional and extrajunctional acetylcholine receptors from rat muscle. Biochemistry 18:3392-3401.

Ngan, J.S.C., and Holloway, A.F., and Cormack, D.V. (1974). Temperature-sensitive mutants of vesicular stomatitis virus: Comparison of the in vitro RNA polymerase defects of group I and group IV mutants. J. Virol. 14:765-772.

Nikodem, V., and Fresco, J.R. (1979). Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr. Anal. Biochem. 97:382-386.

Nivinskas, R.H., Keinien, L.G., Kauzaite, V.V., and Vaiksnoraite, O.M. (1978). Investigation of transmission of bacteriophage T4 amber mutants. 1. Temperature-sensitivity of the multiplication of gene 26 mutant amN131 of phage T4 in Eschericia coli B cells. Genetika (Rus.) 14:695-703.

Noel, K.D., Nikaido, K., Ferro-Luzzi Ames, G. (1979). Single amino acid substitution in a histidine-transport protein drastically alters its mobility in sodium dodecyl sulfate-polysucrylamide gel electrophoresis. Biochemistry 18:4159-4165.

O'Farrell, P.H. (1975). High resolutiohn two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

Omenn, G.S., Fontana, A., and Anfinsen, C.B. (1970). Modification of the single tryptophan residue in Staplylococcal nuclease by a new mild oxidizing agent. J. Biol. Chem. 245:1895-1902.

Panyim, S. and Chalkley, R. (1971). The molecular weights of vertebrate histones exploiting a modified sodium dodecyl sulfate electrophoretic method. J. Biol. Chem. 245:7557-7560.

Pelham, H.R.B., and Jackson, R.J. (1976). An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247-256.

Perrault, J., and Sembler, B.L. (1979). Internal genome deletions in two distinct classes of defective interfering particles of vesicular stomatitis virus. Proc. Natl. Acad. Sci. 76:6191-6195.

Pitt-Rivers, R., and Impiombato, F.S.A. (1968). The binding of sodium dodecyl sulfate to various proteins. Biochem. J. 109:825-830.

Potts, J.T. Berger, A., Cooke, J., and Anfinson, C.B. (1962). A reinvestigation of the sequence of residues 11 to 18 in bovine pancreatic ribonuclease. J. Biol. Chem. 237:1851-1855.

Pringle, C.R. (1970a). The induction and genetic characterization of conditional lethal mutants of vesicular stomatitis virus, in: The Biology of Large RNA Viruses (R.D. Barry and B.W.J. Mahy, eds.), pp. 567-682, Academic Press, New York.

Pringle, C.R. (1970b). Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-flourourical, 5-azacytidine and ethylmethane sulfonate. J. Virol. 5:559-567.

Pringle, C.R. (1977). Genetics of Rhabdoviruses in Comprehensive Virology V.9, p.239-290 (H. Fraenkel-Conrat and R.R. Wagner, Eds.). Plenum Press, New York.

Pringle, C.R., and Duncan, I.B. (1971). Preliminary physiological characteirsation of temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. 8:56-61.

Pringle, C.R., Duncan, I.B., and Stevenson, M. (1971). Isolation and characterization of temperature-sensitive mutants of vesicular stomatitis New Jersey serotype. J. Virol. 8:836-841.

Pringle, C.R., and Wunner, W.H. (1973). Genetic and physiologic properties of ts mutants of Cocal virus. J. Virol. 12:677-683.

Ramig, F.R., White, R.M., and Fields, B.N. (1977). Suppression of the temperature-sensitive phenotype of a mutatn of Reovirus type 3. Science 195:406-407.

Reichmann, R.E., Bishop, D.H.L., Brown, F., Crick, J., Holland, J.J., Kang, C.-Y., Lazzarini, R., Moyer, S., Perrault, J., Prevec, L., Pringle, C.R., Wagner, R.R., Younger, J.S., and Huang, A.S. (1980). Proposal for a uniform nomenclature for defective interfering viruses of vesicular stomatitis virus. J. Virol. 34:792-794.

Reichmann, M.E., Schnitzlein, W.M., Bishop, D.H.L., Lazzarini, R.A., Beatrice, S.T., and Wagner, R.R. (1978). Classification of the New Jersey serotype of vesicular stomatitis virus into two subtypes. J. Virol. 25:446-449.

Repik, P., and Bishop, D.H.L. (1973). Determination of the molecular weight of animal RNA viral genomes by nuclease digestion. J. Virol. 12:969-983.

Repik, P., Flamand, A., and Bishop, D.H.L. (1976). Synthesis of RNA by mutants of vesicular stomatitis virus (Indiana serotype) and the ability of wild-type VSV New Jersey to complement the VSV Indiana ts GI-114 transcription defect. J. Virol. 20:157-169.

Repik, P., Flamand, A., Clark, H.F., Obijeski, J.F., Roy, P., and Bishop, D.H.L. (1974). Detection of homologous RNA seqeunces among six Rhabdovirus genomes. J. Virol. 13:250-252.

Rettenmier, C.W., Dumont, R., and Baltimore, D. (1975). Screening procedure for complementation dependent mutants of vesicular stomatitis virus. J. Virol. 15:41-49.

Reynolds, J.A. and Tanford, C. (1970). The gross conformation of protein-sodium dodecyl sulfate complexes. J. Biol. Chem. 245:5161-5165.

Rhodes, D.P., and Banerjee, A.K. (1976). 5'-terminal sequence of vesicular stomatitis virus in RNAs syntehsized in vitro. J. Virol. 17:33-42.

Rhodes, D.P., and Banerjee, A.K. (1980). Poly(A)-adjacent sequence of the 14.5 S mRNA of vesicular stomatitis virus (New Jersey serotype). Virology 105:297-300.

Roberts, B.E., and Paterson, B.M. (1973). Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. Proc. Natl. Acad. Sci. 66:471-481.

Robinson, A.B., McKerrow, J.H., and Cary, P. (1970). Controlled deamidation of peptides and proteins: an experimental hazard and a possible biological timer. Proc. Natl. Acad. Sci. 66:753-757.

Robinson, N.C., and Tanford, C. (1975). The binding of deoxycholate, triton X-100, sodium dodecyl sulfate, and phosphotidylcholine vesicles to cytochrome b_5. Biochem. 14:369-378.

Rose, J.K. (1980). Complete intergenic and flanking gene seuqences from the genome of vesicular stomatitis virus. Cell 19: 415-421.

Schaffhausen, B.S., Silver, J.E., and Benjamin, T.L. (178). Tumor antigen(s) in cells productively infected by wild-type polyoma virus and mutant NE-18. Proc. Natl. Acad. Sci. 75:79-83.

Schechter, Y. Patchornik, A., and Burstein, Y. (1976). Selective chemical cleavage of tryptophanyl peptide bonds by oxidative chlorination with N-chlorosuccinimide. Biochem. 15:5071-5075.

Schincariol, A.L., and Howatson, A.F. (1972). Replication of vesicular stomatitis virus II. Separation and characterization of virus-specific RNA species. Virology 49:766-783.

Schlesinger, M.J., Schlesinger, S., and Burge, B.W. (1972). Identification of a second glycoprotein in Sindbis virus. Virology 47:539-541.

Schmidt, M.F.E., and Schlesinger, M.J. (1979). Fatty acid binding to vesicular stomatitis virus glycoprotein a new type of post-translational modification of the viral glycoprotein. Cell 17:813-819.

Schnitzer, T.J., Dickson, C., and Weiss, R.A. (1979). Morphological and biochemical characterization of viral particles produced by the ts 045 mutant of vesicular stomatitis virus at restrictive temperature. J. Virol. 29:185-195.

Schnitzer, T.J., and Lodish, H.F. (1979). Noninfectious vesicular stomatitis virus particles deficient in the viral nucleocapsid. J. Virol. 29:443-447.

Schubert, M., Keene, J.D., and Lazzarini, R.A. (1979). A specific internal RNA polymerase recognition site of VSV RNA is invovled in the generation of DI particles. Cell 18:749-757.

Schubert, M., Keene, J.D., Lazzarini, R.A., and Emerson, S.U. (1978). The complete sequence of a unique RNA species synthesized by a DI particle of VSV. Cell 15:103-112.

Schweingruber, M.E., Stewart, J.W., and Sherman, F. (1979). Primary site and second site revertants of missense mutants of the evolutionarily invariant tryptophan 64 in iso-1-cytochrome c from yeast. J. Biol. chem. 254:4132-4143.

Sekellick, M.J., and Marcus, P.I. (1980). Viral interference by defective particles of vesicular stomatitis virus measrued in individual cells. Virology 104:247-252.

Shapiro, A.L., Vinuela, E. and Mazel, J.V., (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. 28:815-820.

Shirahama, K., Tsujii, K., and Takagi, T. (1974). Free-boundary electrophoresis of sodium dodecyl sulfate protein polypeptide complexes with special reference to SDS-polyacrylamide gel electrophoresis. J. Biochem. 75:309-319.

Smith, A.E., and Marcker, K.A. (1970). Cytoplasmic methionine transfer RNAs from eukaryotes. Nature (Lond.) 226:607-610.

Sokol, F., and Clark, H.F. (1973). Phosphoproteins, structural comonents of Rhabdoviruses. Virology 52:246-263.

Stanley, W.M. (1972). Preparation and analysis of L-[^{35}S]-methionine labeled transfer ribonucleic acids from rabbit liver. Anal. Biochem. 48:202-216.

Steers, E., Craven, G.R., and Anfinsen, C.B. (1965). Evidence for nonidentical chains in the β-galactosidase of Escherichia coli k12. J. Biol. Chem. 240:2478-2484.

Sullivan, B., Bonoventura, J., Bonaventura, C., Pennell, C., Elliott, J., Boyum, R., and Lambie, W. (1976). The structure and evolution of parvalbumins. 1. Amino acid compositional studies of parvalbumins from four Perciform species. J. Mol. Evol. 5:103-116.

Takagi, T., Tsujii, K., and Shirahama, K. (1975). Binding isotherms of sodium dodecyl sulfate to protein polypeptides with special reference to SDS-polyacrylamide gel electrophoresis. J. Biochem. 77:939-947.

Testa, D., and Banerjee, A.K. (1979). Initiation of RNA synthesis in vitro by viscular stomatitis virus. J. Biol. Chem. 254:2053-2058.

Testa, D., Chanda, P.K., and Banerjee, A.K. (1980). Unique mode of transcription in vitro by vesicular stomatitis virus. Cell. 21:267-275.

Toneguzzo, F., and Ghosh, H.P., (1978). In vitro syntehei s of vesicular stomatitis virus glycoprotein and insertion into membranes. Proc. Natl. Acad. Sci. 75:715-719.

Tung, J.-S., and Knight, C.A. (1972). Relative importance of some factors affecting the electrophoretic migration of proteins in sodium dodecyul sulfate-polyacrylamide gels. Anal. Biochem. 48:153-163.

Uy, R., and Wold, F. (1977). Posttranslational covalent modification of proteins. Science 198:890-896.

Van der Ouderaa, F.J., DeJong, W.W., Hilderink, A., and Bloemendal, H. (1974). The amino-acid sequence of the αB2 chain of bovine α-crystallin. Eur. J. Biochem. 49:157-168.

von Arx, E., and Neher, R. (1963). Eine multidimensionale techik zur chromatographischen identifizierung von aminosauren. J. Chromatography (German) 12:329-341.

Wagner, R.R. (1975). Reproduction of Rhabdoviruses in Comprehensive Virology V.4, pp. 1-94. (H. Fraenkel-Courat and R.R. Wagner Eds.), Plenum Press, New York and London.

Wagner, R.R., Levy, A.H., Snyder, R.M., Ratcliff, E.A., Jr., and Wyatt, D.F. (1963). Biological properties of two plaque variants of vesicular stomatitis virus (Indiana serotype). J. Immunol. 91:112-122.

Weber, K., and Osborn, M. (1969). The reliability of molecular weight determinations of dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

Weber, K., and Osborn, M. (1975). Proteins and sodium dodecyl sulfate: Molecular weight determination on polyacrylamide gels and related procedures in The Proteins (H. Neurath and R.L. Hill, eds.). Vol. I, pp. 179-223, Academic Press, N.Y.

Weber, L.A., Feman, E.R., and Baglioni, C. (1975). A cell free system from HeLa cells active in initiation of protein synthesis. Biochemsitry 14:5315-5321.

Weigele, M., DeBernardo, S.L., Tengi, J.P., and Leimgruber, W. (1972). A novel reagent for the fluorometric assay of primary amines. J. Am. Chem. Soc. 94:5927-5928.

Wilson, A.C., Corison, S.S., and White, T.J. (1977). Biochemical evolution. Ann. Rev. Biochem. 46:573-639.

Wong, D.K.Y., Holloway, A.F., and Cormack, D.V. (1971). Search for recombination between temperature-sensitive mutants of vesicular stomatitis virus. J. Gen. Virol. 13:477-479.

Wright, A.K., Thompson, M.R. and Miller, R.L. (1975). A study of protein-sodium dodecyul sulfate complexes by transient electric birefringence. Biochem. 14:3224-3228.

Wunner, W.H., and Pringle, C.R. (1974). A temperature-sensitive mutant of vesicular stomatitis virus with two abnormal virus proteins. J. Gen. Virol. 23:97-106.

Yang, Y.J. Stolz, D.B., and Prevec, L. (1969). Growth of vesicular stomatitis virus in a continuous clulture line of Antheraea eucolypti moth cells. J. Gen. Virol. 473-483.

Yang, R.C., Young, A., and Wu, R. (1980). BK virus DNA sequence coding for the t and T antigens and evaluation of methods for determining sequence homology. J. Virol. 34:416-430.

Zavada, J. (1972). Pseudotypes of vesicular stomatitis virus with the coat of murine leukemia and of avian myeloblastosis virus. J. Gen. Virol. 15:183-191.

Zavada, J., and Rosenbergova, M. (1972). Phenotypic mixing of vesicular stomatitis virus with fowl plague virus. Acta Virol. 16:103-114.

Zuckerkandl, E. (1976) Evolutionary processes and evolutionary noise at the molecular level. I. Functional density in proteins J. Mol. Evol. 7:167-183.

Zuckerkandl, E., and Pauling, L. (1965). Molecules as documents of evolutionary history. J. Theoret. Biol. 8:357-366.