STUDIES ON NS THE PHOSPHOPROTEIN OF VESICULAR STOMATITIS VIRUS

by

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STUDIES ON NS THE PHOSPHOPROTEIN OF VESICULAR STOMATITIS VIRUS

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ABSTRACT

The vesicular stomatitis virus phosphoprotein, NS, was the subject of this investigation. Multiple forms of NS protein were identified in Piry infected cells. These multiple species were demonstrated to be related by comparative peptide mapping under conditions of complete or partial digestion. Furthermore, kinetic studies revealed that one of the NS forms (NS_i) could be converted into a mature NS form (NS_v) by a covalent post-translational modification. The nature of this modification was investigated using inhibitors of both phosphorylation and acetylation.

NS protein isolated from cells infected with the Indiana serotype was structurally characterized by a variety of techniques including enzymatic digestion, chemical cleavage and partial acid hydrolysis. The observations presented here indicate that NS exists under denaturing conditions as a monomer and is post-translationally modified by multisite phosphorylation. NS isolated from infected cells appears to be phosphorylated toward the amino terminus of the polypeptide primarily in one large tryptic peptide.

Monospecific antisera were raised against

SDS-polyacrylamide gel purified Indiana virion proteins. These sera were used to investigate viral protein aggregates in both virions and infected cells. In particular it was demonstrated that a protein kinase activity capable of *in vitro* phosphorylation of NS protein could be identified in immunoprecipitates of NS protein.

The significance of these findings with respect to other published observations is discussed.

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ABBREVIATIONS

Ab = antibody

Ag = antigen

Arg = arginine

Asp = aspartic aoid

ATP = adenosine triphosphate

BHK = baby hamster kidney cells

BPB = bromophenol blue

BSA = bovine serum albumin

cAMP = cyclic adenosine monophosphate

Ci = curie

CIT = citrate

CoA = coenzyme A

cpm = counts per minute

CTP = cytidine triphosphate

DNA = deoxyribonucleic acid

DOC = deoxycholate

DTT = dithiothreitol

EDTA = ethylenediaminotetra-acetic acid

eIF = eukaryotic initiation factor

E/M = electron microscope

FMDV = foot and mouth disease virus

fMET = N-formyl-methionine

FV-3 = frog virus 3

Glu = glutamic acid

GTP = guanosine triphosphate

h = hour

Hepes = N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic

HSS = high salt solubilizer

HSV = herpes simplex virus

IEF = isoelectric focussing

Ig = immunoglobulin

IND = Indiana serotype of vesicular stomatitis virus

Lys = lysine

MEM = Joklik modified minimum essential medium

Met = methionine

min = minute

moi = multiplicity of infection

mRNA = messenger ribonucleic acid

NBCS = newborn calf serum

NCS = N-chlorosuccinimide

NEPHGE = non-equilibrium pH gradient electrophoresis

NJ = New Jersey serotype of VSV

NMR = nuclear magnetic resonance

OR	=	origin
OX	=	oxaloacetate
PBS	=	phosphate buffered saline
PFU	=	plaque forming units
Pi	=	inorganic phosphate
pI	=	isoelectric point
PITC	=	phenylisothiocyanate
Pro	=	proline
PS	=	phosphoserine
PT	=	phosphothreonine
PTC	=	phenylthiocarbamyl derivative
PTH	=	phenylthiohydantoin derivative
RIPA	=	radioimmunoprecipitation assay buffer
RMuLV	=	Raucher murine leukemia virus
RNA	=	ribonucleic acid
RNP	=	ribonucleoprotein
rpm	=	revolutions per minute
RSV	=	Rous sarcoma virus
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	=	serine
Ser(P)	=	phosphoserine
STM	=	salt tris magnesium buffer
SUP	=	supernatant

SVPD	=	snake venom phosphodiesterase
TCA	=	trichloracetic acid
TEMED	=	tetramethylethylenediamine
TFA	=	trifluoracetic acid
Thr	=	threonine
Thr(P)	=	phosphothreonine
TPCK	=	tolylsulfonyl phenylalanyl chloromethyl ketone
tris	=	tris (hydroxymethyl) amino-methane
tRNA	=	transfer ribonucleic acid
ts	=	temperature sensitive
U	=	units
UV	=	ultraviolet light
VSV	=	vesicular stomatitis virus
w/v	=	weight to volume
w/w	=	weight to weight

TABLE OF CONTENTS

	Page
DESCRIPTIVE NOTE	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
ABBREVIATIONS	vii
INTRODUCTION	1
1. Vesicular Stomatitis Virus Structure	2
2. VSV Reproductive Cycle	3
3. Cellular Physiological Phosphorylation	10
4. Kinase Encoded by Viruses	13
5. Host Cell Kinases Involved in Viral Reproduction	15
6. Phosphorylation and the Viral Infectious Cycle	18
a) Regulation of Nucleic Acid-Protein Interactions	18
b) Phosphorylation:dephosphorylation Regulating Virus Enzyme Activity	20
c) Site-specific Phosphorylation Leading to Processing	21

TABLE OF CONTENTS (continued)

	Page
7. Characteristics of VSV NS Protein	21
a) Functions of NS in the Infectious Cycle	21
b) Regulation of NS Activity by Phosphorylation:dephosphorylation . . .	23
c) Aberrant Electrophoretic Mobility of NS Protein	25
8. Area of Investigation	27
MATERIALS AND METHODS	29
Materials	29
1. Biochemicals and Reagents	29
2. Solutions	30
3. Source of Cells	38
4. Source of Viruses	38
Methods	39
1. Growth, Labelling and Fractionation of Cells and Viruses	39
1.1 L Cell Growth	39
1.2 Infection and Labelling of Suspension Cultures	39
1.3 Isotopic Labelling of Virus	40
1.4 ^{32}P-labelling of Infected Cells	40
1.5 Pulse-chase Experiments	40

TABLE OF CONTENTS (continued)

		Page
1.6	Virus Purification	41
1.7	High Salt Solubiliser Fractionation of Virus	42
1.8	Preparation of RNP from Piry Infected Cells	42
1.9	Fractionation of Infected Cells	43
2.	Gel Electrophoresis	43
2.1	Sample Preparation for Electrophoresis . . .	43
2.2	Discontinuous SDS Polyacrylamide Gel Electrophoresis (Laemmli, 1970)	44
2.3	Sample Preparation for IEF or NEPHGE	44
2.4	Isoelectric Focussing of Proteins (O'Farrell, 1975)	45
2.5	Non Equilibrium pH Gradient Electrophoresis (NEPHGE)	46
2.6	Fixing and Staining of Gels	46
3.	Peptide Mapping of Viral Proteins	46
3.1	Tryptic Peptide Mapping	46
3.2	Oxidation of Protein	47
3.3	Partial Cleavage at Tryptophan Residues with N-chlorosuccinimide	48
4.	_In Vitro_ Protein Synthesis	48
4.1	Preparation of Infected Cell Cytoplasmic Extracts	48
4.2	Cell Free Translation Using L Cell Extracts	49

TABLE OF CONTENTS (continued)

	Page
4.3 Synthesis of N-formyl-^{35}S-Met-tRNA$_1^{Met}$ . . .	50
4.4 Amino Terminal Labelling *in vitro*	51
5. Phosphodipeptide Analysis	51
5.1 Partial Acid Hydrolysis of Phosphoproteins .	51
5.2 Preparation of Amberlite 1R 120 Resin . . .	51
5.3 Preparation of Dowex-1 Resin	52
5.4 Purification of Phosphodipeptides from Partial Acid Hydrolysates	52
5.5 High Voltage Paper Electrophoresis	53
5.6 Ninhydrin Staining of Paper and Thin Layer Electropherograms	53
5.7 Elution of Radioactive Peptides from Paper Electropherograms	54
5.8 Subtractive Edman Degradation	54
6. Immunoprecipitation and Kinase Assays . . .	55
6.1 Production of Monospecific Antisera	55
6.2 Immunoprecipitation of Viral Proteins . . .	55
6.3 Protein Kinase Assays	56
6.4 Preparation of Denatured Radioactive Viral Protein Antigens	57
RESULTS	58
1. Identification and Characterization of Multiple Forms of Piry NS	58
1.1 Piry Infected Cells Contain Two NS Species Labelled with ^{35}S-methionine	58

TABLE OF CONTENTS (continued)

		Page
1.2	Phosphorylation of Piry NS Proteins	65
1.3	Maturation of NS_i in the Presence of Theophylline	71
1.4	Possible Acetylation of Piry NS Proteins .	75
1.5	Lack of Separation of NS_v and NS_i on NEPHGE	82
2.	Structural Analysis of NS Protein	85
2.1	Amino Terminal Labelling of NS Protein . .	85
2.2	Molecular Weight Estimation of NS Protein .	87
2.3	Phosphodiesterase Treatment of NS Protein .	91
2.4	Linear Mapping of Phosphorylated Residues in NS Protein	98
2.5	Tryptic Digestion of Indiana NS Protein . .	103
2.6	Phosphodipeptide Analysis of Phosphoproteins	111
2.7	Phosphodipeptide Analysis of NS Protein . .	121
2.8	Edman Degradation of (^{32}P) Labelled Phosphodipeptide	127
3.	Protein Kinase Activity Associated with NS Protein	130
3.1	Characterization of Monospecific Antisera .	130
3.2	Kinase Activity Associated with Immunoprecipitates of Infected Cells . . .	142
3.3	Kinase Activity Phosphorylates NS at Serine Residues	144
3.4	Phosphate Does Not Cycle Off in Immunoprecipitates	146

TABLE OF CONTENTS (continued)

	Page
3.5 Kinase Activity of Immunoprecipitates from HSS-treated VSV Virions and Cytoplasmic Extracts	148
DISCUSSION	156
1. Multiple NS Species in Piry Infected Cells .	156
1.1 Isoelectric Focussing of Piry NS	159
1.2 Lack of Detectable Compartmentalization of Piry NS Protein	161
1.3 Possible Acetylation of Piry NS Protein . .	162
2. Structural Analysis of Indiana NS Protein .	163
2.1 Does NS Protein Exist as a Dimer During SDS-PAGE?	163
2.2 NS Phosphorylation Sites Are Predominantly Found on the Amino Terminal NCS Fragment of NS Protein	165
2.3 A Large Tryptic Fragment is Phosphorylated in NS Protein	166
2.4 Phosphodipeptide Analysis of NS Protein . .	168
3. Protein Kinase Associated with Viral Protein Immunoprecipitates	171
3.1 Preparation of Monospecific Antibodies . . .	171
3.2 Coprecipitation of Kinase with Viral Proteins	173

TABLE OF CONTENTS (continued)

Page

SUMMARY. 175

APPENDICIES 178

Appendix I : Amino acid sequence for NS protein, San Juan strain (Gallione _et al_ 1981) 178

Appendix II : Schematic representation of NS dimer and monomer models 180

Appendix III : Theoretical isoelectric point determination of Indiana NS protein 182

REFERENCES 185

LIST OF FIGURES

Figure	Title	Page
1	Comparison of viral proteins in Piry and Indiana VSV infected cells and Piry virions	59
2	Tryptic fingerprint of methione labelled proteins NS_i and NS_v	60
3	Pulse-chase of protein NS_i to NS_v	62
4	Conversion of NS_i to NS_v in the presence and absence of cycloheximide	63
5	Pulse-chase analyses of Piry, Indiana and Cocal infected cells	64
6	Sucrose gradient analysis of Piry infected cells	66
7	SDS-PAGE analysis of fractionated Piry infected cells	67
8	Phosphate-labelled proteins in Piry virus-infected cells	69
9	SDS-PAGE analysis of ^{32}P-labelled virions	70
10	N-chlorosuccinimide cleavage of Piry proteins	72
11	Treatment of Piry-infected L cells with theophylline	74
12	^{3}H-acetate labelling of protein in an S-4 polysomal extract	76
13	Effect of oxaloacetate and citrate synthase on ^{3}H-acetate incorporation in S-4 polysomal extracts	78
14	Effect of oxaloacetate and citrate synthase on NS_i maturation	80
15	Effect of citrate on NS_i conversion	81

LIST OF FIGURES (continued)

Figure	Title	Page
16	Isoelectric and SDS-PAGE analysis of a Piry infected cell extract	83
17	Non equilibrium pH gradient electrophoresis analysis of Piry infected cells	84
18	Two dimensional analysis of Piry-infected L cells	86
19	Tryptic hydrolysis of internally and end-labelled Indiana NS protein	88
20	Snake venom phosphodiesterase treatment of an Indiana infected cell extract	92
21	Snake venom phosphodiesterase treatment of Indiana infected cell extracts	94
22	Snake venom phosphodiesterase treatment of immunoprecipitates	95
23	Snake venom phosphodiesterase treatment of N-formyl-^{35}S-methionine labelled Indiana proteins	97
24	N-chlorosuccinimide treatment of purified Indiana NS protein	99
25	N-chlorosuccinimide cleavage of Indiana NS protein	101
26	N-chlorosuccinimide cleavage of Piry NS proteins	102
27	N-chlorosuccinimide cleavage of Indiana NS protein produced in human KB cells	104
28	N-chlorosuccinimide cleavage of Indiana NS protein produced in L cells	105
29	Tryptic fingerprint of ^{35}S-methionine labelled Indiana NS protein	106

LIST OF FIGURES (continued)

Figure	Title	Page
30	Thin layer electrophoresis of ^{32}P-labelled tryptic peptides	108
31	SDS-PAGE analysis of trypsin hydrolysed ^{32}P-labelled Indiana NS protein	109
32	High voltage paper electrophoresis of α-casein and BSA acid hydrolysates	115
33	High voltage paper electrophoresis of α-casein acid hydrolysate fractionated on a Dowex-1 anion exchange column	118
34	High voltage paper electrophoresis of phosphodipeptides after Edman degradation	119
35	Two dimensional analysis of α-casein phosphodipeptides	122
36	High voltage paper electrophoresis of ^{32}P-labelled NS protein acid hydrolysates	123
37	Three dimensional analysis of acid hydrolysed Indiana NS protein	125
38	Edman degradation of ^{32}P-labelled Indiana phosphodipeptides	129
39	Specificity of rabbit anti-NS and anti-N sera	131
40	Reactivity of monospecific antisera with denatured viral proteins	133
41	Reactivity of monospecific antisera with denatured viral proteins	134
42	Antibody precipitation of disrupted VSV virions	136
43	Antibody treatment of ^{35}S-methionine labelled infected cell extracts	137

LIST OF FIGURES (continued).

Figure	Title	Page
44	Antibody treatment of ^{35}S-methionine labelled infected and uninfected L cells	138
45	Fractionation of infected cell cytoplasmic extracts	140
46	Immunoprecipitates from supernatant and pellet fractions of infected cells and their associated protein kinase activity	141
47	Kinase activity associated with anti-NS and anti-N immunoprecipitates	143
48	Products *in vitro* kinase reaction analysed on a 0.26% bis acrylamide gel	145
49	Identification of the amino acid phosphorylated in the protein kinase assay of anti-NS immunoprecipitate	147
50	*In vitro* pulse-chase experiments with ^{32}P-γ-ATP	149
51	Protein kinase activity identified in anti-NS immunoprecipitate of virion proteins having sarc-type activity	151
52	Antibody precipitates of HSS treated infected cell extracts	153
53	Kinase activity associated with immunoprecipitates of HSS treated infected cell extracts	155
54	Calculated net charge of Indiana NS protein as a function of pH	183

LIST OF TABLES

Number	Title	Page
1	Approximation of Molecular Weight of NS Protein	90
2	Automated sequence Analysis of ^{35}S-methionine Labelled NS Protein	112
3	Charged Residues and Their pK_a Values for Indiana NS	183

INTRODUCTION

In recent years it has been recognized that a protein's function may be regulated by reversible covalent modification of that protein's primary structure (Cohen, 1976; Wold, 1981). The original observation which generated this concept was made in 1956 by Krebs and Fischer when they demonstrated the existence of two interconvertible forms of mammalian glycogen phosphorylase, an active phosphorylated "a" form and an inactive dephosphorylated "b" form. Now many years later although a multitude of systems appear to be controlled in a similar fashion the complexities of this type of regulation are still incompletely understood. One approach which has frequently been used to unravel complex cellular problems is to look at simpler isolated viral systems. In this respect a well defined, self contained virus enzyme system controlled by phosphorylation:dephosphorylation could serve as a useful model for studying this mode of regulation.

The work carried out in this thesis was directed toward further characterization of NS the phosphoprotein of vesicular stomatitis virus (VSV). The information gleaned from this project will aid in the understanding of the role of phosphorylation in the regulation of NS protein activity. In turn, this knowledge may eventually

lead to the development of the afore mentioned viral model system.

In the following is provided a brief description of vesicular stomatitis virus and a summary of what is known of the various steps in the viral reproductive cycle. Evidence is then presented supporting the central role of phosphorylation in a wide variety of cellular physiological events. Following this is a section dealing with viral strategies with respect to phosphorylation and a presentation of a number of specific examples of viral proteins regulated by phosphorylation/dephosphorylation mechanisms. The final portion of this section contains a synopsis of some of the unique characteristics of VSV NS protein.

1. Vesicular Stomatitis Virus Structure

Vesicular stomatitis virus (VSV), a member of the rhabdovirus group is a bullet shaped, lipoprotein enveloped particle containing a single strand RNA genome. The negative sense (non-infectious) genome has a molecular weight of $4X10^6$ Daltons (Huang and Wagner, 1966; Kang and Prevec, 1971) and is capable of coding for five distinct proteins. All five gene products are components of the mature virion. The glycoprotein, G, protrudes from the viral envelope while the matrix protein M, lies just under the viral membrane possibly interacting with both the ribonucleoprotein core and transmembraneous

G protein (Mudd and Swanson, 1978). The polymerase protein L, phosphoprotein NS and nucleocapsid protein N are all intimately associated with the viral genome in a ribonucleoprotein complex (Emerson and Yu, 1975).

2. VSV Reproductive Cycle

VSV appears to be a very promiscuous virus capable of infecting a wide variety of insect, avian, marsupial and mammalian hosts (Yang *et al*., 1969; Murphy and Shope, 1971; Pringle, 1978). The infectious process is probably initiated by either virus:host membrane fusion or by a pinocytotic event (Simpson *et al*., 1969; Heine and Schnaitman, 1971). Electron microscopic evidence suggests that while both phenomena occur (i.e. fusion and pinocytosis) the predominant mechanism is viropexis (Dahlberg, 1974). Fan and Sefton (1978) using immunolysis of infected cells as an indicator of viral:host membrane fusion confirmed the E/M studies. Their work indicates that high multiplicities membrane fusion can occur, however, the major (and probably physiological) mode of entry is by viropexis.

In vitro transcription studies suggest that primary transcription can occur only if the viral membrane is disrupted (Emerson and Wagner, 1972). Therefore the next step in infection following viropexis must be removal of the virion envelope (i.e. virus uncoating). Witt *et al*., (1981) recently provided evidence for a kinase activated

uncoating of VSV particles.

Since the VSV genome is of negative polarity the subsequent step to uncoating is the primary transcription of single strand anti-sense RNA to yield five translatable messenger RNA species. The final product of this process is five monocistronic mRNAs each with a 5' methylated cap and a 3' poly A tail (Banerjee, 1980; Ehrenfeld and Summers, 1972). As well a 47 nucleotide leader RNA is synthesized from the 3' extreme end of the genome (Colonno and Banerjee, 1978).

The polymerase responsible for primary transcription of the ribonucleoprotein template is composed of proteins L, NS and possibly unidentified host cell factors (Emerson and Yu, 1975; Naito and Ishihama, 1976; Pringle, 1978). Naito <u>et al</u>., (1976) have evidence which suggests that the species of holoenzyme with maximum <u>in vitro</u> transcriptional activity contains equimolar amounts of L and NS proteins. Mellon and Emerson (1978) using <u>in vitro</u> reconstitution studies have demonstrated that the polymerase binds via its NS component to the viral template. Keene <u>et al</u>., (1981) using methylation protection studies identified specific nucleotide sequences in the leader gene where NS apparently binds, possibly initiating transcription.

The exact mechanism of primary transcription is still unclear. Ball and White (1976) demonstrated by UV irradiation of the VSV genome that transcription of

mature mRNAs occur in the obligate order N-NS-M-G-L. This reflects the physical arrangement of the cistrons on the genome and implies there is either one initiation site for transcription or one binding site for polymerase at the 3' end of the genome. This work has been supported by kinetic studies of mRNA synthesis (Iverson and Rose, 1981). One model to rationalize the existence of a single initiation site and five distinct mRNA products is a processing scheme where transcription begins at the 3' end of the genome and can potentially progress to the 5' end without interruption (Banerjee et al., 1977; Ball and Wertz, 1981). Each individual RNA product (leader or mRNA) is then derived from the polycistronic transcript by cleavage and processing (i.e. 5' methylated cap and 3' polyadenylated tail). Theoretically the precursor molecule can be processed before it is genome length and the observed in vivo gradient of mature products (Schincariol and Howatson, 1972) represents initiation at the 3' end and falling off of the polymerase holoenzyme at various positions along the template. If this were the actual mechanism one might predict that it would be possible to isolate precursor polycistronic mRNA species. In fact Herman et al., (1978) have identified polycistronic mRNA species which are linked by tracts of poly A suggesting that VSV polymerase can readthrough intercistronic boundaries and may be involved in polyadenylation. Furthermore, transcripts containing

leader RNA joined to N mRNA have been isolated (Herman and Lazzarini, 1981). A true precursor product relationship between these polycistronic transcripts and mRNA molecules has not yet been established.

An alternative explanation for Ball's UV experimental observations is that polymerase must enter at the 3' end of the genome but is subsequently capable of initiating at the beginning of each gene (Banerjee et al., 1977; Ball and Wertz, 1981). One would predict that if this were the case it should be possible to identify mRNA molecules that contain 5' triphosphates instead of 5' methylated caps. Rose (1975) has shown that approximately 10-15% of in vivo synthesized mRNA species do contain 5' triphosphates as opposed to methylated caps. In vitro transcription products which contain di or tri phosphorylated 5' termini have been identified by Testa et al., (1980), as being complementary to the 3' ends of the N and NS genes. These short RNA species have very small UV target sizes and are synthesized prior to the sequential appearance of mature mRNA products indicating that they are internal initiating events. However Lazzarini et al., (1982) have shown that these short RNA products accumulate for 5 hours in in vitro reactions and 90% of them are released from the transcriptional complex into a stable non productive pool. Their work suggests that the majority of these independent internal initiation events lead primarily to abortive transcription. Similarly

Chanda and Banerjee (1981) have been unable to chase these potential precursors into mature mRNA products.

Taken together, the above evidence appears insufficient to support or refute either a sequential readthrough or internal initiation model for VSV transcription.

Once VSV has successfully produced primary transcripts, translation of the viral mRNAs occurs on host polysomes. The viral protein products then effect replication and secondary transcription of the viral genome.

Perlman and Huang, (1973) have shown that the same viral polymerase components are involved in replication and transcription. The mechanism of polymerase switching from transcriptase to replicase is currently under investigation by several groups. Testa _et al._, (1980) have shown that _in vitro_ synthesis of preinitiated RNA species in the presence of a γ imido analog of ATP results in synthetic products, 50% of which are genome size transcripts. This suggests that protein kinase activity (which relies on cleavage of the γ phosphodiester bond) is somehow involved in regulation of polymerase switching between transcription and replication functions. As will be discussed in detail later, several other groups are now actively investigating the relationship between kinase and polymerase activity in VSV (Clinton _et al._, 1978; Witt and Summers, 1980; Hsu _et al._, 1982). It seems unlikely

that phosphorylation of the polymerase alone is sufficient for conversion from replicase to transcriptase since several groups have demonstrated a strict requirement for ongoing protein synthesis during replication (Wertz and Levine, 1973). This finding may imply that either a stoichiometric amount of one or more viral proteins or an unstable catalytic protein is required for maintenance of the replicative complex.

Blumberg et al., (1981) favour the idea that stoichiometric amounts of N protein are necessary for replication to occur. In their model, during primary transcription the polymerase initiates at the 3' end of the genome and begins to synthesize leader sequences. At a particular site the polymerase reads a strong stop and a short leader transcript is released. The polymerase now resumes transcription, possibly by one of the two previously described mechanisms, generating the five mRNA species. Once sufficient N message has been produced and translated N protein begins to accumulate and binds site specifically to nascent unreleased leader transcripts. Co-operative binding of more N protein facilitates encapsidation of RNA and readthrough of the attenuation signal resulting in genome length ribonucleoprotein complexes. Blumberg et al., (1981) feel their model explains some of the known properties of VSV replication. For instance, it explains why full length transcripts are

rapidly encapsidated while mRNAs lack significant N protein binding. They argue that only transcripts contiguous with leader RNA (which contains the presumed nucleation site) can be encapsidated. Furthermore, their model explains the strict requirement for continuous protein synthesis during replication.

One possible flaw in their model of regulation is the finding that free intracellular leader RNAs are encapsidated by N protein (Blumberg and Kolakofsky, 1981). This suggests that either N binding alone is insufficient to always override attenuation or alternatively that leader RNP structures are formed after release from template.

At some point in infection secondary transcription off negative strands must be inhibited as this form of ribonucleoprotein is eventually packaged into mature virions. Perrault and Kingsbury, (1974) identified a viral envelope associated protein (not G protein) which at high concentrations could inhibit VSV transcription. More recently, Martinette et al., (1979) demonstrated that a VSV mutant (ts 023) with a defect in M protein overproduces VSV mRNA at the non permissive temperature. Carrol and Wagner, (1979) using purified wild type M protein were able to inhibit in vitro transcription. The inhibitory affect of M is manifested only at high protein concentrations (as one would find late in infection). Newcomb and Brown (1981) were able to demonstrate by specific

removal of M protein from purified virions that M is involved in maintaining VSV nucleocapsids in the compact form found in native virions. Taken together these experimental observations suggest that M protein is instrumental in some of the final steps of VSV propagation. That is, high concentrations of M late in infection may initiate condensation of nucleocapsids simultaneously shutting down transcription and preparing viral genomes for the process of budding (Simons and Garoff, 1980).

3. Cellular Physiological Phosphorylation

Finn Wold, (1981) has cited examples of 125 different covalent post-translational modifications that occur *in vivo*. Of the 125, only six have been demonstrated to be reversible (Krebs and Beavo, 1979). Phosphorylation, adenylation, acetylation, methylation, uridylylation and S/SH interconversions are all reversible and therefore postulated to be involved in regulatory pathways. The central role of phosphorylation:dephosphorylation in a broad spectrum of physiological events suggests that it is indeed a fundamental method of control in the cell.

For example, Plet *et al.*, (1982) have shown that one of the very early steps (within three hours of induction) in the differentiation of mouse teratocarcinoma cells is an alteration in the levels of cAMP dependent kinase. This finding suggests that an early, important event in

differentiation is a change in phosphorylation patterns of the pluripotent cell.

In the developing oocyte of <u>Xenopus</u> <u>laevius</u>, site specific phosphorylation of the 40S ribosomal protein, S-6, appears to be related to specific translation of a limited class of mRNA species required for development. Translation in rabbit reticulocytes can be inhibited by glutathione, an effect which is mediated by cAMP independent kinase phosphorylation of the α subunit of eIF-2 (Ernst <u>et</u> <u>al</u>., 1978).

Eukaryotic transcription is also regulated, at least in part, by phosphorylation:dephosphorylation mechanisms. The DNA dependent RNA polymerase II of calf thymus is phosphorylated and activated by both cAMP dependent and independent kinases (Krebs and Beavo, 1979). Lincoln <u>et</u> <u>al</u>., (1977), have also demonstrated that multiple kinases are responsible for phosphorylation of calf thymus RNA pol II suggesting that <u>multisite</u> phosphorylation of this enzyme may be important in its regulation as has been demonstrated for several other proteins (Cohen, 1976).

Chromosomal replication in dividing cells has been correlated with phosphorylation of essentially all histone F_1 molecules bound to chromatin structures (Langan and Hohmann, 1974). In contrast glucagon treatment of rat liver cells induces phosphorylation of only about 1% of histone F_1 molecules (Langan, 1969). The phosphorylation takes place at sites on histone F_1

other than those modified in dividing cells and reduces the ability of F_1 to cause structural changes in double stranded DNA (Langan, 1971). Together these findings suggest that differential phosphorylation of one class of histone molecules may be related to gross replication of DNA or finely tuned transcription of portions of DNA.

Phosphorylation may also play a role in neuronal activity. Greengard and Kebabian (1974), have implicated phosphorylation of specific synaptic membrane proteins with regulation of post synaptic membrane permeability.

Finally, in a recent review, Krebs and Beavo, (1979) have listed 22 metabolic enzymes which undergo specific phosphorylation:dephosphorylation reactions.

Taken together, these examples serve to illustrate that phosphorylation:dephosphorylation is an ubiquitous mode of regulation linked to modulation of gene expression, translation, transcription, DNA replication, membrane permeability and general cell metabolism. Since these are all phenomena with which an infecting virus must contend in its attempt to commandeer or parasitize a cell, one would expect that viruses have evolved mechanisms that allow them to integrate into pre-existing phosphorylation: dephosphorylation regulatory pathways. In this respect, three possible strategies viruses could adopt are:

(1) viruses could encode their own kinases to phosphorylate viral and/or host proteins.

(2) viruses could encode proteins which can act as substrates for cellular kinases.

(3) viruses could encode a regulatory protein(s) which could alter the specificity of particular cellular kinases.

In the following will be presented specific examples which support the idea that several viruses practice strategies (1) and (2). Although I find the third strategy appealing, to my knowledge there have been no documented examples of this phenomenon.

4. Kinases Encoded by Viruses

Transcriptional control of *E. coli* DNA dependent RNA polymerase has shown to be affected by a T_7 phage protein kinase (Zillig *et al.*, 1975). Apparently early in infection (3 minutes post infection) the phage specified kinase phosphorylates two subunits of the host coded polymerase resulting in shut down of both host RNA synthesis and early T_7 transcription.

Silberstein and August, (1976), have clearly shown that the genome of frog virus 3 codes for a protein kinase activity. This kinase is found both in viral capsids and the infected cell. Gravell and Cromeans, (1972) demonstrated that the FV-3 kinase is necessary for viral infectivity by co-infecting mutant FV-3 particles which lack kinase activity and UV inactivated wild type FV-3 particles. In this complementation experiment the virion bound kinase of UV inactivated wild type particles was able to

restore infectivity to the mutant strain by transphosphorylation within the infected cell.

Rous sarcoma virus specifies a tyrosine specific protein kinase activity in its src gene (Collett and Erikson, 1978). Levinson _et al._, (1978) demonstrated that the kinase, pp60^{src} can be found associated with a cellular phosphoprotein and Willingham _et al._, (1979) have shown that some of the pp60^{src} is tightly bound to the internal surface of the plasma membrane. The amount of total cellular phosphotyrosine can be raised from 0.03% to 0.3%, a tenfold increase following transformation by RNA tumor viruses (Sefton _et al._, 1980). Recently it has been demonstrated that a host cell protein, vinculin, is an _in vivo_ substrate for the viral kinase (Sefton _et al._, 1981).. Finally viral mutants, ts in the src gene, have been identified as temperature sensitive for both transformation and kinase activity.

Polyoma virus relies on its hr-t gene products for induction of malignant transformations (Schaffhausen and Benjamin, 1981). There is now growing evidence to suggest that one of these products, mid T, like pp60^{src} is a virally coded kinase. Immunoprecipitates containing multiple forms of mid T (i.e. 56K and 58K) show _in vitro_ phosphorylation of mid T tyrosine residues (Smith _et al._, 1979). With appropriate antisera (i.e. rat but not hamster) one sees phosphorylation of Ig heavy chain in mid T immunoprecipitates

(Smith *et al.*, 1979). As with $pp60^{src}$, mid T associated kinase activity is bound to internal plasma membrane surfaces (Ito *et al.*, 1977). Finally, a single insertion mutant in the hr-t gene (NG-59) results in concurrent loss of kinase activity and transforming ability (Schaffhausen and Benjamin, 1979; Staneloni *et al.*, 1977). The fact that a single amino acid insertion abrogates kinase activity suggests, but does not prove, that mid T is not merely a substrate for a coprecipitating tyrosine kinase but is itself a phosphorylating enzyme.

As pointed out by Schaffhausen *et al.*, (1981) two quite diverse virus systems (R.S.V. and Polyoma) appear to induce malignant transformation by a single gene and in both cases the gene product may be a protein kinase. It may be argued that since viable virus mutants defective in both src and hr-t functions can be isolated these virally coded kinases may not be required for virus replication per se. However, the role of transformation in the etiology of viral propagation in the whole animal is unclear and it may be that viruses coding for kinases of this type have a selective advantage in some natural situations.

5. Host Cell Kinases Involved in Viral Reproduction

Grubman *et al.*, (1981) have recently demonstrated that purified foot and mouth disease virus contains kinase

activity. The enzyme has been shown to phosphorylate viral structural proteins VP_2 and VP_3 probably at serine residues. Since the level of kinase activity is dependent upon the host cell in which the virus is propagated, these authors believe it is probably a host coded enzyme.

A similar type of evidence first led Imblum and Wagner (1974) to postulate that the virion bound kinase of VSV was not virally coded. Their work revealed that actinomycin D treatment of cells prior to infection (act D inhibits DNA dependent RNA synthesis but not viral transcription) resulted in lower levels of kinase in progeny particles. The kinase packaged into virions was able to phosphorylate VSV proteins and Watanabe _et al_., (1974) demonstrated that primary transcription of viral genomes was dependent upon continuing kinase activity. Witt and Summers, (1980) observed a similar kinase activity requirement for primary transcription _in vitro_ using disrupted VSV virions. Witt _et al_., (1981), also suggest that phosphorylation, in particular of M protein by the virion bound kinase may facilitate virus uncoating.

Clinton _et al_., (1982), have provided the most definitive evidence yet that viruses package cellular kinases. Using purified VSV virions they were able to demonstrate the presence of BHK endogenous sarc protein in viral envelope and RNP fractions. _In vitro_ kinase reactions with disrupted virions resulted in phosphorylation of

M protein at tyrosine residues, (sarc is a tyrosine specific kinase). Since earlier work (Clinton and Huang, 1981) had demonstrated that M can be phosphorylated *in vivo* at tyrosine residues it seems reasonable to suggest that virion sarc is not merely a fortuitously trapped kinase but may be involved in the viral replicative cycle.

Lamb, (1975) identified a kinase activity in Sendai virions which, as with FMDV, showed differential activity depending on the cell type in which the virus had been grown. The virion kinase phosphorylated the same spectrum of viral proteins *in vitro* as had been observed to be phosphorylated *in vivo*. Lamb made the unique observation that the predominant (if not only) amino acid labelled with γ-^{32}P-ATP *in vitro* by the virion kinase was threonine. In retrospect, however, Lamb performed his phosphoamino acid analysis using a system which would later prove to be inadequate for separation of phosphothreonine from phosphotyrosine (Clinton and Huang, 1981). Furthermore, the Sendai kinase showed a similar virion location to that identified for VSV virion sarc by Clinton *et al*., (1982). Taken together these results may suggest that the presumptive host kinase packaged into Sendai virions may in fact be the avian sarc equivalent.

A recent paper by Cajean-Feroldi *et al*., (1981) illustrates the direction that investigations into virus: host kinase research is beginning to take. Their approach

has been to isolate viral phosphoproteins from infected cells and look for tight associations between these polypeptides and cellular kinases. Working with adenovirus 72K single stranded DNA binding protein these authors have identified two distinct cellular kinases which co-purify with 72K on DNA cellulose and by acrylamide agarose gel filtration. The normal substrates for these kinases have yet to be identified.

6. Phosphorylation and the Viral Infectious Cycle

In the following will be presented specific examples of different ways viruses use phosphorylation to regulate protein activity during the infectious cycle.

a) Regulation of Nucleic Acid-Protein Interactions

Sen *et al.*, (1977) have investigated the relationship between the phosphorylation status of Rauscher murine leukemia viruses phosphoprotein p12 and the ability of the polypeptide to bind to homologous viral RNA. Five species of p12 apparently differing only with respect to the number of covalently bound phosphate moieties can be isolated from purified RMuLV virions. In *in vitro* reconstitution experiments with homologous 70S viral RNA only one species of p12 which contained relatively few phosphate groups could bind to genomic RNA. When RNP complexes were isolated from UV stabilized viral genomes again only the species which bound *in vitro* to RNA was specifically bound *in vivo*.

The role of p12 protein in the infectious cycle is still not clear, however what is evident is that the association of a viral RNA binding protein with homologous RNA is regulated by a phosphorylation:dephosphorylation mechanism. One question that comes to mind is: why are there so many forms of p12 when only one type is associated with homologous RNA? The answer may be trivial, that is hyperphosphorylated nonfunctional species are merely packaged into virions by chance. Alternatively there may be other roles played by p12 besides its functional association with RNA.

Walter et al., (1981) showed that the SV-40 large T antigen is phosphorylated in vivo both at threonine and serine residues. Edwards et al., (1979) further demonstrated that the covalently bound phosphate groups cycle on and off of large T during the infectious cycle suggesting a regulatory role for phosphorylation: dephosphorylation. Since large T is a DNA binding protein it seemed reasonable to Montenarh and Henning, (1980) to suggest that the DNA binding activity may be altered depending upon the protein phosphorylation status. Their investigation did reveal a direct relationship between the degree of SV-40 large T phosphorylation and its ability to bind to DNA cellulose. Shaw et al., (1981) believe that if in fact the large T DNA binding activity is controlled by phosphorylation it must be effected through modification of threonine residues. These authors were

able to show that enzymatic removal of phosphate from all serine residues in the large T molecule did not alter its ability to bind to the SV-40 origin of replication (the natural substrate for large T). They were unable to dephosphorylate threonine residues however and therefore their role in DNA binding is unclear. Since large T is a multi-functional enzyme (Tooze, 1980) it may be that phosphorylation of different domains of the molecule (multisite phosphorylation) may affect its activity in several different functions.

Wilcox et al., (1980) have shown that in HSV 1 and 2 infected cells virally coded polypeptides are phosphorylated in a cyclic fashion. Further some polypeptides (i.e. ICP-4) are rapidly labelled with ^{32}P-orthophosphate while others are phosphorylated only after long periods of labelling. The DNA binding properties of some of these polypeptides is altered by phosphorylation. For example ICP-29 binds more efficiently to DNA when phosphorylated while ICP-6 binds DNA only in the dephosphorylated form.

b) Phosphorylation:dephosphorylation Regulating Virus Enzyme Activity

It has been known for some time that the stability and activity of RNA dependent DNA polymerase is affected by post-translational phosphorylation of the virally coded enzyme (Lee et al., 1975). In more recent work Schiff and Grandgenett (1980), believe that the target of this

specific modification event is pp32 one of the components of the reverse transcriptase holoenzyme. It is believed that pp32 contains the amino acid sequences which code for a superhelical DNA endonuclease activity.

c) Site-specific Phosphorylation Leading to Processing

Yoshinaka and Luftig, (1982) have identified an immature virion associated kinase which specifically phosphorylates the Pr 65^{gag} protein of MuLV. These authors and others (Naso *et al.*, 1979) believe that phosphorylation of Pr 65^{gag} is responsible for triggering the processing events leading to the generation of internal structural proteins (p30, p15, p12 and p19) and subsequent virion maturation.

7. Characteristics of VSV NS Protein

a) Functions of NS in the Infectious Cycle

Genetic studies with the New Jersey serotype of VSV (Evans *et al.*, 1979; Szilagyi *et al.*, 1979) indicate that the phosphoprotein NS is a multifunctional polypeptide. The ts E1 NS mutant of New Jersey is defective for *in vitro* RNA synthesis at the non permissive temperature (39°C) confirming earlier studies that NS is an integral component of the transcriptional complex (Emerson and Yu, 1975). A second NS mutant ts E3 is capable of synthesizing mRNA *in vitro* at 39°C however *in vivo* studies have shown that replication of virion RNA is defective in these mutants implicating NS in the RNA replicative process (Pringle *et al.*, 1971).

The third member of the complementation group, ts E2, is capable of synthesizing both mRNA and genome length RNA species at 39°C, raising the possibility that NS is involved in some other late viral maturation process. In this respect it is notable that although the most extensively studied function of NS is as a component of the polymerase, approximately 75% of all NS produced in the infected cell is never associated with cytoplasmic templates or virions (Hsu et al., 1979; Kang and Prevec, 1971).

The exact role of NS in replication or transcription is unclear. As mentioned earlier NS may facilitate the binding of the transcriptase to viral genomes through sequence specific contacts in the leader gene (Mellon and Emerson, 1978; Keene et al., 1981). Thus NS may be involved in the initiation step of RNA synthesis. Monospecific antibody directed against NS immediately switches off RNA synthesis *in vitro* which suggests that NS is also somehow involved in RNA chain elongation (Imblum and Wagner, 1975). Other events which are tightly linked to the NS-L polymerase include capping, methylation and polyadenylation of mRNAs.

Recently (Rubio et al., 1980) have suggested that the VSV polymerase (NS-L) is not merely a RNA synthesizing complex but in fact is a "ribonucleoprotein synthesizing enzyme". Their kinetic studies demonstrated that an active pool of NS, L and N proteins present in infected

cells allowed RNP replication to proceed for 20 minutes in the absence of protein synthesis. They argue that all three proteins may be necessary for encapsidation of nascent genome length RNA or that co-assembly of N with L and NS may confer some secondary structure on the RNA, possibly leading to packaging. In support of this latter hypothesis Mellon and Emerson, (1978) have quantitated the numbers of NS and L molecules packaged into virus and have shown this to be well over 100 of each polypeptide per virion. Since there are probably very few sites of initiation for transcription, these excess enzyme complexes if evenly spaced along the genome could function as scaffolding for the coiled RNP. Furthermore, Witt and Summers, (1980) have demonstrated that phosphorylation of virion bound NS *in vitro* resulted in release from RNP templates of NS, L and N proteins presumably leading to RNP conformational alterations prerequisite for transcription.

b) Regulation of NS Activity by Phosphorylation: dephosphorylation

Taken together the above findings suggest that NS is a multi-functional protein involved in several facets of the viral replicative cycle. Since NS is also a phosphoprotein many research groups have investigated the relationship between this post-translational modification and NS protein activity. As stated earlier Watanabe *et al.*, (1974) have demonstrated a strict kinase activity requirement for

activation in vitro of VSV polymerase. Witt and Summers, (1980) have shown that continual kinase activity is required for RNA chain elongation. In the latter study, NS was identified as one of the targets of this kinase activity.

If multisite phosphorylation is in fact a regulatory mechanism in the VSV system then it should be possible to isolate species of NS which differ both in phosphorylation status and functional activity. Kingsford and Emerson, (1980) identified multiple phosphorylated forms of NS from infected cells and virions. Soluble cytoplasmic NS was the least phosphorylated species and by itself was inactive in in vitro transcription assays. Virion NS could be resolved into at least four species by coupling column chromatography with polyacrylamide gel electrophoresis. The most phosphorylated NS forms (NS II) were very active in in vitro transcriptional assays while the poorly phosphorylated species (NS I) exhibited little activity. When NS I or cellular NS molecules were added to a transcriptional system containing NS II the activity of the total system was constitutively enhanced. These findings suggest that functional activity of NS and its phosphorylated state are tightly coupled. Hsu et al., (1982) have recently shown by enzymatic removal of phosphate residues that the rate of in vitro transcriptional activity is decreased when NS protein is specifically dephosphorylated. These

authors also identified two domains in the NS protein where multisite phosphorylation can occur. Presumably one domain regulates as yet unidentified functions of NS while the other seems to be related to high levels of transcriptional activity.

In contrast to the studies mentioned above, the findings of Clinton et al., (1978) infer that the more poorly phosphorylated NS species of infected cells and virions is involved in the transcriptional process. In their hands the heavily phosphorylated species of NS were not bound to RNP while NS proteins with fewer phosphates were tightly associated with templates. These contradictory findings are difficult to compare however, because Clinton et al., (1978) reported only quantitive and not qualitative phosphorylation differences. That is, although NS may be phosphorylated at up to 21 different sites (Hsu et al., 1982) possibly only a few of these residues are functionally relevant.

c) Aberrant Electrophoretic Mobility of NS Protein

The aberrant mobility of NS protein in SDS polyacrylamide gels has been documented in several studies (Obijeski et al., 1974; Mudd and Summers, 1970; Stampfer and Baltimore, 1973; Knipe et al., 1975). One reproducible observation is that while the mRNA for NS appears capable of coding for a protein with a molecular weight of 25,110 Daltons (Gallione et al., 1981) the mature polypeptide

migrates in conventional gel systems with the mobility of a 39-54K Dalton protein (Knipe et al., 1975; Brown, 1981).

Gallione et al., (1981) suggest that NS must either run in SDS-PAGE gels as a dimer or the monomer has some unusual SDS binding characteristics. In their analysis of the nucleotide sequence of NS mRNA these authors predict that the nascent protein should contain a large tryptic fragment (7.5K Daltons) which contains 18 negatively charged and no positively charged residues. They argue that this large negatively charged domain may not bind SDS efficiently and therefore NS will migrate with an aberrantly slow mobility.

Evans et al., (1979) identified three NS mutants of VSV which migrated in SDS gels with an aberrant electrophoretic mobility compared to wild type NS protein. Each of these mutants upon reversion to wild type growth characteristics possessed NS polypeptides which comigrated with wild type NS. Since gross differences in phosphorylation were probably not responsible for the mobility differences between mutant and wild type polypeptides these authors suggest that secondary structure of NS may affect its migration in SDS gels. Furthermore, they propose that intramolecular phosphodiester bridges are essential for maintaining NS conformation and that increases in mutant polypeptide electrophoretic mobility represent point

mutations which disrupt these intramolecular linkages.

Working with ts El mutants Maack *et al.*, (1980) conclude that alterations in NS mobility following reversion represent synthesis of a significantly larger NS protein. This conclusion was based on their finding an extra ^{3}H-labelled tryptic peptide present in revertants but absent in mutants.

Brown (1981) has shown that the mobility of New Jersey Concan NS protein relative to other viral proteins can be manipulated simply by changing the percentage of crosslinker (i.e. N'N' methylene bisacrylamide) in the resolving gel. The reason for this unique NS electrophoretic behaviour is unclear.

8. Area of Investigation

The work reported in this thesis is presented in three sections. The first section deals with the identification and partial characterization of multiple NS species found in cells infected with the Piry serotype of vesicular stomatitis virus. Observations by Takayesu and Prevec (unpublished results) suggested that at least two forms of Piry NS can be resolved in SDS polyacrylamide gels. By analogy with the work by Kingsford *et al.*, (1980) with the Indiana serotype it was hoped that characterization of different NS forms would shed light on function and regulation of NS in the infected cell.

The second section contains experiments designed

to probe the primary structure of NS protein in the infected cell. In particular, emphasis was placed on mapping the sites of phosphorylation within the NS polypeptide.

Presented in the final section are experiments directed toward identification of the kinase responsible for NS phosphorylation in the infected cell. The approach to this problem involved the use of monospecific antibodies and therefore their production and specificity are also documented.

MATERIALS AND METHODS

MATERIALS

1. Biochemicals and Reagents

Protein A sepharose CL-4B was a product of Pharmacia, Sweden. Citrate synthase, oxaloacetate and α-casein were purchased from Sigma Chemical Co., St. Louis, Missouri. Cell culture media and solutions were products of Grand Island Biochemical Co. Freund's complete adjeuvant was from Difco Chemical. Rabbit reticulocyte translation extract was from New England Nuclear. TPCK trypsin (247 U/mgm) and snake venom phosphodiesterase were products of Worthington Biochemical Corp. Snake venomphosphodiesterase was also obtained from P-L Biochemical and Sigma Chemical Co. Theophylline was a gift from Dr. Haslam while Dr. P. Branton kindly provided cordycepin. N,N,N',N'-tetramethylethylenediamine (TEMED), N,N'-methylenebisacrylamide and ammonium peroxydisulfate were purchased from Eastman Organic; acrylamide from Biorad Laboratories; ampholytes from LKB; MN-400 thin layer cellulose plates from Brinkman Instruments Inc.; ultra pure urea from Schwartz-Mann; ^{35}S-methionine, ^{3}H-acetate, ^{32}P-γ-ATP and ^{32}P-orthophosphoric acid from New England Nuclear and Amersham. N-hydroxysuccinimide, N-chlorosuccinimide and amino acids were purchased from Sigma Chemical Co. Wheat embryo tRNA was a gift of Dr. S. T. Bayley.

NN'-dicyclohexylcarbodiimide was obtained from Aldrich Chemical Co. Amberlyte 1R 120 sulfonated polystyrene beads were a product of BDH Chemicals. Phenol, pyridine and HCl were all redistilled at 182°C, 115.5°C and 108°C respectively.

2. Solutions

2.1 Phosphate Buffer Saline (PBS)

NaCl	8 gm
KCl	2 gm
$NaHPO_4$	1.15 gm
KH_2PO_4	2 gm

made up to one litre with double distilled water pH 7.5.

2.2 Salt Tris Magnesium Buffer (STM)

NaCl	0.01M
$MgCl_2$	0.001M
Tris Base	0.005M

adjusted to pH 7.5 with concentrated HCl.

2.3 Solutions for SDS-PAGE

2.3.1 Stock Acrylamide Solution

Acrylamide	30 gm
N,N Methylenebisacrylamide	0.4 gm or 0.8 gm

made up to 100 ml with double distilled water.

2.3.2 Running Gel Buffer (pH 8.9) (8X)

Tris Base	36.6 gm
1N HCl	48 ml

made up to 100 ml with double distilled water.

2.3.3 Spacer Gel Buffer (pH 6.7) (8X)

Tris Base	5.98 gm
1N HCl	48 ml

made up to 100 ml with double distilled water.

2.3.4 Tank Buffer

Tris Base	6.32 gm
Glycine	3.99 gm
SDS	1.0 gm

made up to one litre with double distilled water pH 8.9.

2.3.5 Sample Buffer

SDS (20%)	2.5 ml
Mercaptoethanol	0.5 ml
Glycerol	1.1 ml
Urea	7.2 gm
Spacer Gel Buffer (2.3.3)	1.0 ml
2% Bromophenol Blue	0.1 ml

made up to 10 ml with double distilled water.

2.3.6 Gel Fixing Solution

H_2O	100 ml
Methanol	100 ml
Glacial Acetic Acid	14 ml

2.3.7 Resolving Gel Solution

Different amounts of solution 2.3.1 were added to produce gels of different concentrations.

Solution 2.3.1

for a 10% acrylamide solution	33.3 ml

for a 15% acrylamide solution	50.0 ml
Solution 2.3.2	12.5 ml
10% (w/v) SDS	1 ml
10% (w/v) ammonium persulfate	0.3 ml
TEMED	0.05 ml

water was added to give a total volume of 100 ml.

2.3.8 Stacking Gel Solution

Solution 2.3.1	1.25 ml
Solution 2.3.3	1.25 ml
10% SDS	0.1 ml
10% ammonium persulfate	0.1 ml
TEMED	0.02 ml

water was added to give a total volume of 10 ml.

2.4 Solutions for Isoelectric Focussing

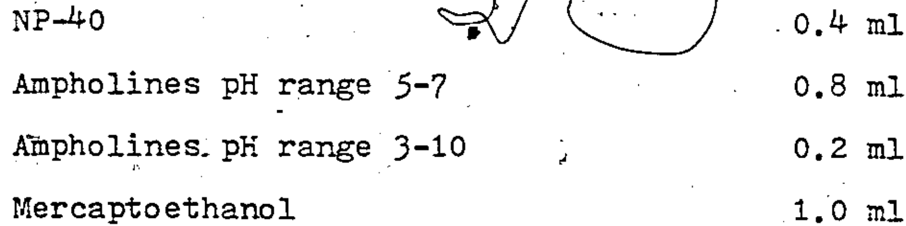
2.4.1 Acrylamide Gel Solution for Isoelectric Focussing

Acrylamide	3 gm
N,N Methylenebisacrylamide	0.162 gm
Ampholines pH range 5-7	0.8 ml
Ampholines pH range 3-10	0.2 ml
Urea	11 gm
NP-40	0.4 ml
10% (w/v) ammonium persulfate	0.2 ml
TEMED	0.05 ml

water was added to give a total of 20 ml.

2.4.2 Lysis Buffer

Urea	11.4 gm



NP-40	0.4 ml
Ampholines pH range 5-7	0.8 ml
Ampholines pH range 3-10	0.2 ml
Mercaptoethanol	1.0 ml

made up to 20 ml with double distilled water.

2.4.3 Sample Overlay Solution

Urea	10.8 gm
Ampholines pH range 5-7	0.4 ml
Ampholines pH range 3-10	0.1 ml

made up to 20 ml with double distilled water.

2.5 Cell Free Translation Solutions (Ghosh et al., 1977)

2.5.1 Washing Buffer pH 7.6

NaCl	8.52 gm
Tris HCl	4.24 gm

made up to one litre with double distilled water autoclaved and then stored at 5°C.

2.5.2 Extraction Buffer

Hepes K	4.76 gm
KCl	8.94 gm
Mg Acetate	1.07 gm

made up to one litre with double distilled water, adjusted to pH 7.6 with KOH and then autoclaved. After cooling, the solution was made 0.06 M in mercaptoethanol and then stored at 5°C.

2.5.3 A-Component of Premix

1 M Hepes (pH 7.0)	0.4 ml

2 M KCl	0.6 ml

2.5.4 B-Component of Premix

0.1 M ATP	0.2 ml
25 mM GTP	0.05 ml
200 mgm/ml creatine phosphate	0.1 ml
60 mM spermine (pH 7)	0.013 ml
Wheat germ tRNA (100 od/ml)	0.02 ml
H_2O	0.02 ml

2.5.5 Premix N

Solution 2.5.3	50 µl
Solution 2.5.4	40 µl
1 M DTT	5 µl
Creatine kinase	1 µl
ddH_2O	4 µl

2.5.6 Cell Free Translation Mix

Solution 2.5.5	5 µl
20 µM of 19 amino acids without methionine	5 µl
(^{35}S) Met 1 µCi/µl	10 µl
Infected L cell cytoplasm	15 µl
20 mM magnesium acetate	5 µl
ddH_2O	10 µl

2.6 Solutions for Tryptic Mapping

2.6.1 50 mM Ammonium Bicarbonate (pH 8.5)

30% ammonia	6.58 ml

add water to one litre and bubble CO_2 through the solution until the pH reaches 8.5.

2.6.2 Performic Acid

Formic Acid	1.9 ml
30% hydrogen peroxide	0.1 ml

incubate at room temperature for 1 hour.

2.6.3 Pyridine Acetate Buffer (pH 3.5)

Pyridine	1 ml
Glacial acetic acid	10 ml
ddH_2O	100 ml

2.6.4 Tryptic Peptide Chromatography Buffer

n-Butanol	204 ml
Pyridine	143 ml
Acetic acid	50 ml
ddH_2O	143 ml

2.7 Formate-Acetate Buffer (pH 1.9)

Formic acid	2.5 ml
Acetic acid	7.5 ml
ddH_2O	90 ml

2.8 Radioimmunoprecipitation (RIPA) Buffer

Triton X-100	1 ml
3 M NaCl	10 ml
1% SDS (w/v)	1 ml
10% DOC	10 ml
1 M tris HCl (pH 8.0)	10 ml

add water to 100 ml.

2.9 Scintillation Fluids

2.9.1 Triton-Xylene Based Fluors

Xylene	750 ml
PPO	4.94 gm
POPOP	0.063 gm
Triton X114	250 ml

2.9.2 Toluene Based Fluors

PPO	4 gm
POPOP	0.3 gm
Toluene	1000 ml

2.10 Protein Elution Buffer

2-mercaptoethanol	0.1 ml
10% SDS	0.1 ml
1 M tris-HCl (pH 8.9)	5 ml

add water up to 100 ml.

2.11 Urea Elution Buffer

2-mercaptoethanol	1 ml
Urea	24 gm
Glycerol	10 ml
10% SDS	1 ml
1 M tris HCl (pH 8.9)	1 ml

2.12 Phosphodipeptide Chromatography Buffer

Isopropyl alcohol	40 ml
Formic acid	2 ml
0.01 M Na_3PO_4	10 ml

2.13 Protein Kinase Buffer

0.1 M tris HCl (pH 8.0)	1 ml
0.1 M $MgCl_2$	1 ml
add water up to 10 ml.	

2.14 High Salt Solubilizer (Emerson and Yu, 1975)

Triton X-100	1.87 ml
1.44 M NaCl	50 ml
1 M DTT	.06 ml
add water up to 100 ml.	

2.15 Synthesis of N-formyl-(^{35}S) Methionyl-tRNA (Brown, 1981)

2.15.1 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.	

2.15.2 Amino Acylation Reaction Mix

Solution 1.15.1	120 µl
Wheat embryo tRNA 10 mgm/ 1	60 µl
(^{35}S) Met 1200-1300 Ci/mmole	600 µCi
E.coli amino acyl synthetase 100 /ml	12 µl
add water to give a 600 1 total volume.	

2.15.3 Triethylamine Acetate Buffer (pH 8.0)

1 M $MgCl_2$	2.0 ml
Triethylamine	2.8 ml

ddH_2O	150 ml

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

2.15.4 Preparation of N-Hydroxysuccinimide Formyl Ester

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

add (a) and (b) to a conical tube then add (c) with stirring. Seal with a stopper and incubate for 3 hours at room temperature. Centrifuge 2K rpm for 10 minutes to remove dicyclohexylurea crystals.

3. Source of Cells

The mouse L cells used throughout these studies were a subline, L 60, of Earle's mouse L cells. Human KB cells were obtained from the lab of Dr. S. Mak while BHK-21 cells were provided by Dr. F. Graham.

4. Source of Viruses

The Indiana standard strain was obtained from Dr. T. Nakai while the Piry and Cocal serotypes were provided by Dr. J. Obijeski, Atlanta, Georgia.

METHODS

1. Growth, Labelling and Fractionation of Cells and Viruses

1.1 L Cell Growth

Mouse L Cells were grown in suspension culture in Joklik's modified minimum essential medium (MEM) supplemented with 5% new born calf serum (NBCS). Cells were kept in suspension by a rotating teflon bar driven by an external magnet. The cultures were kept in the exponential phase of growth by daily dilutions maintained in a 37°C warm room.

1.2 Infection and Labelling of Suspension Cultures

Some 1.5 X 10^8 growing L cells were pelleted from suspension culture by centrifugation at 800 rpm for 10 minutes and then resuspended in 4 ml of virus diluted to yield an moi of 30 PFU/cell. The infected culture was incubated at 37°C for ½ hour to allow virus adsorption. The preparation was then diluted with 17 ml of MEM (supplemented with 5% NBCS) and maintained in suspension culture at 37°C for 3½ hours. By this time cellular protein synthesis has been almost completely inhibited by the viral infection. At 3.5 hours cells were again pelleted by centrifugation (10 minutes at 800 rpm) and then suspended in growth medium containing 1/20 the normal amino acid complement and 2% NBCS. ^{35}S-methionine, at a final concentration of 10 μCi/ml, was added to the cultures

for 0.5 to 1.0 hours, four hours post infection. In all but pulse chase experiments the cells were harvested after the labelling period.

1.3 Isotopic Labelling of Virus

L cells grown in suspension were infected at an moi of 20 PFU/cell. One hour post infection the cells were concentrated by centrifugation at 500 rpm for 10 minutes and then resuspended at 10^6 cells/ml in MEM lacking either methionine or phosphate but supplemented with 2% NBCS. Either ^{35}S-methionine at 1 μCi/ml or ^{32}P-orthophosphoric acid at 20 μCi/ml was added at this point and then incubated an additional 18 hours. Virus was then purified as described in section 1.6.

1.4 ^{32}P-labelling of Infected Cells

Monolayers of 5 X 10^6 L cells infected with virus at an moi of 50 PFU per cell were incubated, 3 hours post infection, in MEM lacking phosphate ($-PO_4$ MEM) for ½ hour. The medium was then removed from the cells and the monolayers washed once with $-PO_4$ MEM and then incubated in $-PO_4$ MEM plus ^{32}P-orthophosphate at a final concentration of 200 μCi/ml. The cells were harvested 90 minutes later.

1.5 Pulse-chase Experiments

Virus was adsorbed to monolayers of 5 X 10^6 L cells for ½ hour at an moi of 50 PFU/cell (37°C). After infection cells were incubated 3 hours in MEM (supplemented with 5%

NBCS) and then an additional ½ hour in MEM growth medium containing 1/20 the normal amino acid complement and 2% NBCS. ^{35}S-methionine was added to the culture for a pulse period of 5 to 15 minutes at a final concentration of 250 µCi/ml in 1/20 amino acid medium plus 2% NBCS. After the pulse the radioactive medium was removed and monolayers were either rapidly frozen in a methanol/dry ice mixture or washed twice with PBS, once with MEM and then chased with MEM plus 5% NBCS for varying lengths of time. In some experiments during the chase period cycloheximide was added to a final concentration of 50 µgm/ml.

1.6 Virus Purification

Cultures of L cells were infected with virus at an moi of 0.1 PFU/cell. Twenty-four hours post infection the infected cell culture medium was clarified by centrifugation at 8K rpm for 20 minutes in a Sorvall GSA rotor. Virus pellets were obtained by centrifugation of the medium at 18K rpm for 180 minutes in a Beckman T 19 rotor. PBS was used to resuspend the viral pellet which was then layered on top of a linear 5-40% sucrose gradient. Centrifugation at 24K rpm for 35 minutes in a SW27 rotor generated an opalescent band approximately half way down the gradient. The virus in this region was removed by side puncture with a needle and syringe and then concentrated by centrifugation at 39K rpm for 90 minutes through a 20% glycerol pad.

Following resuspension of the pellet with PBS the entire purification cycle was repeated.

1.7 High Salt Solubiliser Fractionation of Virus

^{35}S-methionine labelled virus, twice banded on linear 5-40% sucrose gradients (24K rpm for 25 minutes; SW-50.1 rotor), was pelleted through a 40% glycerol pad (40K rpm for 120 minutes; SW-50.1 rotor). The pellet was resuspended in high salt solubiliser (solution 2.14) and centrifuged at 40K for 120 minutes. The material which remained on top of the pad was designated the HSS supernatant and material which pelleted through the glycerol as the HSS RNP fraction.

1.8 Preparation of RNP from Piry Infected Cells

L cells infected at an moi of 25 PFU/cell with Piry virus were labelled four hours post infection with ^{35}S-methionine and then disrupted in STM (solution 2.2) by homogenization with a Dounce type homogenizer. Nuclei and whole cells were removed from the homogenate by centrifugation at 800 rpm for five minutes. The supernatant was then treated with 1% DOC; 1% NP-40 and 20 μM EDTA and then spun for 90 minutes at 38K rpm in an SW-50L rotor. The pellet containing RNP and supernatant containing soluble proteins were lyophilized and then prepared for electrophoresis.

1.9 Fractionation of Infected Cells

Infected cells labelled with ^{35}S-methionine were pelleted in conical glass tubes at 1000 rpm for 15 minutes in a Damon preparative centrifuge. The supernatant was retained for total counts and analysis on SDS polyacrylamide gels while the cellular pellet was resuspended with 2 mls of STM buffer and the cells disrupted by subjecting them to 25 strokes of a Dounce homogenizer (the efficiency of homogenization was monitored by examining the cells using a phase contrast microscope). Intact nuclei and the small number of remaining whole cells were pelleted in conical glass tubes by centrifugation for 5 minutes at 800 rpm. The nuclear pellet was tested for total TCA precipitable counts and the supernatant retained for further fractionation.

The supernatant fraction was layered over a linear 7-47% sucrose gradient in STM buffer and spun at 13,000 rpm for 16 hours using an SW27.1 rotor and Beckman ultracentrifuge. Gradients were collected in 50 drop fractions and appropriate aliquots TCA precipitated for scintillation counting or analysis by SDS-PAGE.

2. Gel Electrophoresis

2.1 Sample Preparation for Electrophoresis

Fifty microlitres of sample was added to 50 μl of sample buffer and the solution disrupted for 60 seconds

with a Bronwill Scientific (Biosonik III) Sonicator. The resultant homogenate was then heated at 100°C for one minute then drawn up and down a fine syringe to shear the viscous DNA. Samples were then stored at -60°C until time of use.

2.2 Discontinuous SDS Polyacrylamide Gel Electrophoresis (Laemmli, 1970)

Slab gels (2 mm thick) were preformed between (19 X 16 cm) glass plates using 30 ml of acrylamide gel solution (2.3.7). In some cases double gels were preformed using three glass plates, one notched plate sandwiched between a notched and an unnotched plate. A spacer gel was polymerized from spacer gel solution (2.3.8) over the running gel just prior to use. Gels were electrophoresed at 200 volts until the BPB marker reached the bottom of the gel then processed appropriately. That is, analytical gels were fixed and when indicated impregnated with a PPO-POPOP mixture as described in section 2.7. Gels were subsequently dried under vacuum in steam heat and then subjected to autoradiography. Preparative gels were generally exposed to X-ray film (Kodak XAR-5 or XRP-1 while still wet and unfixed.

2.3 Sample Preparation for IEF or NEPHGE

Radioactive protein extracts were prepared in ddH_2O and then lyophilized to dryness. Lysis buffer (2.4.2) was

used to solubilize the sample. If necessary the dissolution of the sample was aided by agitation, heating above 37°C was avoided.

2.4 Isoelectric Focussing of Proteins (O'Farrell, 1975)

Using isoelectric focussing solution (2.4.1) polyacrylamide slab gels were cast between glass plates with a sample well spacer in position. Following polymerization the sample spacer was removed and wells were filled with lysis buffer (2.4.2). After 2 hours of incubation fresh lysis buffer was added before generating the pH gradient by electrophoresis. The upper reservoir of the gel apparatus was made 20 mM NaOH by dropwise addition of 100x stock to degassed double distilled water. The anode tank was filled with 10 mM phosphoric acid just prior to preequilibration using an electric field. The sequence of equilibration was begun with 200V for 15 minutes followed by 300V for 30 minutes and concluded by electrophoresis at 400V for 30 minutes. The wells were then emptied, refilled with solubilized samples and then overlayed with sample overlay buffer (2.4.3). Electrophoresis was conducted at 400V for 10,000 volt hours and then terminated after a final hour at 800V. The gel was then either processed for autoradiography or analysis in a second dimension by SDS-PAGE.

2.5 Non Equilibrium pH Gradient Electrophoresis (NEPHGE)

Isoelectric slab gels were cast between glass plates using solution (2.4.1). The lower, cathode chamber of the electrophoresis apparatus was filled with 20 mM NaOH and 400 ml of ddH_2O was added to the upper (anode) chamber. Samples were dissolved in solution and applied to the top of the gel and then overlayed with solution (2.4.3). Four ml of 2N phosphoric acid was added to the upper tank and then electrophoresis was carried out for 4 hours at 500 volts. The pH gradient in the gel was determined by soaking 0.5 cm fractions in 1.5 ml of ddH_2O overnight and then measuring the pH of each fraction using Fisher Accumet pH meter.

Individual channels were sliced from the isoelectric slab and run in the second dimension on SDS-PAGE.

2.6 Fixing and Staining of Gels

Gels were incubated in gel fixation solution (2.3.6) with shaking for one hour and then stained using fixative supplemented with 0.1% coomassie brilliant blue for 2-3 hours. Destaining was accomplished by shaking the gel in several changes of gel fixation solution (2.3.6) until the background of the gel was stain free.

3. Peptide Mapping of Viral Proteins

3.1 Tryptic Peptide Mapping

Appropriate protein bands located by autoradiography were excised and eluted from the gel in solution (2.10)

with BSA as carrier (final protein concentration 100 μgm/ml) concentrated by TCA precipitation (20% TCA) at 12,000 x g for 20 minutes. TCA was removed from the pellet by repeated acetone washes and the resultant protein was oxidized for 3 hours at 0°C in performic acid (2.6.2). After three cycles of water washing and lyophilization the protein was digested by adding TPCK trypsin (1 μgm/1 μl in 0.05 M NH_4HCO_3, pH 8.5) for 1 hour at 37°C. A second portion of trypsin was added at this time and the reaction continued for three more hours at 37°C. The peptides were lyophilized and dissolved in ddH_2O twice before 2D analysis on cellulose thin layers. Essentially the tryptic digests were spotted onto MN 300 thin layers by repeated application of small amounts of sample and blow drying. The first dimensional chromatographic analysis was carried until the buffer (solution 2.6.4) had ascended to the top of the thin layer. The thin layer sheet was removed and air dried overnight before the second dimension, thin layer electrophoresis, was performed. The entire thin layer was wetted with pyridine acetate buffer (solution 2.6.3) and placed on the cooling block of a Brinkman thin layer apparatus and connected to tanks by paper wicks. Electrophoresis was conducted for 1.5 hours at 400 volts.

3.2 Oxidation of Protein

Samples were dissolved in 200 μl of fresh performic acid (solution 2.6.2) and incubated at 0°C for 3 hours. The

oxidized proteins were diluted with ddH_2O and then lyophilized to dryness.

3.3 Partial Cleavage at Tryptophan Residues with N-chlorosuccinimide

Proteins were purified by SDS-PAGE and passively eluted in buffer (2.10) for 12 hours at 37°C with agitation. The eluate was lyophilized to dryness and then resuspended in 1 ml of 0.5 M acetic acid and 0.25 ml of 75 mM N-chlorosuccinimide in dimethyl formamide. Partial cleavage was effected by incubation with shaking at room temperature for 2 hours. BSA was added at this time as a carrier and the cleavage products were precipitated with ice cold acetone at -20°C overnight. Centrifugation at 10K rpm for 20 minutes resulted in formation of a precipitate which was subsequently dissolved in sample buffer (solution 2.3.5) and analysed on a 15% acrylamide gel containing 0.26% crosslinker.

4. In Vitro Protein Synthesis

4.1 Preparation of Infected Cell Cytoplasmic Extracts

All glassware used in this procedure was soaked in sulphuric acid: nitric acid (3:1) washed in ddH_2O and then baked at 110°C overnight. Plastic ware was washed with 10% SDS and then rinsed with sterilized ddH_2O.

An S-4 polysomal extract was prepared as described by Toneguzzo and Ghosh (1976). 1.5 litres of L cells in

the exponential phase of growth were infected at a multiplicity of infection of 25 PFU/cell. Four and one half hours post infection 0.5 litres of frozen PBS was immersed in the cell suspension with the whole solution in an ice water bath. The infected cells were then pelleted by 5 minutes of centrifugation at 1000 rpm and subsequently resuspended with washing buffer (2.5.1). This procedure was repeated three times using a decreasing resuspension volume for each cycle. The final pellet was then resuspended with 2 volumes of extraction buffer and then subjected to twenty slow strokes with a Dounce homogenizer kept constantly at ice bath temperature. The extract was now subjected to centrifugation at 4000 x g for 10 minutes and the resultant supernatant dialysed for [illegible]5 hours against extraction buffer (2.5.2) stored in an ice bath. Aliquots of the extract were then rapidly frozen with liquid nitrogen and stored at -60°C.

4.2 Cell Free Translation Using L Cell Extracts

Cell free translation with L cell extracts was performed at 32°C for varying lengths of time. Essentially the infected L cell extract was mixed with other components of the cell free system in the ratios presented under solution 2.5.6. The volume of water added to the system was variable depending upon whether solutions of oxaloacetate, citrate synthase or citrate were included in the mix.

4.3 Synthesis of N-formyl-^{35}S-Met-$tRNA_1^{Met}$

Amino acylation of wheat embryo initiator $tRNA_1^{Met}$ was accomplished by incubation of the mixture listed under solution (2.15.2) for 15 minutes at 37°C. Four volumes of 2% potassium acetate (pH 5.4) were added to the mixture at this point followed by hot phenol extraction. Essentially the solution was mixed with equal volumes of water saturated phenol and chloroform containing 4% isoamyl alcohol. Following incubation for 2 minutes at 50°C with shaking, the phases were separated by centrifugation at 2.5K rpm for 10 minutes. The aqueous phase was removed and the phenol was reextracted with an additional volume of 2% potassium acetate (pH 5.4). Following pooling of the aqueous phase and extraction with ether the RNA was precipitated by the addition of 2.5 volumes of cold ethanol, overnight incubation at -20°C and centrifugation at 8K rpm for 20 minutes.

The formylating reagent was prepared as described under Solutions (2.15.4). The ^{35}S-Met-$tRNA_1^{Met}$ ethanol precipitate was dissolved in 1 ml of 0.1 M triethylamine acetate (pH 8.0), 0.5 ml of the formylating reagent and a predetermined volume of 0.2 M KOH to bring the pH up to 8.0. Following ten minutes of incubation at 0°C, 0.2 ml of potassium acetate (pH 5.4) was added and the formylated product precipitated with ethanol.

4.4 Amino Terminal Labelling *in vitro*

To label at the amino terminus with N-formyl-^{35}S-methionine a commercially prepared reticulocyte extract was used. The reaction mixture was prepared with: 40 μl of reticulocyte lysate; 8 μl of potassium acetate; 8 μl of cocktail mixture; 44 μl of a mixture of N-formyl-^{35}S-methionine; 10^3 fold excess of cold methionine; infected L cell extract. The ratio of components and the latter extract was optimized for each experiment.

5. Phosphodipeptide Analysis

5.1 Partial Acid Hydrolysis of Phosphoproteins

Samples to be hydrolysed were suspended in 30% aqueous pyridine and transferred into glass freeze drying ampoules. The samples were reduced to dryness by lyophilization resuspended in 2N HCl sealed under a reduced nitrogen atmosphere and then heated at 110°C for four hours. Following hydrolysis the sample was filtered through a glass fibre filter and concentrated by lyophilization.

5.2 Preparation of Amberlite 1R 120 Resin

The beads were washed with several volumes of 4N ammonium hydroxide by filtration of the resin in a filter paper lined Buchner funnel. This was followed by 2 litres of ddH_2O passed through the filter and then washing with 1 litre of 4N formic acid. Again 2 litres of ddH_2O was

used to wash the beads before final equilibration of the resin with pH 1.9 buffer (solution 2.7).

5.3 Preparation of Dowex-1 Resin

The anion exchange resin was washed with 1 litre of 4N formic acid and then 2 litres of ddH_2O in a filter paper lined Buchner funnel. One litre of 4N ammonium hydroxide was passed through the resin followed by 4 litres of ddH_2O. The resin equilibrated with ddH_2O was then loaded into a 10 ml syringe which had been plugged with a glass fibre filter.

5.4 Purification of Phosphodipeptides from Partial Acid Hydrolysates

Acid hydrolysates reduced to dryness by lyophilization were resuspended in pH 1.9 buffer (2.7) and then mixed with preequilibrated Amberlite 1R 120 beads in a 20 ml beaker. A teflon coated magnetic stirring bar was added to the slurry to effect mixing for 1 hour at room temperature. The contents of the beaker were poured into a 10 ml syringe plugged with a glass fibre filter. The buffer was drained from the beads and then concentrated by lyophilization. Material bound to the beads was eluted with 1 N ammonium hydroxide.

Following lyophilization the pH 1.7 eluate was resuspended in ddH_2O and bound to a preequilibrated Dowex-1 column. Increasing concentrations of formate buffer were

added to the column in twenty ml volumes to effect elution of bound material. Eluates were concentrated by lyophilization.

5.5 High Voltage Paper Electrophoresis

Hydrolysates to be analysed were spotted in the middle of a piece of Whatman 3M paper. The electropherogram was wetted with pH 1.9 buffer (solution 2.7) and subsequently subjected to electrophoresis at 2000 volts for 90 minutes. The electropherogram was dried with a hair dryer and then stained with ninhydrin as described in section 5.6.

5.6 Ninhydrin Staining of Paper and Thin Layer Electropherograms

To stain paper electropherograms a solution of 0.1% (w/v) ninhydrin in acetone was liberally applied to the sheet and allowed to evaporate at room temperature. Development of ninhydrin positive spots was carried out in a 110°C oven for 5-10 minutes. Phosphoamino acids appeared as pink spots while other amino acids and peptides were detected as purple spots.

Thin layer cellulose sheets were stained with 10 mgm of ninhydrin dissolved in a solution made from 7.5 ml of absolute ethanol, 2.5 ml of glacial acetic acid and 0.05 ml pyridine. Development of ninhydrin positive spots was carried out in a 110°C oven for 5-10 minutes.

5.7 Elution of Radioactive Peptides from Paper Electropherograms

(^{32}P)-labelled peptides were detected by autoradiography and the appropriate area cut from the electropherogram. The radioactive peptides were then eluted by incubating the slice of paper in 1 ml of ddH_2O with shaking for 12 hours at room temperature. The eluate was filtered through a glass fibre filter and concentrated by lyophilization.

5.8 Subtractive Edman Degradation

Partial acid hydrolysates were divided into two equal halves and reduced to dryness by lyophilization in 1.5 ml plastic Eppendorf centrifuge tubes. Ninety microlitres of 60% aqueous pyridine were added to each tube. To one tube was added an additional 10 μl of aqueous pyridine while the other received 10 μl of phenylisothiocyanate (PITC). Both tubes were then flushed with N_2 and incubated with rotation at 37°C for 2 hours. Following the coupling reaction volatile reagents were removed under vacuum. Cleavage was effected by incubation of both samples in anhydrous trifluoracetic acid (TFA) under a nitrogen atmosphere at 37°C for 30 minutes. TFA was removed under vacuum and the remaining residue was dissolved in 200 μl of ddH_2O. Three cycles of ethyl acetate extraction (200 1/cycle) were used to remove

PTC-derivatives. The organic phases were pooled and dried under a nitrogen stream. The aqueous phase which contains the shortened peptide was concentrated by lyophilization.

6. Immunoprecipitation and Kinase Assays

6.1 Production of Monospecific Antisera

The sucrose gradient purified virus from 10^{10} L cells, was suspended in sample buffer (solution 2.3.5) and then run on preparative polyacrylamide gels to resolve the five viral proteins. The appropriate bands located by Coomassie brilliant blue staining, were excised from the wet gels and macerated. The viral proteins were then eluted passively in elution buffer (solution 2.10). The eluate was filtered through glass wool, dialysed against double distilled water for twelve hours and then lyophilized to dryness. After resuspension in ddH_2O one third of each antigen sample was mixed with an equal volume of complete Freund's adjeuvant to form an emulsion and injected intramuscularly into a rabbit's thigh. The remaining antigen samples were injected subcutaneously at two week intervals. One week after the final injection, the rabbits were bled and serum collected. All antisera were heat treated at 56°C for thirty minutes.

6.2 Immunoprecipitation of Viral Proteins

Infected L cells were suspended in STM (solution 2.2)

for ten minutes at 0°C and then homogenized in a Dounce ball-type homogenizer. Nuclei and debris were pelleted by sedimentation at 2K rpm for ten minutes and the supernatant was mixed with an equal volume of 2X RIPA buffer (solution 2.8). One ml of the extract was mixed with 10 μl of antiserum, 40 μl of protein A sepharose CL 4B beads and then incubated with rotation for 16 h at 5°C. After adsorption the beads were centrifuged at 1000 rpm for one minute and the supernatant removed. The beads were then washed at least eight times with cold RIPA buffer and then either analysed by SDS-PAGE or used in protein kinase assays. In some experiments STM or HSS lysates were fractionated into supernatant and RNP fractions by ultracentrifugation through an appropriately buffered 40% glycerol pad before immunoprecipitation.

6.3 Protein Kinase Assays

Immunoprecipitates bound to protein A sepharose beads were washed three times with cold protein kinase buffer (solution 2.13). Assays were initiated by the addition to beads of 50 μCi of ^{32}P-γ-ATP in 30 μl of kinase buffer followed by incubation at 31°C for 30 minutes. The beads were then resuspended in ice cold RIPA buffer and washed a further three times to remove excess ^{32}P-γ-ATP. Finally the immunoprecipitates were resuspended in 50 μl of sample buffer and heated for one minute at 100°C

before analysis by SDS-PAGE and autoradiography.

6.4 <u>Preparation of Denatured Radioactive Viral Protein Antigens</u>

^{35}S-methionine labelled, infected L cell extracts were analysed by preparative SDS gel electrophoresis and autoradiography of the wet gel. Using the autoradiograph as a template, the desired viral bands were excised and electroeluted (50 volts, for 4 hours) into dialysis tubing using urea elution buffer (solution 2.11) and 100 μgm of BSA as carrier. The eluate was dialysed against RIPA buffer for twelve hours and then tested with antisera as described.

RESULTS

1. Identification and Characterization of Multiple Forms of Piry NS

1.1 Piry Infected Cells Contain Two NS Species Labelled with ^{35}S-methionine

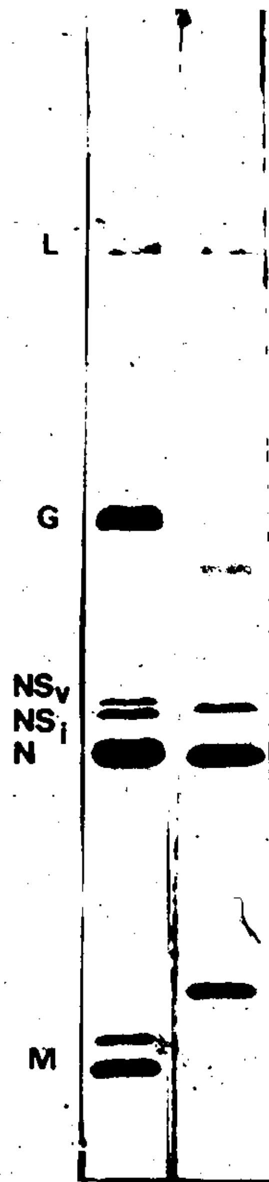
^{35}S-methionine-labelled extracts from Piry virus-infected cells contain two distinct protein bands migrating in the region of NS protein on SDS-PAGE (figure 1). In contrast under identical conditions cells infected with Indiana VSV contain only one NS species migrating in this region. Although both forms of Piry NS appear to be associated with ribonucleoprotein structures (figure 1, INF CELL) only the slower migrating form (NS_v) was detected in the preparation of purified Piry virions, (figure 1, VIRION).

The structural relatedness of the two Piry NS polypeptides was confirmed by fingerprinting of the methionine-labelled tryptic peptides of each band after separation, excision and elution from a preparative SDS-gel. Most of the features of the tryptic fingerprint are common to both proteins (figure 2).

The possible precursor-product relationship of the two proteins was demonstrated by pulse-chase experiments.

Figure 1. Comparison of viral proteins in Piry and Indiana VSV infected cells and Piry virions

Piry virus and Indiana (IND) virus infected cells (as indicated) were labelled with ^{35}S-methionine for 30 min some 4 hours post infection. The infected (INF) cell protein was analysed on SDS-PAGE. The location of the two NS bands in the Piry extract is indicated in the left hand panel. Piry virions purified on sucrose gradients after overnight growth with ^{35}S-methionine label were analysed on SDS-PAGE in the right hand panel, with a control marker of ribonucleoprotein extract from pulse labelled Piry infected cells.


L
G
NSv
NSi
N
M
PIRY
IND

INF
CELL
VIRION

Figure 2. <u>Tryptic fingerprint of methionine labelled proteins NS_i and NS_v</u>

^{35}S-methionine labelled NS_i and NS_v were purified by preparative SDS-PAGE from Piry infected cell extracts labelled for 5 min and 30 min, respectively, some 4 hours post infection. The protein bands were excised from the gel, oxidized, and trypsinized as described in Materials and Methods. Two dimensional analysis by electrophoresis and chromatography was carried out on cellulose thin layer.

electrophoresis
NS
chromatography

Piry virus infected cells were pulsed with ^{35}S-methionine followed by incubation in the presence of excess unlabelled methionine. Samples removed at various times during the chase period were analysed on SDS-PAGE. Chasing after either 5 or 15 minutes of labelling resulted in progressive decrease in radioactivity in the faster band (NS_i) and a corresponding increase in the radioactivity of the NS_v protein (figure 3). A double band in the M protein region was observed in the fifteen minute pulse chase experiment (figure 3) however it was not the subject of further investigation.

The apparent conversion of NS_i to NS_v is independent of continued protein synthesis since the conversion occurs at the same rate in the presence of cycloheximide as in its absence, (figure 4).

From the preceding results I conclude that the protein NS_i is converted by post-translational modification to a protein of greater apparent molecular weight, NS_v.

This precursor-product relationship seems to be a phenomenon restricted to the Piry serotype. Pulse chase experiments performed with either Indiana, Cocal or Piry virus infected cells revealed that at either time only Piry infected cells show the double NS form, (figure 5). This finding suggests either that Piry NS protein is unique in its maturation or that the gel system employed

Figure 3. Pulse-chase of protein NS_i to NS_v

^{35}S-methionine label was added to Piry virus infected L cells at 4 hours post infection. After a labelling period of 5 or 15 min, the radioactive medium was removed and some of the cultures were incubated in excess unlabelled methionine, as described in Materials and Methods. Samples were taken at the indicated times. The infected cells were harvested and analysed on SDS-PAGE. The figure describes the length of the radioactive pulse plus the length of the subsequent chase. The reason for the double-M protein in the 15 min label experiment is not known. An extract of labelled uninfected cells is presented in the central well (CNT).

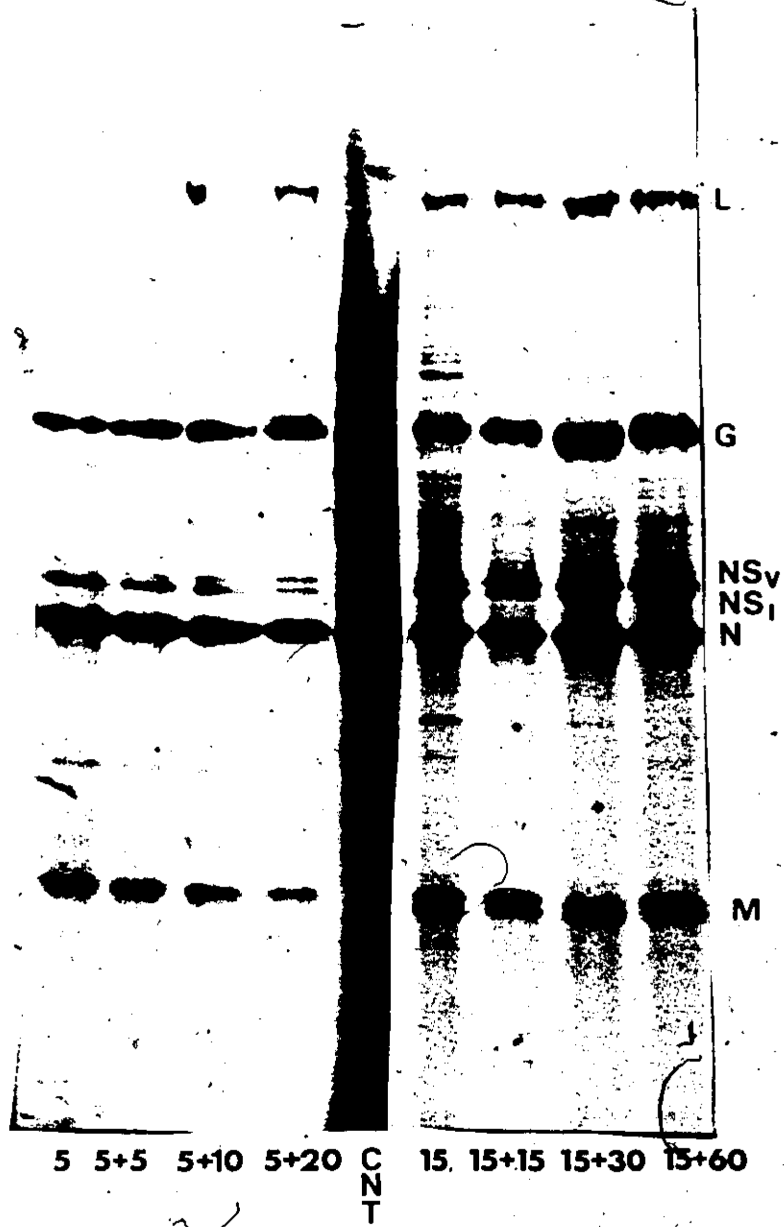

L
G
NSv
NS1
N
M
5
5+5
5+10
5+20
CNT
15.
15+15
15+30
15+60

Figure 4. Conversion of NS_i to NS_v in the presence and absence of cycloheximide

Piry virus infected cells at 4 hours post infection were pulse labelled with ^{35}S-methionine for 5 min and chased with excess unlabelled methionine. The chase medium for one experiment contained 50 μg of cycloheximide per ml. Samples were taken at the indicated times and analysed by SDS-PAGE. The sample well labelled (P) contains an infected cell extract kept in ^{35}S-methionine for the full 20 min period.

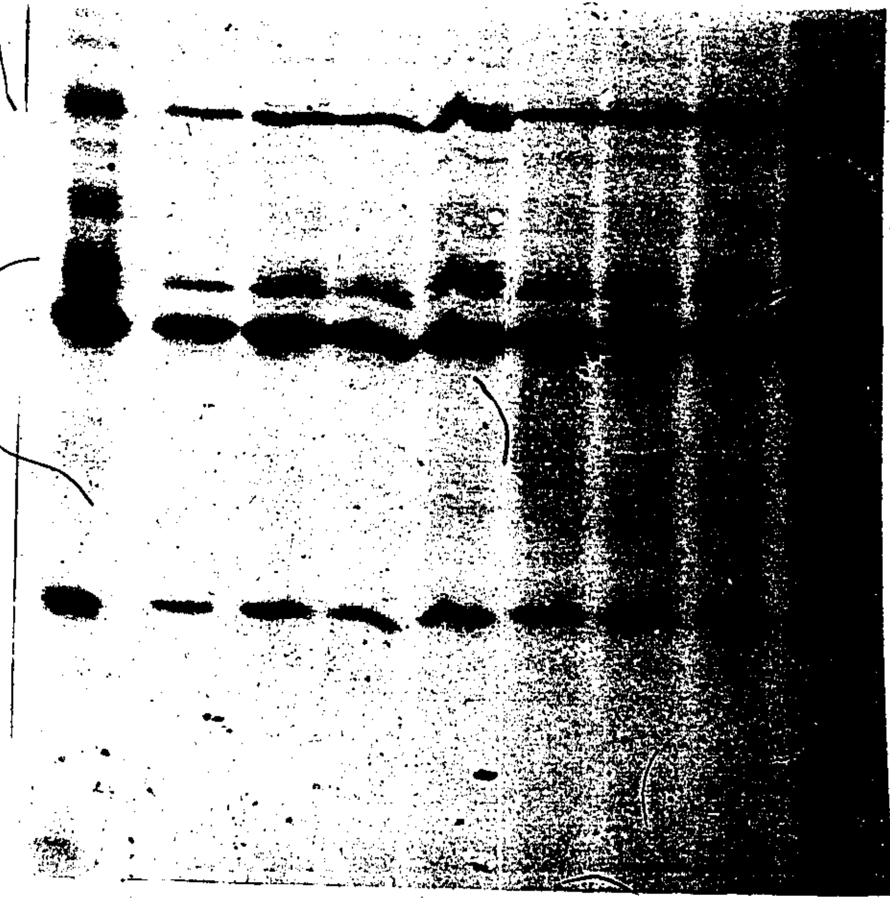

NS
N
P
5
5+5
5+10
5+20
5
5+5
5+10
5+20
CONTROL
CYCLOHEXIMIDE

Figure 5. Pulse-chase analyses of Piry, Indiana and Cocal infected cells

Monolayers of L cells were infected with Piry, Indiana (IND) or Cocal (Coc) virus at a multiplicity of infection of PFU/cell. Monolayers were then labelled with ^{35}S-methionine (200 μCi/ml) for a ten minute pulse, washed with PBS and then incubated with whole minimum essential medium for a thirty minute chase. Samples solubilized with SDS sample buffer were analysed by SDS-PAGE and autoradiography. (10) ten minute pulse; (10+30), ten minute pulse plus a 30 minute chase.

NSv
NSi
N

NS
N

NS
N

10 10+30
PIRY

10 10+30
IND

10 10+30
COCCAL

is incapable of resolving multiple forms of Indiana and Cocal NS protein.

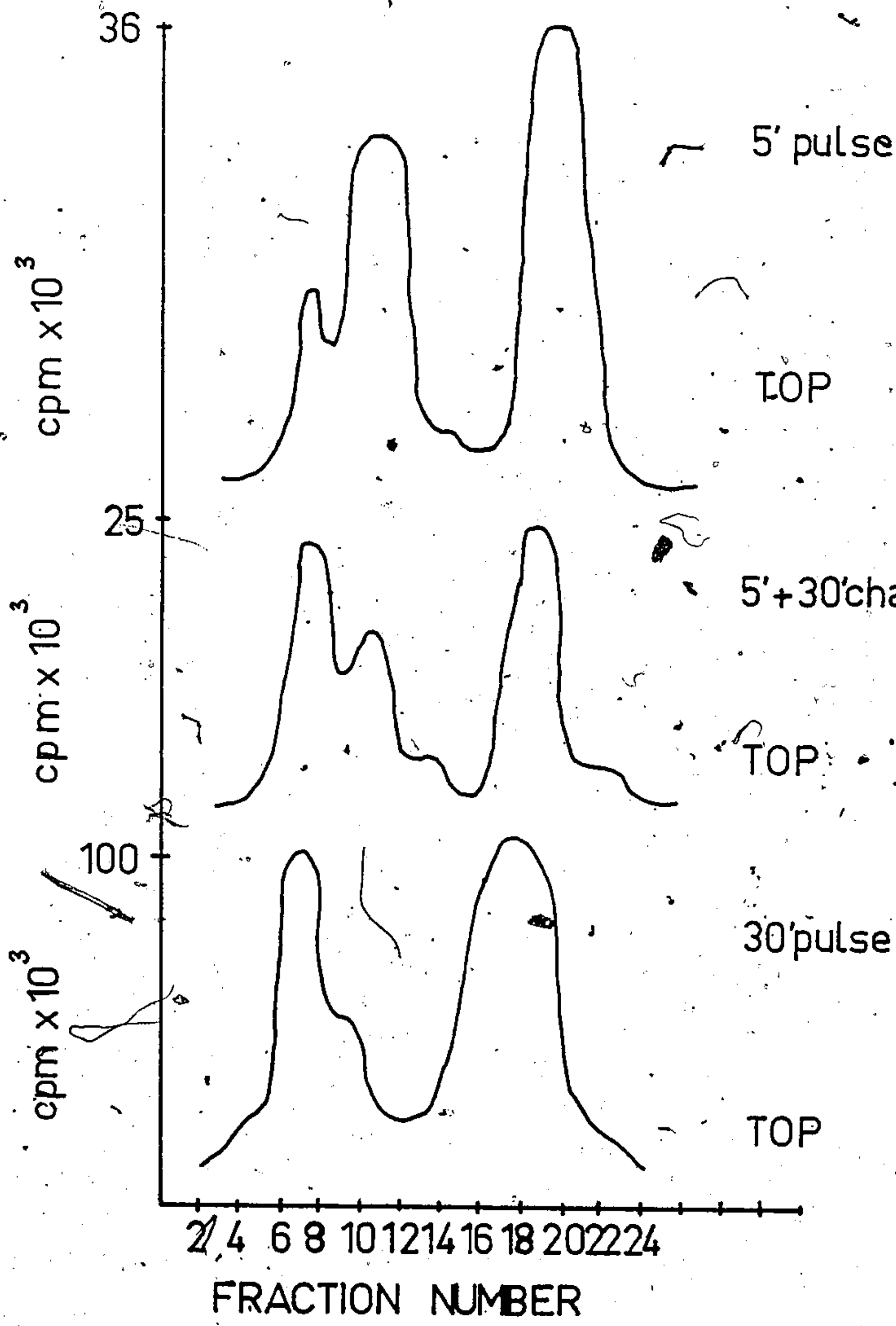
Since there appeared to be a discrimination in the packaging of NS into mature Piry virions, (i.e. NS_v packaged, NS_i excluded; figure 1) I attempted to fractionate Piry infected cells to determine if there was preferential sequestering of the two species within the cell. Cells labelled with ^{35}S-methionine either for 5 min or for 5 min followed by a 20 min chase were disrupted by 20 strokes with a Dounce type homogenizer and then fractionated on a 5-40% sucrose gradient as described in Materials and Methods. A plot of the TCA precipitable counts from the gradient fractions is shown in figure 6. The two large peaks and the pellet were then separately precipitated with 10% TCA and analysed by SDS gel electrophoresis. From figure 7 it is clear that both species of NS are found in all fractions of the cytoplasm including the presumptive ribonucleoprotein pellet. This suggests that the conversion of NS_i to NS_v does not affect the ability of Piry NS to associate with viral or cellular components.

1.2 Phosphorylation of Piry NS Proteins

Sokol and Clark (1973) had demonstrated earlier that NS protein is phosphorylated in rhabdoviruses and since NS_i matured to NS_v by some post-translational mechanism

Figure 6. Sucrose gradient analysis of Piry infected cells

Piry infected L cells were labelled with ^{35}S-methionine for varying time periods four hours post infection. Cells were swollen in STM and disrupted with 25 strokes of a Dounce type homogenizer. Intact nuclei and any remaining whole cells were pelleted by centrifugation for five minutes at 800 rpm. The STM lysate was centrifuged on a 5-40% sucrose gradient (13,000 rpm, 16 hours, SW27.1 rotor) and then fractionated by collection of 50 drop aliquots. Representative samples of each fraction were TCA precipitated on nitrocellulose filters and quantitated by scintillation counting.



36
25
100
cpm x 10^3
cpm x 10^3
cpm x 10^3
5' pulse
TOP
5'+30'chase
TOP
30'pulse
TOP
2 4 6 8 10 12 14 16 18 20 22 24
FRACTION NUMBER

Figure 7. SDS-PAGE analysis of fractionated Piry infected cells

Appropriate fractions collected from the sucrose gradient profile shown in the preceding figure were pooled, TCA precipitated, acetone washed and finally dissolved in SDS sample buffer. Samples were then resolved by SDS-PAGE and autoradiography. (1-13), fractions 1-13 from figure 6; (14-24), fractions 14-24 from figure 6; (PELLET), pellet from the sucrose gradient described in figure 6; (5), five minute pulse; (5+20) five minute pulse plus a twenty minute chase; (30), thirty minute pulse.

5 30 5+20 5 30 5+20 5 30 5+20

1-13 14-24 PELLET

(figure 4), phosphorylation seemed a reasonable explanatior.

To test this possibility, Piry infected L cells were labelled with radioactive inorganic phosphate and analysed on SDS-PAGE. Figure 8 shows the result of this experiment. Clearly there is ^{32}P-label associated with NS_v however it is difficult to determine if NS_i is phosphorylated since the much more heavily labelled NS_v overshadows this region. In my hands, short pulses (5-30 minutes) with inorganic $^{32}PO_4$ were ineffective in labelling viral or cellular proteins probably due to the very large inorganic phosphate pools within the cell. The lack of the detectable label associated with NS_i in these experiments is likely due to the large excess of NS_v and slow utilization of exogenous radioactive phosphate due to large internal pools. In fact, more recent work in Dr. Prevec's laboratory clearly demonstrated inorganic phosphate labelling of NS_i (DeTina, 1980).

A second major phosphorylated protein present in Piry infected cells and absent in uninfected cells (figure 8) migrates just slightly faster than Piry N protein. The idea that this protein is functionally significant to the Piry infectious cycle is reinforced by the finding that it is incorporated into mature virions (figure 9). A comparison of the structural relatedness of this protein to other Piry proteins is presented in figure 10.

Figure 8. Phosphate-labelled proteins in Piry virus-infected cells

Piry virus-infected cell cultures were labelled with ^{32}P-orthophosphate from 3.5 to 5 hours post infection as described in Materials and Methods, (central well). An infected culture labelled over the same time interval with ^{35}S-methionine (left well) and an uninfected cell culture labelled for 90 min with ^{32}P-orthophosphate (right well) are included as controls. The labelled cell extracts were analysed by SDS-PAGE.

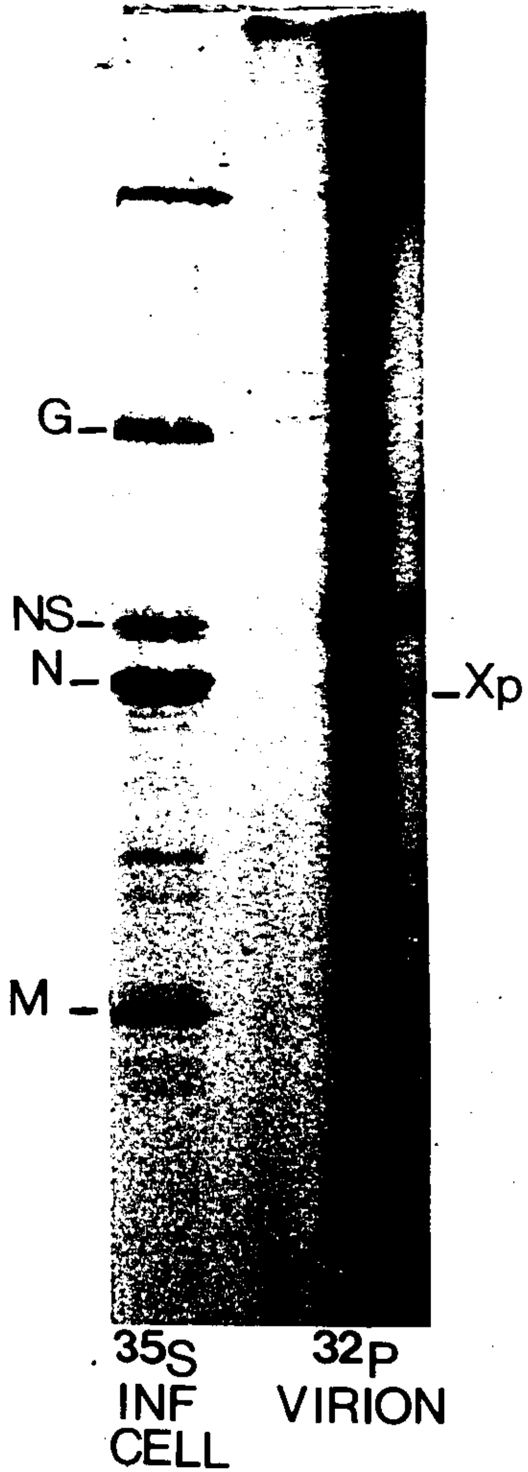
NS$_v$
NS$_i$
N

^{35}S ^{32}P ^{32}P

INF INF UNINF

Figure 9. SDS-PAGE analysis of ^{32}P-labelled virions

^{32}P-labelled Piry virions were purified by two successive runs on 5-40% sucrose gradients as described in Materials and Methods. An aliquot of the ^{32}P-labelled virus along with a ^{35}S-methionine labelled infected cell marker was analysed by SDS polyacrylamide gel electrophoresis and autoradiography.


G
NS
N
M
Xp
35S INF CELL
32P VIRION

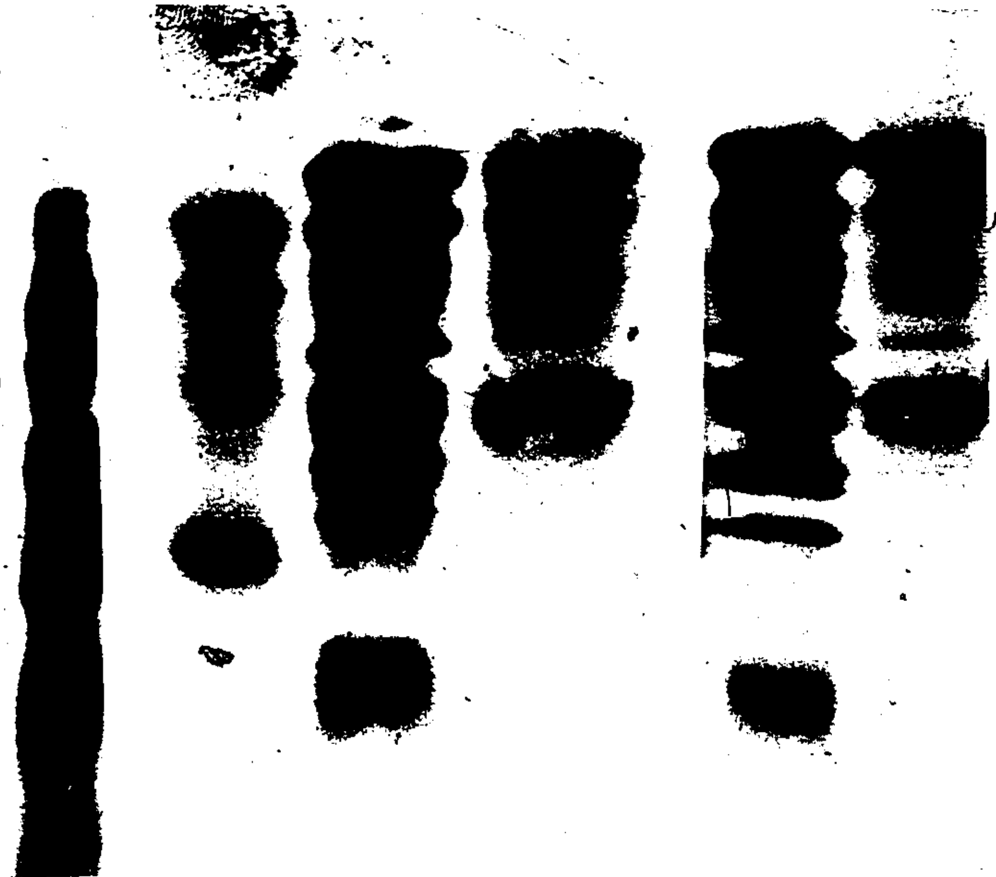
In these experiments the chemical cleavage agent N-chlorosuccinimide (NCS) was used to generate partial cleavage maps of gel purified labelled proteins. The conditions outlined in Materials and Methods (Brown and Prevec, 1979) for NCS cleavage facilitate fragmentation at tryptophan residues. The problem encountered in doing this analysis was that there was no ^{35}S-methionine labelled counterpart for Xp and therefore while I could directly compare ^{32}P-labelled patterns between NS and Xp, the comparison between N and Xp had to be made between ^{35}S-labelled material (N protein) and ^{32}P-labelled material (Xp). Figure 10 shows the partial cleavage pattern comparison between Xp and N protein. Although these digests were carried out under identical conditions, the starting N material was much more susceptible to digestion than whole Xp. This was a repeatable finding and I think suggestive evidence that Xp is not a phosphorylated form of N protein. A more plausible explanation would be that Xp is another species of Piry NS protein. Consistent with this idea is the finding that the cleavage patterns of phosphate labelled NS and Xp are similar both in terms of numbers of bands and their relative intensities.

1.3 Maturation of NS_i in the Presence of Theophylline

If the conversion of NS_i to NS_v was dependent on phosphorylation, inhibitors of protein kinase activity

Figure 10. N-chlorosuccinimide cleavage of Piry proteins

Piry infected L cells were labelled with ^{35}S-methionine or ^{32}P-orthophosphate as described in Materials and Methods. Infected cell extracts were prepared and analysed by preparative SDS-PAGE and autoradiography. Appropriate protein bands were excised, eluted and then incubated with N-chlorosuccinimide as described in Materials and Methods. The partial cleavage maps were then resolved by analysis of the chemical digests on 15% SDS polyacrylamide gels and autoradiography (^{35}S,N), ^{35}S-methionine labelled Piry N protein; (^{32}P,Xp) ^{32}P-labelled Piry Xp protein; (^{35}S,NS_v) ^{35}S-methionine labelled Piry NS_v protein; (^{32}P, NS_v) ^{32}P-labelled Piry NS_v protein.

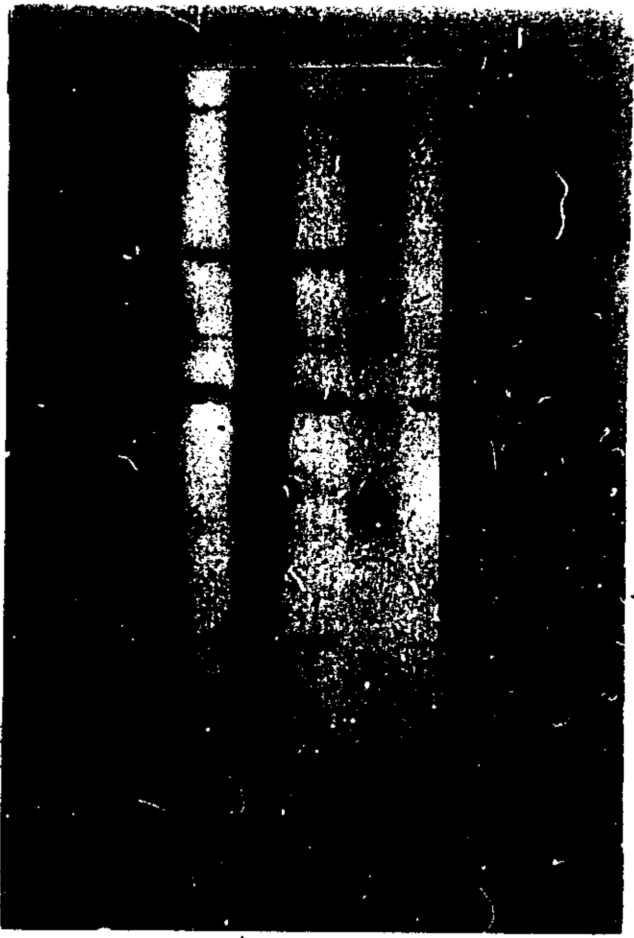


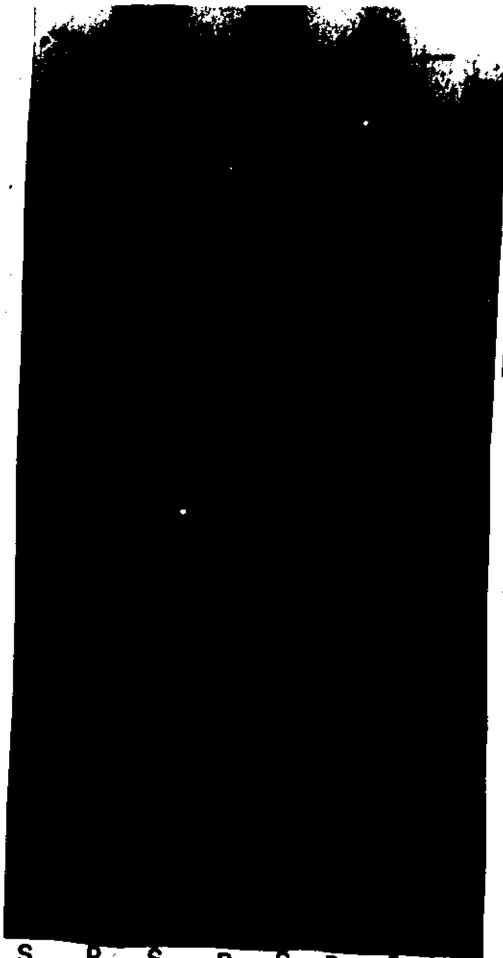
^{35}S ^{32}P ^{35}S ^{32}P ^{35}S ^{32}P
N Xp NSv NSv NSv NSv

might reduce or prevent this conversion. The following experiment was designed to see if theophylline while inhibiting protein kinase activity would also concomitantly prevent the conversion of NS_i to NS_v. Monolayers of L cells infected with Piry virus were treated with theophylline at the concentrations indicated in the legend to figure 11. Three hours post-infection the monolayers were labelled with either ^{32}P-orthophosphate or ^{35}S-methionine for an additional 1.5 hours in the continued presence of theophylline. Extracts were prepared and appropriate aliquots were analysed by SDS-PAGE (figure 11). The result of this experiment demonstrates that, though theophylline causes a general reduction in the amount of radioactive phosphate and ^{35}S-methionine incorporated into proteins there was no concurrent reduction in the relative rate of conversion of NS_i to NS_v (see ^{35}S-methionine result, figure 11). The adenosine analog cordycepin, had essentially the same effect as theophylline, that is, it inhibited phosphorylation without preventing the conversion of NS_i to NS_v (figure 11). These results are consistent with the idea that NS_i can be converted to NS_v in the absence of protein kinase activity. I cannot rule out however, the possibility that the decreased ^{32}P-labelling seen during treatment of these cells with inhibitors really represents decreased uptake of isotope and that a kinase responsible for NS phosphorylation is still functioning.

Figure 11. Treatment of Piry-infected L cells with theophylline

Monolayers of 5 X 10^6 L cells were infected with Piry virus at an moi of 100 PFU/cell as described in Materials and Methods and then overlayed with MEM containing 1:20 normal amino acid complement for ^{35}S-methionine labelling or with -PO_4 MEM for labelling with ^{32}P-orthophosphate. At this time, theophylline was added to each plate to a final concentration of 10^{-3} M, 10^{-4} M or 10^{-5} M. At 3 hours post infection, the overlay was removed and medium containing either ^{35}S-methionine (30μCi/ml) or ^{32}P-orthophosphate (200μCi/ml) was added to appropriate plates along with the inhibitor theophylline. Labelling was allowed to continue for 1.5 hours at which time the samples were prepared for electrophoresis as described in the text. P, labelled with ^{32}P-orthophosphate; S, labelled with ^{35}S-methionine.




NSv
N
S P S P S P S P
CNT
10^{-5} 10^{-4} 10^{-3}
THEOPHILLINE

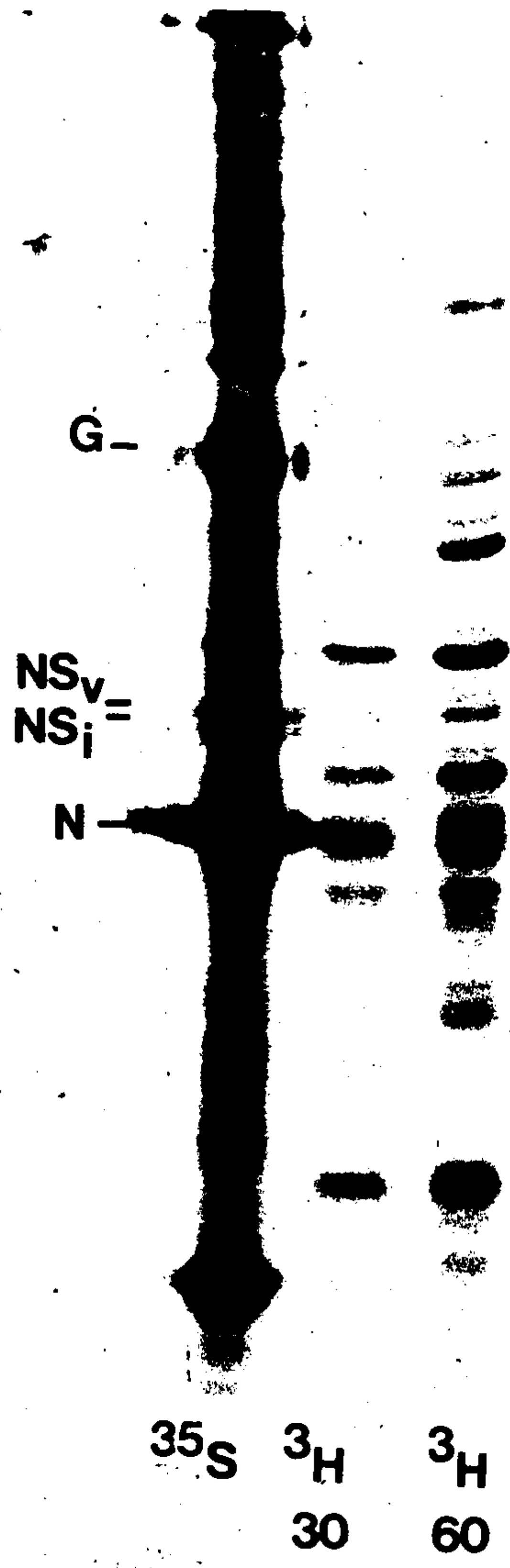
1.4 Possible Acetylation of Piry NS Proteins

Acetylation is a relatively common post-translational modification of certain proteins including phosphorylated histones (Schaffhausen and Benjamin, 1976). Stegink *et al.*, (1970) demonstrated that acetylation of human fetal hemoglobin causes a change in electrophoretic mobility of the α subunit analogous to the shift I observed during conversion of NS_i to NS_v.

As a first approach to test the possibility that NS_v may be an acetylated form of NS_i an S-4 polysomal extract prepared from Piry infected cells as described in Materials and Methods was incubated under cell free protein synthesis conditions in the presence of ^{3}H-acetate and exogenously added coenzyme A (0.4 mM final concentration). At 30 and 60 minutes, aliquots were removed and analysed by SDS gel electrophoresis. Following fluorography, the gels were exposed to X-ray film for ninety days and the resultant autoradiogram shown in figure 12. From the figure it appears as if there is ^{3}H-labelled material comigrating with ^{35}S-labelled NS_v. The observation that ^{3}H-label is incorporated in the presence of the normal amino acid complement and is not found in all viral proteins (e.g. compare M and NS) is consistent with the idea that the ^{3}H-acetate is added post-translationally to the proteins (i.e. not through a biosynthetic pathway). Under the

Figure 12. ^{3}H-acetate labelling of protein in an S-4 polysomal extract

A Piry infected S-4 polysomal extract prepared as described in Materials and Methods was incubated in the presence of ^{3}H acetate and coenzyme A. At appropriate intervals (30 or 60 minutes) aliquots were removed and analysed by SDS-PAGE and fluorography. (^{35}S), ^{35}S-methionine labelled infected cell extract; (^{3}H, 30), ^{3}H acetate incubated for 30 minutes in an S-4 polysomal extract; (^{3}H, 60), ^{3}H acetate incubated for 60 minutes in an S-4 polysomal extract.


G
NS$_v$
NS$_i$
N
^{35}S
^{3}H
30
^{3}H
60

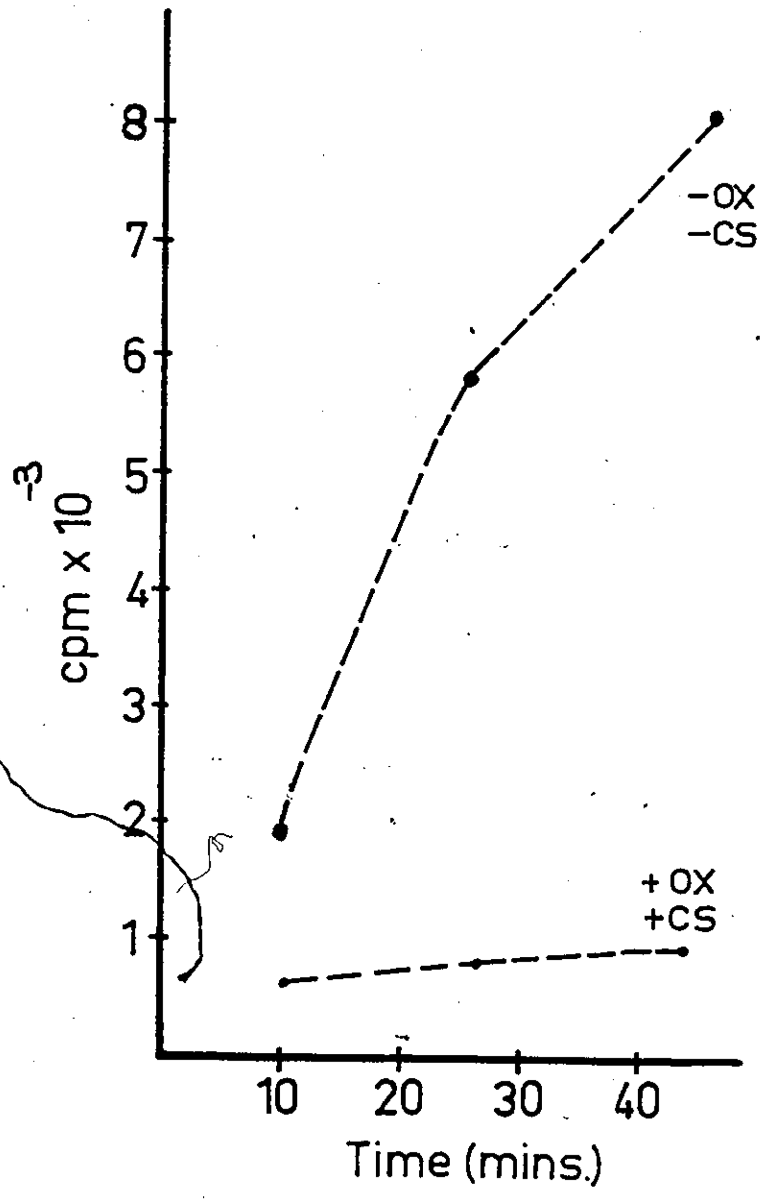
conditions of this experiment the lack of label associated with NS_i may either indicate that NS_i is not acetylated or simply reflect the smaller pool size of NS_i species.

Following the same tack as with phosphorylation experiments it was decided to attempt to prevent the conversion of NS_i to NS_v by inhibiting the acetylation process. Palmiter, (1977) demonstrated that the inclusion of excess oxaloacetate and citrate synthase in cell free protein synthesis systems prevented protein acetylation by depletion of the acetate donor, acetyl CoA. In figure 13 the incorporation of ^{3}H-acetate into TCA precipitable material in the presence and absence of oxaloacetate and citrate synthase is shown for the polyribosomal extract prepared from Piry infected cells. The presence of the competing system effectively prevented significant ^{3}H-acetate incorporation into protein (figure 13).

The effect of oxaloacetate and citrate synthase on the conversion of NS_i to the NS_v was examined as follows. Cell free protein synthesis by the Piry infected L cell extract was carried out for 40 minutes in the presence of ^{35}S-methionine label. Excess unlabelled methionine plus cycloheximide was added at this point and the mixture incubated a further 90 minutes. In figure 14 the labelled proteins NS_i and NS_v are present in approximately equivalent amounts at the end of the 40 minute pulse period. Following

Figure 13. Effect of oxaloacetate and citrate synthase on ^{3}H-acetate incorporation in S-4 polysomal extracts

Piry infected S-4 polysomal extracts were incubated with ^{3}H acetate and coenzyme A under protein synthesis conditions with or without oxaloacetate and citrate synthase. At the indicated times aliquots were removed TCA precipitated onto nitrocellulose filters and quantitated by scintillation counting. (-OX,-CS), without oxaloacetate and citrate synthase; (+OX,+CS), with oxaloacetate and citrate synthase.



cpm x 10^-3
8
7
6
5
4
3
2
1
10
20
30
40
Time (mins.)
−OX
−CS
+OX
+CS

the 90 minute chase essentially all the NS_i has been converted to NS_v. When oxaloacetate and citrate synthase are present throughout the incubation there is little or no apparent conversion of NS_i to NS_v during the 90 minute chase. These results are consistent with the idea that acetylation may play a part in the conversion of NS_i to NS_v.

The following observations however cast some doubt upon this interpretation of the experimental results. Firstly, oxaloacetate in the absence of exogenous citrate synthase is still an effective inhibitor of the conversion of NS_i to NS_v (figure 14). This may be explained by the presence of endogenous citrate synthase in the L cell extract as has been described earlier for the rabbit reticulocyte system (Palmiter, 1977). Alternatively the maturation of NS protein may be a phenomenon independent of acetylation which is inhibited by high levels of oxaloacetate or end product, citrate. To test this possibility the extract was incubated in the presence of excess citrate during the pulse and cycloheximide chase. In figure 15 it is evident that the inclusion of citrate alone is sufficient to inhibit the conversion of NS_i to NS_v.

The results presented in this section neither prove nor disprove that acetylation is the post-translational modification of NS protein responsible for its decreased electrophoretic mobility in the NS_v form. At this point

Figure 14. Effect of oxaloacetate and citrate synthase on NS_i maturation

A Piry infected S-4 polysomal extract was pulsed with ^{35}S-methionine for 40 minutes and then chased an additional 90 minutes with excess cold methionine and cycloheximide.. In the indicated experiments either citrate synthase and oxaloacetate or oxaloacetate alone were included in the incubation mixture. (P), pulse; (C), chase; (-CS,-OX), no oxaloacetate or citrate synthase; (+CS, +OX), both oxaloacetate and citrate synthase; (-CS, +OX), oxaloacetate alone.

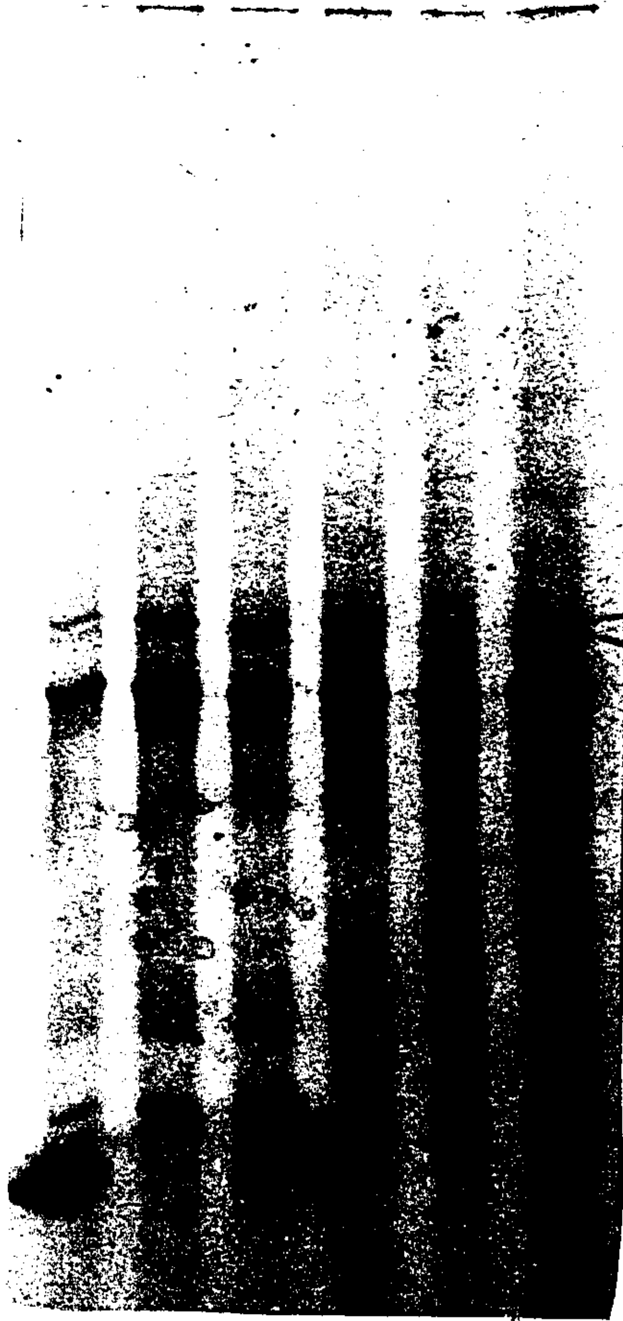
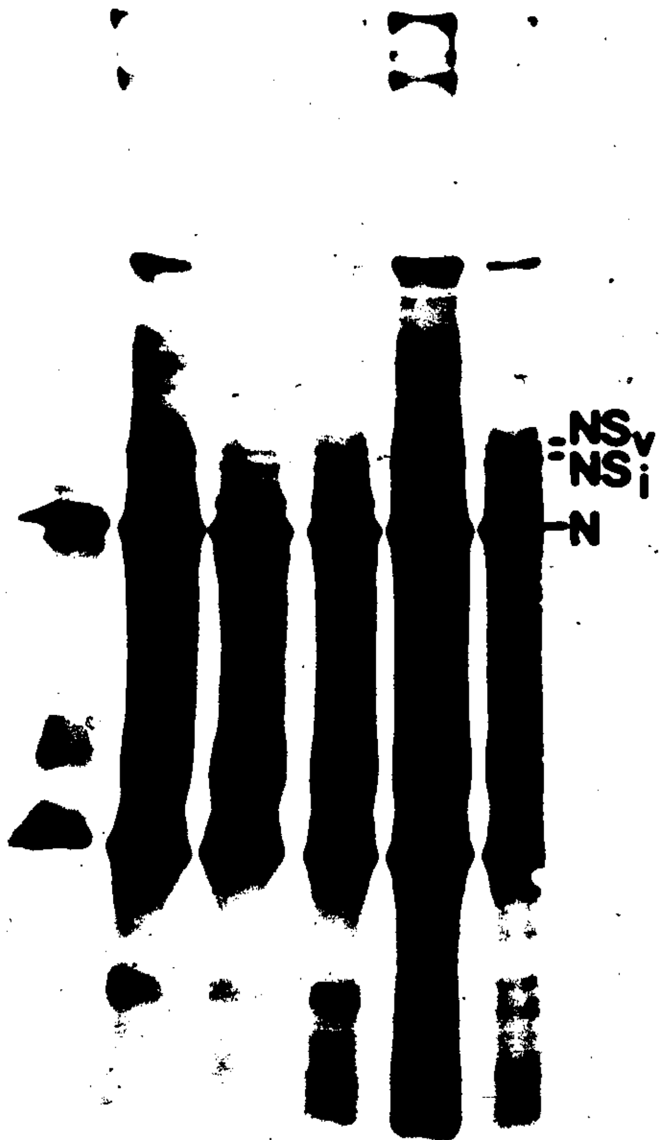

NS$_V$
NS$_I$
P C P C P C
−CS +CS −CS
−OX +OX +OX

Figure 15. Effect of citrate on NS_i conversion

A Piry infected S-4 polysomal extract was pulsed with ^{35}S-methionine for 40 minutes and then chased an additional 90 minutes with excess cold methionine and cycloheximide. In the indicated experiments either oxaloacetate or citrate were included in the incubation mixture. (P), pulse; (C), chase; (CNT), standard reaction mixture; (OX), oxaloacetate included in the reaction mixture; (CIT), citrate included in the reaction mixture.


NSv
NSi
N
P C
CNT
P C
OX
P C
CIT

there seemed no obvious way of determining where the citrate inhibitory effect was taking place (i.e. was citrate inhibiting a putative acetylating enzyme or some other post-translational modification?) and therefore this line of research was terminated.

1.5 Lack of Separation of NS_v and NS_i on NEPHGE

In an attempt to resolve further subsets of Piry NS proteins I performed 2D electrophoresis of infected cell extracts as described by O'Farrell, (1975). An infected cell extract labelled with ^{35}S-methionine was resolved in an isoelectric slab gel as described in Materials and Methods and then subjected to SDS-PAGE in the second dimension (figure 16). Employing this system I was able to resolve multiple G species and two spots comigrating with Piry N protein however no radioactive material could be detected in the region of NS protein.

Since Piry NS is a phosphorylated protein it seemed possible that it was not remaining in the first, isoelectric focussing dimension because it was too acidic. In an attempt to overcome this problem I employed the technique of non-equilibrium pH gradient electrophoresis (NEPHGE) developed by O'Farrell et al., (1977) which resolves highly acidic or basic proteins. Figure 17 shows the result of a non-equilibrium pH gradient slab gel analysis of Piry infected L cells. Multiple bands

Figure 16. Isoelectric and SDS-PAGE analysis of a Piry infected cell extract

Piry infected L cells were labelled with ^{35}S-methionine four hours post infection for 30 minutes. A cytoplasmic extract was prepared, lyophilized and then resuspended in isoelectric focussing lysis buffer as described in Materials and Methods. An isoelectric slab gel was prepared and prefocussed exactly as described by O'Farrell (1975), (see Materials and Methods) before loading of the infected cell sample and isoelectric focussing. An appropriate channel was cut from the first dimension slab gel and then immediately prepared for and run in the second dimension (i.e. SDS-PAGE, exactly as described by O'Farrell, 1975). The positions of NS, G and N protein were verified by co-analysis of an infected cell extract in the second dimension.

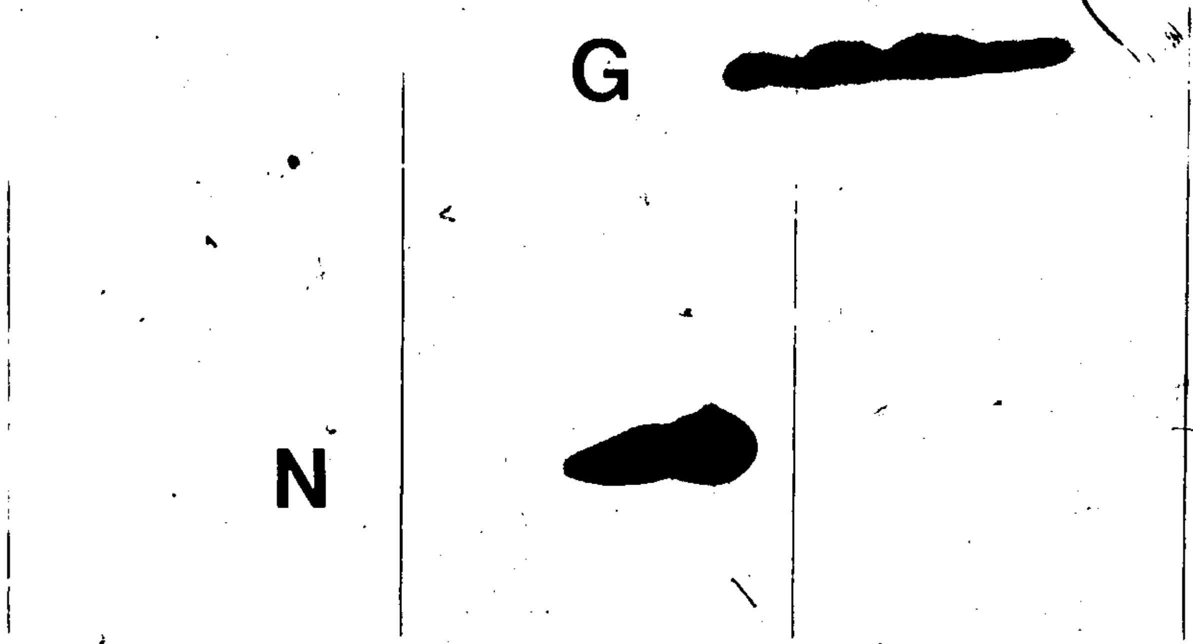

acidic
IEF
basic
G
N
SDS

Figure 17. <u>Non equilibrium pH gradient electrophoresis analysis of Piry infected cells</u>

Infected monolayers were pulse-labelled with ^{35}S-methionine, 4 hours post infection either for 5 minutes or 30 minutes as described in Materials and Methods. Samples were prepared for NEPHGE applied to the top of an isoelectric slab gel and then electrophoresed for 4 hours at 500 volts. Positions of NS, N, G and M proteins were determined by analysis of the individual lanes in the second SDS-PAGE dimension. (30), thirty minute pulse label; (5+30), mixture of 5 minute and 30 minute pulse; (5), five minute pulse label.

NS
NS
N

G

M
30 5+30 5

migrating to the acidic side of N protein (i.e. top of the gel) are detected in both a 5 minute pulse and a 30 minute labelling of Piry infected L cells. In a two dimensional analysis (i.e. NEPHGE followed by SDS-PAGE, figure 18) only one of these bands migrates to the position of NS protein either in the five minute pulse or 30 minute labelling period. Therefore, within the resolution capacity of this two dimensional gel system, I could detect only one species of NS_i or NS_v protein. Furthermore, under the conditions of NEPHGE there appears to be no charge difference between NS_i and NS_v as I would have predicted if the two species differed in acetylation or phosphorylation status.

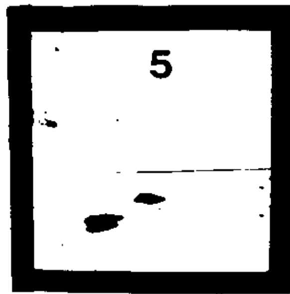
2.0 Structural Analysis of NS Protein

2.1 Amino Terminal Labelling of NS Protein

For several of the experiments described in the following sections I made use of NS protein labelled specifically at the amino terminus of the polypeptide with N-formyl-^{35}S-methionine. The protocol used for labelling of VSV proteins was exactly as described by Brown and Prevec (1981). Briefly, wheat germ initiator methionyl tRNA was charged with ^{35}S-methionine using *E.coli* aminoacyl-tRNA synthetases and then chemically formylated by the method of Gillam *et al.*, (1968). N-formyl-^{35}S-methionine and excess cold methionine was

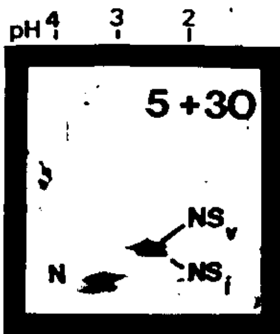
Figure 18. Two dimensional analysis of Piry-infected L cells

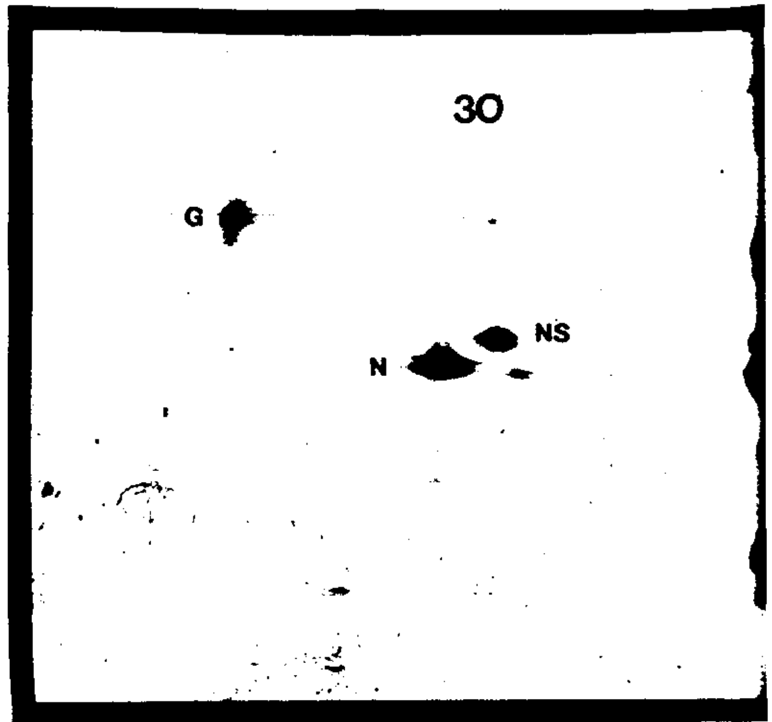
Infected monolayers were pulse labelled with ^{35}S-methionine, 4 hours post infection, either for 5 min or 30 min as described in the text. Samples, prepared for NEPHGE, were applied to the top of isoelectric slab gels and run for 4 hours at 500 V. Appropriate channels were then immediately prepared for and run in the second dimension in SDS-PAGE, as described by O'Farrell (1975). Presented in this figure: 5 min pulse label; 30 min pulse label: mixture of a 5 and 30 min pulse label.



IF →

SDS ↓





then added to a cell free protein synthesis system composed of a commercial rabbit reticulocyte extract programmed with a virus infected L cell cytoplasmic extract. Labelled NS protein was resolved by preparative SDS-PAGE and purified as described in Materials and Methods. To ensure that label was incorporated only at the amino terminus and not at internal methionine sites I performed tryptic digestion of the end-labelled material. Figure 19 shows a comparison of a tryptic digest of NS protein labelled _in vitro_ either with N-formyl-^{35}S-methionine or unmodified methionine. Clearly NS synthesized in the presence of ^{35}S-methionine (MET) generated three major tryptic peptides while N-formyl tagged NS protein (fMET) is labelled predominantly in one peptide, the amino terminal tryptic peptide.

2.2 Molecular Weight Estimation of NS Protein

At the time of these experiments the complete nucleotide sequence of NS mRNA which predicts that the phosphoprotein should have a molecular weight of 25,110 Daltons had not been published. (Gallione _et al._, 1981). As pointed out in the Introduction (see section 7 (c)) although NS has an electrophoretic mobility in SDS-PAGE consistent with a protein of molecular weight 39-54K Daltons the NS mRNA is capable of coding for a protein of no more than 30K Daltons. As a first attempt to resolve this dichotomy, I tried to estimate the actual size of

Figure 19. Tryptic hydrolysis of internally and end-labelled Indiana NS protein

Indiana NS protein was labelled *in vitro* either with N-formyl-^{35}S-methionine or unblocked ^{35}S-methionine. The NS protein were then separately resolved by SDS preparative gel electrophoresis and autoradiography. Eluted NS protein was oxidized, trypsinized and electrophoresed on cellulose CEL 300 thin layers as described in Materials and Methods.

OR
MET
fMET

an NS protein monomer by quantitation of the $^3H/^{35}S$ ratio in molecules labelled uniformly with ^3H and at the amino terminus with ^{35}S. To accomplish this, cell free protein synthesis of VSV polypeptides was carried out with a cell free polysomal extract using ^3H amino acids and N-formyl-^{35}S-methionine. Dually labelled proteins N, M and NS synthesized under these conditions were separately purified by SDS-PAGE, autoradiography and elution all as described in Materials and Methods.

The $^3H/^{35}S$ ratio in each of the purified proteins was determined by scintillation counting and the results are shown in Table 1. Of the three proteins, NS has the lowest $^3H/^{35}S$ ratio indicating that either the N-terminal methionine is occasionally removed (i.e. post-translational processing) or that the nascent NS polypeptide has a lower molecular weight than either M or N. The latter explanation is probably the correct one since NS labelled with <u>unblocked</u> methionine does <u>not</u> have an underrepresentation of the amino terminal tryptic peptide as one would expect if NS were processed at the amino terminus. Also shown in Table 1 is the estimated molecular weight of NS protein determined by comparison of the $^3H/^{35}S$ ratios and molecular weights of N and M proteins (determined by co-analysis with cold marker proteins, see legend to Table 1).

Table 1

Approximation of Molecular Weight of NS Protein

Protein	Uniform ^{3}H cpm	N-formyl-^{35}S-methionine cpm	$^{3}H/^{35}S$	Molecular Weight X 10^{3}
N	889	205	4.3	50
M	639	231	2.7	30
NS	857	486	1.8	20

The samples were each counted twice for 100 minutes and the averaged cpm values were corrected for background radiation. The molecular weight values for N and M proteins were determined by co-analysis of labelled infected cell extract and cold marker proteins (i.e. bovine serum albumin, ovalbumin, trypsin and lysozyme). The molecular weight value for NS was then estimated by comparison of the $^{3}H/^{35}S$ ratio to those of N and M.

2.3 Phosphodiesterase Treatment of NS Protein

One explanation for the large difference in NS protein molecular weight and its mobility in SDS-PAGE could be that NS exists as a dimer formed by an intermolecular bridge resistant to reducing agents, heat and detergent. Since NS is also a phosphoprotein it seemed reasonable to suggest that such a bridge could be formed by an intermolecular phosphodiester linkage. This suggestion was not without precedent as Veis and Schluetter (1963) had earlier suggested the possibility of this type of crosslinking in dentine collagen and more recently Evans et al., (1979) had postulated the existence of intramolecular phosphodiester bonds to explain the anomalous mobility of NS mutants and revertants of New Jersey serotype. Edmondson and James (1979) believe that their ^{31}P NMR studies of flavodoxin provide the first direct evidence that a phosphodiester linkage probably between serine residues actually occurs in vivo.

If a phosphodiester bond did exist one might expect it to be susceptible to phosphodiesterases and therefore I attempted to alter the mobility of NS protein by treatment with snake venom phosphodiesterase (SVPD).

Initial experiments were very encouraging as illustrated in figure 20. A ^{35}S-methionine labelled Indiana infected cell extract was treated with 10 units of SVPD

Figure 20. Snake venom phosphodiesterase treatment of an Indiana infected cell extract

An Indiana infected cell extract was labelled with ^{35}S-methionine 4 hours post infection for 30 minutes. Cytoplasmic extracts were prepared as described in Materials and Methods and then incubated with SVPD (Worthington enzymes) 50 μgm/ml or an equal volume of reaction buffer as described. The reaction was terminated by the addition of SDS sample buffer and heating. Samples were then analysed by SDS-PAGE and autoradiography. (CNT), infected cell extract incubated without SVPD; (SVPD), infected cell extract incubated with SVPD.

L-

G-

NS-
N-

M-

CNT SVPD

purchased from Worthington biochemicals and run on SDS-PAGE with a parallel extract treated identically except that SVPD was omitted. Clearly, treatment with SVPD results in a loss of labelled material at the normal NS position and a formation of a new band running below M with an estimated molecular weight of 19.5K Daltons. Subtle changes in background bands were also observed however these may have represented other host proteins modified with phosphodiester linkages. Two other sources of SVPD were used on infected cell extracts, however one of these (from P-L biochemicals) gave no discrete product on digestion and the other (from Sigma) seemed to cause generalized proteolysis (figure 21) therefore the Worthington enzyme was used in all subsequent experiments. In the following experiments the anti-NS and anti-M antisera characterized in section 3.0 were used to try and eliminate confusion due to changes in host cell proteins during enzyme treatment. A high speed supernatant from a ^{35}S-methionine labelled infected cell extract (see section 1.7 Materials and Methods) was immunoprecipitated with combined anti-NS and anti-M sera to give a preparation enriched in NS but still containing M and N proteins as internal controls, (figure 22, Ab cnt). When this immunoprecipitate was treated with SVPD the NS band is lost and a new labelled band appears as shown previously (figure 20, SVPD).

Figure 21. Snake venom phosphodiesterase treatment of Indiana infected cell extracts

Indiana infected cell extracts were labelled with ^{35}S-methionine four hours post infection for 30 minutes. Cytoplasmic extracts were prepared as described in Materials and Methods and then incubated with SVPD (either Sigma or P-L biochemicals 50 μgm/ml) or an equal volume of reaction buffer as described. The reaction was terminated by the addition of SDS sample buffer and heating. Samples were then analysed by SDS-PAGE and autoradiography. (C), infected cell extract incubated without SVPD; (P-L), SVPD treatment of an infected cell extract with enzyme purchased from P-L biochemicals; (SIGMA), SVPD treatment of an infected cell extract with enzyme purchased from SIGMA biochemicals.

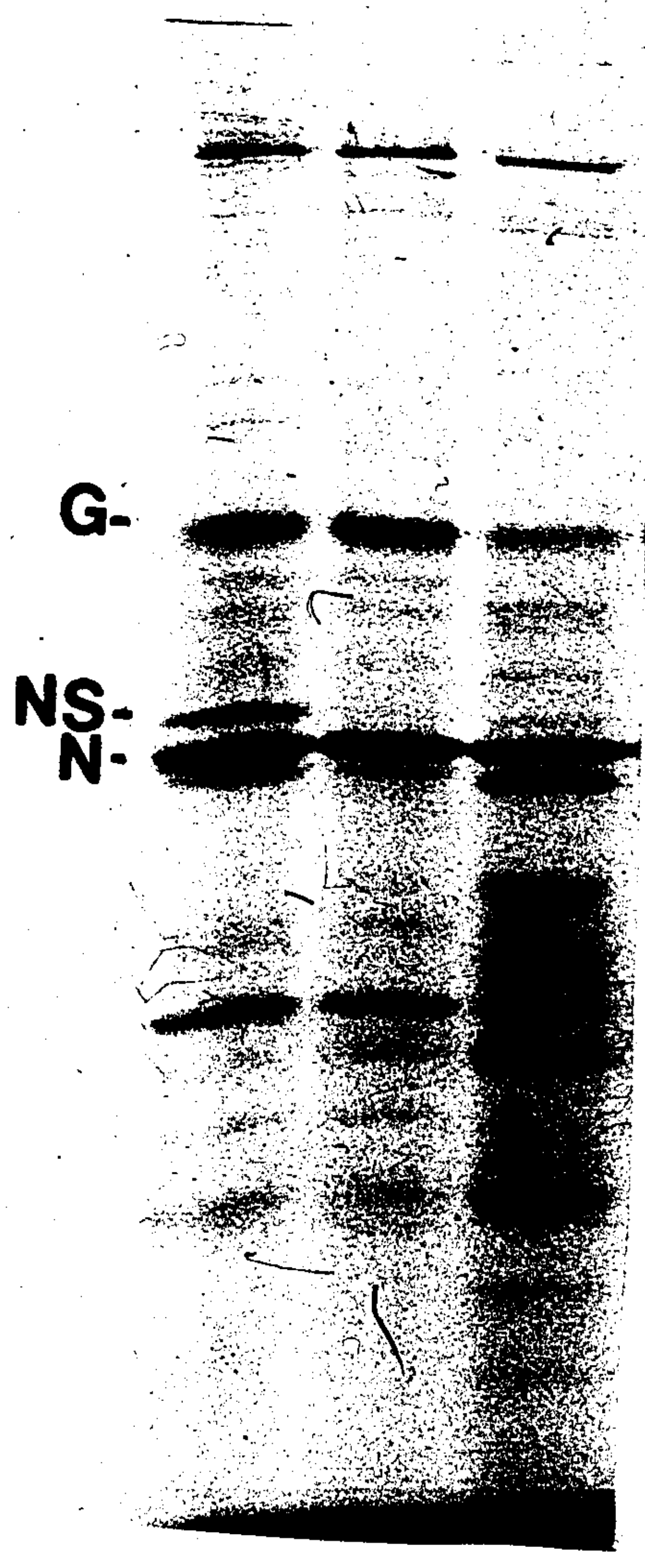
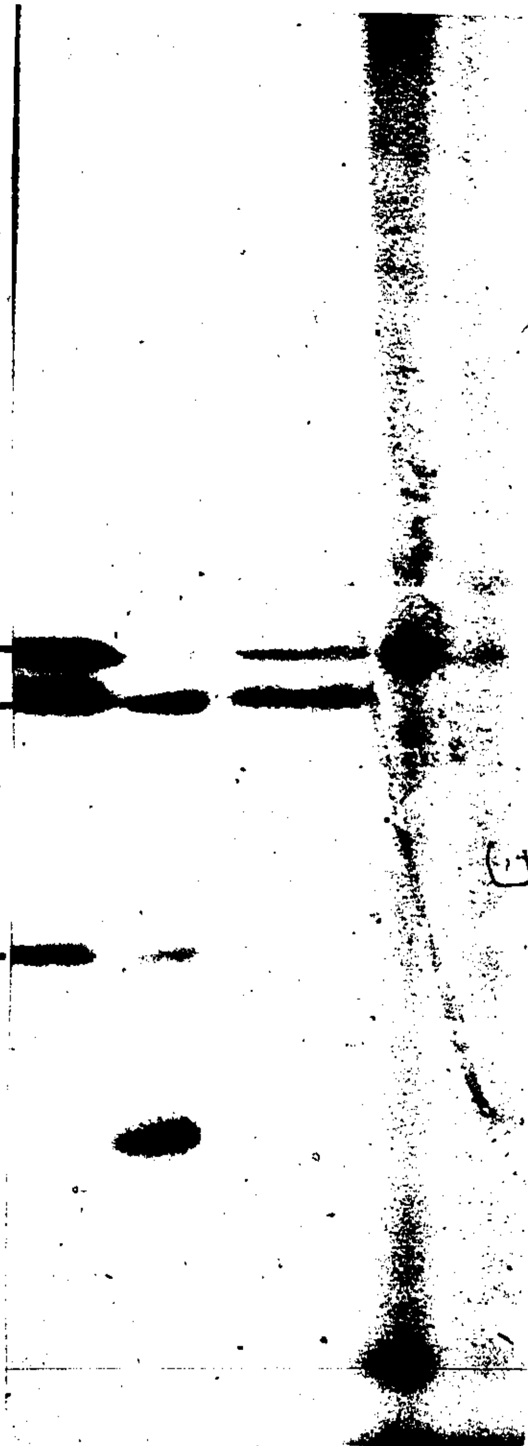

G-
NS-
N-
C
P-L
SIGMA

Figure 22. Snake venom phosphodiesterase treatment of immunoprecipitates

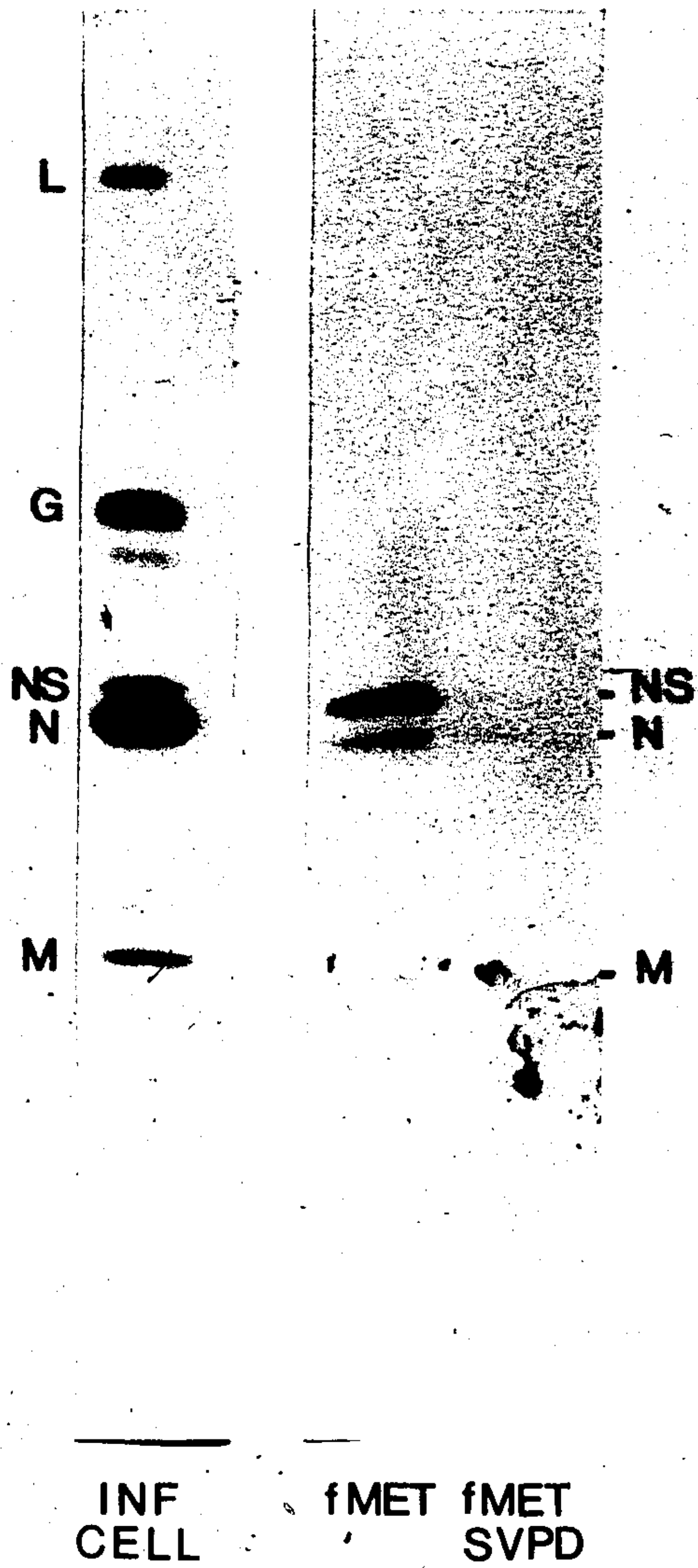
Infected cell extracts labelled 4 hours post infection with either ^{35}S-methionine or ^{32}P-orthophosphate were subjected to high speed ultracentrifugation as described in section 1.7 of Materials and Methods. The high speed supernatant was immunoprecipitated with combined anti-NS and anti-M serum as described in section 3.0. The immunoprecipitates were then washed once with reaction buffer and then reacted with snake venom phosphodiesterase as described in Materials and Methods. (Ab cnt), immunoprecipitate from infected cell supernatant untreated; (Ab svpd), snake venom phosphodiesterase treatment of the immunoprecipitate; (SUP), high speed supernatant which served as the source of ^{35}S-methionine antigen.


NS
N
M
Ab cnt
Ab svpd
sup
Ab cnt
Ab svpd
35S
32p

In a parallel experiment the enzyme SVPD was incubated with antibody precipitated ^{32}P-labelled material. Figure 22 shows that the only ^{32}P-labelled band immunoprecipitated with the combined sera is NS and upon treatment with SVPD no discrete bands are formed. This finding was discouraging since from the known action of SVPD I predicted that one half of a dimer held together by a phosphodiester linkage should remain labelled with ^{32}P after digestion. The complete loss of ^{32}P could be rationalized if the enzyme preparation was contaminated with a phosphatase or alternatively the SVPD preparation may have contained some protease activity which was responsible for the change in NS mobility. To try and resolve this issue the following experiment was designed. Since, in my hands, the phosphorylated sites in NS protein appear grouped toward the amino terminus of the molecule (see section 2.4) then, if the loss of ^{32}P is the result of proteolysis one would expect to see the loss of the amino terminal methionine containing peptide. On the other hand if the ^{32}P-label was lost because of the action of SVPD (or a phosphatase or the combination of both), I predicted that NS labelled with N-formyl-^{35}S-methionine after treatment with SVPD should migrate with a mobility of approximately 20K Daltons. In figure 23 the result of this experiment is presented.

Figure 23. Snake venom phosphodiesterase treatment of N-formyl-^{35}S-methionine labelled Indiana proteins

A cell free extract was incubated with N-formyl-^{35}S-methionine to label viral proteins NS, N and M as described in Materials and Methods. The extract was divided into two parts, half incubated with SVPD the other half incubated with buffer. Samples were analysed by SDS-PAGE and autoradiography. (INF CELL), infected cell marker; (fMET), N-formyl-^{35}S-methionine labelled extract incubated with buffer; (fMET SVPD) N-formyl-^{35}S-methionine labelled extract incubated with SVPD (50 μgm/ml.



L
G
NS
N
M
NS
N
M
INF
CELL
fMET
fMET
SVPD

The lane designated fMET shows the SDS-PAGE profile of material synthesized <u>in vitro</u> in the presence of N-formyl-^{35}S-methionine and the lane, fMET SVPD, shows the same material after treatment with SVPD. Clearly all labelled material comigrating with NS protein is lost after the enzymatic treatment however no discrete bands appear at lower positions in the gel. Taken together I think these findings indicate that the effect of SVPD on NS mobility is most likely due to protease contaminating the enzyme preparation.

2.4 Linear Mapping of Phosphorylated Residues in NS Protein

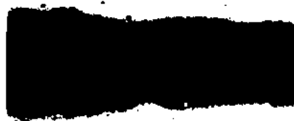
The partial cleavage mapping experiments described in this section were designed to provide preliminary information in the search for the exact sites of NS phosphorylation. The technique used was developed by Brown and Prevec (1982) and it allowed me to locate the phosphorylated residues in NS with respect to its tryptophan sites and the protein's amino terminus. To perform this analysis three types of labelled NS protein were prepared. Labelled, Indiana infected L cell extracts were used as a source of either ^{32}P or ^{35}S-methionine metabolically labelled NS protein, while <u>in vitro</u> synthesized NS protein was labelled only at the amino terminus as described in section 2.1 of the Results.

The three independently labelled NS species were

Figure 24. <u>N-chlorosuccinimide treatment of purified Indiana NS protein</u>

Indiana infected L cell extracts were labelled with ^{35}S-methionine as described in Materials and Methods. Infected cell extracts were prepared and analysed by preparative SDS-PAGE and autoradiography. The NS band was excised eluted and then either incubated with N-chlorosuccinimide plus solvents (A) or solvents alone (B) as described in Materials and Methods. The molecular weights of the indicated fragments (given in Daltons) was determined by co-analysis with cold marker proteins, as described in legend to Table 1.

45K



25K

14K

A B

then reacted with N-chlorosuccinimide under conditions which yield partial digestion at tryptophan residues. As a control, to assure that cleavage was being effected by NCS and not simply by the reagents which are required for the reaction, purified ^{35}S-methionine labelled NS was incubated either with solvents alone or solvents plus NCS. The result of this experiment is shown in figure 24. Clearly, only when NCS is included in the reaction mix is there significant digestion of pure NS protein.

The partial digests of the three differentially labelled NS species are shown in figure 25. Eight well defined fragments are generated by partial digestion of ^{35}S-methionine labelled NS protein produced in whole cells, (figure 25, lane A). The four slowest migrating fragments are detected when end-labelled material is digested with NCS demonstrating that these are the fragments which contain the amino terminus of the original nascent protein, (lane C). Material labelled with ^{32}P, when digested with NCS, gave a partial digestion pattern identical to that of end-labelled material, (lane B). This clearly demonstrates that Indiana NS protein synthesized in infected cells is phosphorylated at sites amino to NS tryptophan residues. This same pattern of labelling is also seen in the Piry serotype of VSV, (see figures 10 and 26). When human KB cells are used as the host for an Indiana infection

Figure 25. <u>N-chlorosuccinimide cleavage of Indiana NS protein</u>

^{35}S-methionine labelled and ^{32}P-labelled Indiana NS protein was purified from infected L cells by SDS-PAGE, autoradiography and elution all as described in Materials and Methods. N-formyl-^{35}S-methionine labelled NS protein was synthesized *in vitro* and purified as described in Materials and Methods. The purified proteins were separately treated with N-chlorosuccinimide and the partial cleavage maps resolved by analysis of the chemical digests on 15% SDS polyacrylamide gels and autoradiography. (A), metabolically ^{35}S-methionine labelled NS protein; (B), metabolically ^{32}P-orthophosphate labelled NS protein; (C), N-formyl-^{35}S-methionine labelled NS protein. The molecular weights of the indicated fragments (given in Daltons) was determined by co-analysis with cold marker proteins, as described in legend to Table 1.

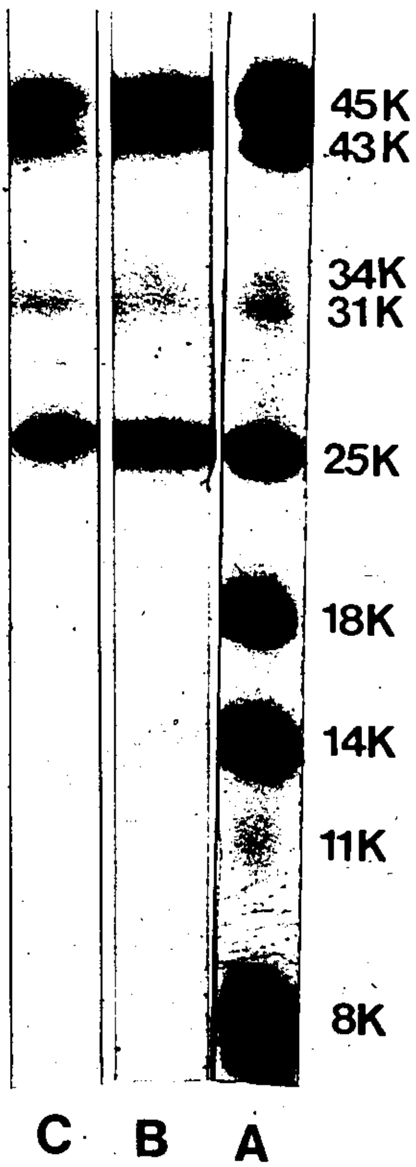

45K
43K
34K
31K
25K
18K
14K
11K
8K
C
B
A

Figure 26. N-chlorosuccinimide cleavage of Piry NS proteins

^{35}S-methionine labelled and ^{32}P-labelled Piry NS protein was prepared from infected L cells by SDS-PAGE, autoradiography and elution all as described in Materials and Methods. N-formyl-^{35}S-methionine labelled NS protein was synthesized *in vitro* and purified as described in Materials and Methods. The purified proteins were separately treated with N-chlorosuccinimide and the partial cleavage maps, resolved by analysis of the chemical digests on 15% SDS polyacrylamide gels and autoradiography. (^{35}S), ^{35}S-methionine labelled NS protein; (fMET), N-formyl-^{35}S-methionine labelled NS protein; (^{32}P), metabolically ^{32}P-orthophosphate labelled NS protein. The molecular weights of the indicated fragments (given in Daltons) was determined by co-analysis with cold marker proteins, as described in legend to Table 1.

54K

31K

^{35}S fMET ^{32}P

PIRY

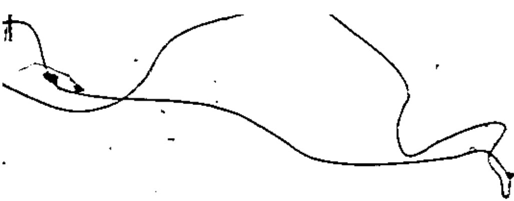
(i.e. as opposed to mouse L cells) again the most heavily labelled fragments are those which contain the amino terminus (figure 27). These results provide good evidence that NS is phosphorylated toward the amino terminus of the protein. The fact that this pattern is preserved in two different serotypes (Indiana and Piry) and in different host cells (mouse and human) suggests that this pattern of phosphorylation may be functionally significant to the activity of NS protein _in vivo_. In some experiments, figures 27 and 28, in addition to the primary phosphorylation sites located in the amino terminal fragments I detected a minor amount of phosphorylation in fragments which are carboxyl to the tryptophan sites.

2.5 Tryptic Digestion of Indiana NS Protein

In an attempt to dissect out the exact locations of phosphate residues in NS protein, I subjected it to two dimensional tryptic mapping. When ^{35}S-methionine labelled material is digested with trypsin and analysed on cellulose thin layers three clearly defined peptides are resolved (figure 29). This finding is in exact agreement with the amino acid sequence prediction for NS protein by Gallione _et al._, (1981) and suggests that the experimental conditions result in complete tryptic digestion of the protein (see Appendix I). In contrast in a parallel 2D tryptic analysis of ^{32}P-labelled NS protein essentially

Figure 27. N-chlorosuccinimide cleavage of Indiana NS protein produced in human KB cells

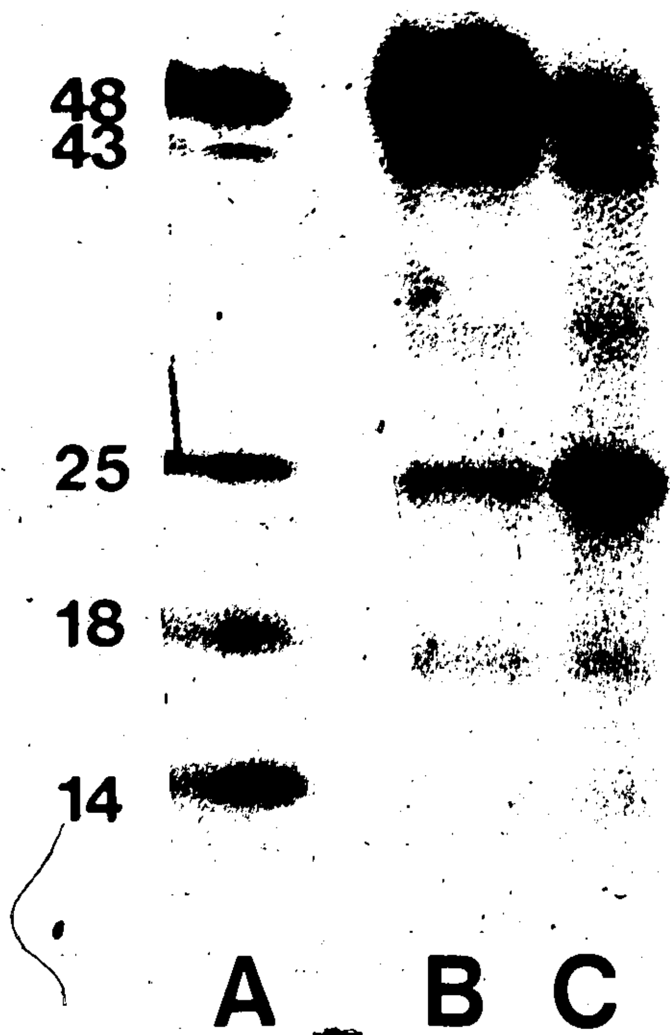
Indiana infected KB cells were labelled with either ^{35}S-methionine or ^{32}P-orthophosphate as described in Materials and Methods. Infected cell extracts were prepared and analysed by preparative SDS-PAGE and autoradiography. NS protein bands were excised, eluted and then incubated with N-chlorosuccinimide as described in Materials and Methods. The partial cleavage maps were resolved by analysis of the chemical digests on 15% SDS polyacrylamide gels and autoradiography. (NS, ^{35}S), purified ^{35}S-methionine labelled NS protein reacted with NCS; (NS, ^{32}P), purified ^{32}P-labelled NS protein reacted with NCS. The molecular weights of the indicated fragments (given in Daltons) was determined by co-analysis with cold marker proteins, as described in legend to Table 1.



45K
25K
14K
NS NS
^{35}S ^{32}P

Figure 28. N-chlorosuccinimide cleavage of Indiana NS protein produced in L cells

Indiana infected L cells were labelled with ^{35}S-methionine or ^{32}P-orthophosphate as described in Materials and Methods. Infected cell extracts were prepared and analysed by preparative SDS-PAGE and autoradiography. NS protein bands were excised eluted and then incubated with N-chlorosuccinimide as described in Materials and Methods. The partial cleavage maps were resolved by analysis of the chemical digests on 15% SDS polyacrylamide gels and autoradiography (A), ^{35}S-methionine labelled Indiana NS protein; (B), ^{32}P-labelled Indiana NS protein treated with NCS for ten minutes; (C) ^{32}P-labelled Indiana NS protein treated with NCS for ninety minutes. The molecular weights of the indicated fragments (given in Daltons) was determined by co-analysis with cold marker proteins, as described in legend to Table 1.


48
43
25
18
14
A
B
C

C

Figure 29. Tryptic fingerprint of ^{35}S-methionine labelled Indiana NS protein

^{35}S-methionine labelled NS protein was purified by preparative SDS-PAGE from Indiana infected cell extracts labelled for 30 minutes some four hours post infection. The NS band was excised from the gel, oxidized and trypsinized (1 μgm/μl) as described in Materials and Methods. Two dimensional analysis by electrophoresis and chromatography was performed on cellulose CEL 300 thin layers.

chromatography

electrophoresis

all radioactive material remains at the origin of application with slight smearing in both dimensions. A similar finding has been reported by Hsu et al., (1982) using the San Juan strain of Indiana. One dimensional thin layer electrophoretic analysis of trypsinized ^{32}P-labelled NS protein is shown in figure 30. At pH 2 most of the applied sample remains at the origin with only slight streaking toward the cathode. At pH 6, the material migrates toward the anode as a single spot with low mobility. One explanation for this low electrophoretic behaviour on thin layers is that the ^{32}P-labelled tryptic peptides are large fragments. Alternatively they may be peptides which are poorly charged and have a high affinity for the cellulose matrix. To differentiate between these possibilities I scraped the ^{32}P-labelled material from the thin layers shown in figure 30, solubilized it in SDS sample buffer and then applied it to a 15% SDS polyacrylamide gel. In my hands, the ^{35}S-methionine labelled NS tryptic peptides are too small to be resolved on a gel of this type and run with the bromophenol blue marker. I predicted that if the ^{32}P-labelled tryptic peptides were large fragments they may be resolved as bands migrating behind the front (bromophenol blue marker). Figure 31 shows the result of this experiment and clearly there is a radioactive band (TF) migrating behind the front (F). The band runs in the gel

Figure 30. Thin layer electrophoresis of ^{32}P-labelled tryptic peptides

^{32}P-labelled NS protein was purified by preparative SDS-PAGE from Indiana infected cell extracts labelled for 1.5 hours, four hours post infection with ^{32}P-orthophosphate (200 μCi/ml). The NS band was excised from the gel, oxidized and trypsinized exactly as described in figure 29. Electrophoresis was performed on cellulose CEL 300 thin layers at pH 2 or pH 6 for 2 hours at 400 volts.

—
origin
+
ph 2
ph6

Figure 31. SDS-PAGE analysis of trypsin hydrolysed ^{32}P-labelled Indiana NS protein

The ^{32}P-labelled material that remained at the origin of electrophoresis in figure 30 was scraped from the thin layer mixed with SDS sample buffer and analysed by SDS-PAGE (15% acrylamide) and autoradiography. A ^{35}S-methionine labelled cell extract was included as a marker in the left hand lane; (N), Indiana N protein; (M), Indiana M protein; (F) gel front identified by bromophenol blue marker; (TF), ^{32}P-labelled tryptic fragment from figure 30.

N

M

TF

F

with an estimated molecular weight of 5.5K Daltons. This result along with the data provided in the linear mapping experiments (section 2.4) suggest that at least one site of phosphorylation in NS protein is in a large tryptic peptide located on the amino terminal side of all NS tryptophan residues. This finding is supported by the amino acid sequence prediction of Gallione et al., (1981). From their nucleotide sequence data these authors predict that NS contains a large 7.5K Dalton tryptic peptide which contains serine and threonine residues both of which are phosphorylated in NS protein (Clinton et al., 1979). Furthermore, all of the potential sites of phosphorylation in this peptide are located on the amino terminal side of all tryptophan residues (see Appendix I). Although the above data are consistent with phosphorylation occurring within the large tryptic fragment they do not exclude the possibility of additional sites of phosphorylation located between the amino terminus of the protein and the large tryptic peptide. As pointed out above, the radioactive material in figure 30 may be a heterogeneous population of peptides, some large and immobile while others are small but poorly charged. These small peptides would not be resolved on the gel (figure 31) and therefore go undetected.

2.6 Phosphodipeptide Analysis of Phosphoproteins

Several possible approaches may be used to identify the exact sites of phosphorylation within a protein.

I had hoped to use sequential Edman degradation of SDS-PAGE purified protein as a direct method for identification of the *in vivo* sites of phosphorylation in NS. In preliminary experiments using ^{35}S-methionine labelled Indiana protein it became evident that SDS-PAGE purified NS was blocked at its amino terminus (Table 2). Recall that the amino acid prediction of Gallione *et al.*, (1981) and the N-formyl-^{35}S-methionine tryptic maps demonstrated that NS has at its amino terminus a methionine residue. In the experiment presented in Table 2 although the internal standard lysozyme was correctly sequenced there was no significant release of radioactive material from ^{35}S-methionine labelled NS. One possible interpretation of this finding is that NS is blocked at its amino terminus by post-translational acetylation as was earlier suggested for Piry NS (see section 1.4). Furthermore Annan *et al.*, (1982) point out that Edman degradation, to determine the sites of phosphorylation can be an unsatisfactory technique since (a) no PTH derivative of phosphoserine is generated (b) the yield of PTH-serine is often low and variable.

Enzymatic cleavage of the phosphoprotein to generate labelled peptides is the most common approach and especially

TABLE 2

Automated Sequence Analysis of ^{35}S-methionine Labelled NS Protein

Cycle #	Radioactivity Loaded	Theoretical ^{35}S-PTH-Methionine	Observed ^{35}S-PTH-Methionine	Observed Lysozyme PTH-Derivative
1	100,000 cpm	33,000 cpm	900 cpm	Lysine
2			40 cpm	Valine
3			70 cpm	Phenylalanine

NS protein purified by SDS-PAGE and eluted as described in Materials and Methods was loaded along with 5 mgm of lysozyme into a Beckman automated sequenator. Sequence analysis was carried out using a 0.1 M Quadrol program. An initial cycle was carried out without the inclusion of phenylisothiocyanate. The anilinothiozoline derivatives were converted to phenylthiohydantoins (PTH) of amino acids by treatment with 1 M HCl at 80°C for 10 minutes and then identified by chromatography using silica N-HR thin layers. ^{35}S-methionine labelled PTH-methionine was identified by elution of authentic PTH-methionine spots and scintillation counting. Following the three sequencing cycles the cup was washed with 10% SDS and the retrieved material was TCA precipitated

(Table 2 continued)

and subjected to scintillation counting. Approximately 92% of the radioactivity applied to the cup was recovered by this protocol indicating that while lysozyme was correctly sequenced (see above) less than 3% of the potential amino terminal methionine residues reacted with PITC.

useful if the protein's sequence is known. A recent technique developed by Jones and Olson (1980) allows nearest neighbour analysis of the phosphorylated residue in the native protein or peptide. These authors demonstrated that one of the products of limited acid hydrolysis of proteins is a phosphodipeptide of the structure X-Ser(P) or X-Thr(P) (where X represents any amino acid). If this technique is coupled with enzymatic peptide mapping it should be possible to unequivocably determine the site or sites of phosphorylation in a protein of known sequence.

I therefore attempted to apply this technique of nearest neighbour analysis to NS protein. In the preliminary experiments described below the milk protein α-casein was used as a model phosphoprotein to optimize the conditions of analysis.

As a first approach 150 mgm of either bovine serum albumin (BSA) or α-casein was subjected to acid hydrolysis using the protocol described by Jones and Olson (see legend to figure 32). The two hydrolysates were filtered through glass wool, lyophilized, spotted in the middle of a piece of Whatman 3M paper and then subjected to high voltage paper electrophoresis, (2000 volts, 1 hour pH 1.9. The electropherogram was stained with ninhydrin to identify the products of hydrolysis. Figure 32 shows

Figure 32. <u>High voltage paper electrophoresis of α-casein and BSA acid hydrolysates</u>

BSA and α-casein were acid hydrolysed as described in Materials and Methods and then analysed by high voltage paper electrophoresis (2000 volts, 30 minutes, pH 1.9). Presented in this figure is a photograph of the ninhydrin stained electropherogram. (PS), phosphoserine marker; (A), BSA hydrolysate after passage through cation beads at pH 1.9; (B), BSA hydrolysate prior to passage through cation beads; (C) α-casein hydrolysate after passage through cation beads at pH 1.9; (D); α-casein hydrolysate prior to passage through cation beads. (OR), site of sample application.

+
OR
−
PS
A
B
C
D

the result of this analysis. In the case of BSA (lane B), all ninhydrin staining material runs in a smear toward the cathode as would be expected since at pH 1.9 all common amino acids are fully protonated. In contrast, the hydrolysate of the phosphoprotein α-casein contains in addition to positively charged ninhydrin staining material, negatively charged phosphoserine and ninhydrin positive material which remains at the origin of application (figure 32, lane D). It was the material which remained at the origin in a nonhistone nuclear protein acid hydrolysate which Jones and Olson (1980) identified as phosphodipeptides. Since these putative phosphodipeptides were either poorly charged or neutral at pH 1.9 I attempted to enrich for them by mixing the hydrolysate with cation exchange beads (amberlite 1R-120(H). The rational here was that at pH 1.9 the positively charged amino acids and peptides, which made up the bulk of the hydrolysate, would bind to the beads while the neutral and negatively charged molecules would remain soluble. The result of this experiment is presented in figure 32. Lane C shows the material in the α-casein hydrolysate which would <u>not</u> bind to cation exchange beads at pH 1.9. Clearly, there is an enrichment in this fraction of both the phosphodipeptide and phosphoserine. In addition there is some ninhydrin positive material which runs ahead of

phosphoserine however this species has not been characterized. The BSA hydrolysate even after enrichment shows no material comigrating with putative phosphodipeptide (figure 32, lane A).

The phosphodipeptides were further purified by passage through an anion exchange column as described by Jones and Olson (1980). Essentially, the wash from the cation beads (figure 32, lane C) was reduced to dryness by lyophilization and resuspended in ddH_2O. This material was applied to a Dowex-1 anion exchange column which had been preequilibrated with ddH_2O. The bound material was eluted by stepwise increments in the concentration of a formic acid wash. The separate fractions were concentrated by lyophilization and analysed by high voltage paper electrophoresis. Figure 33 is a photograph of the ninhydrin stained electropherogram and evidently this fractionation provided me with essentially pure putative phosphodipeptide material in the 0.3N formic acid wash.

To verify that this material did represent phosphodipeptides I submitted the 0.3N formic acid wash to one cycle of Edman degradation as described by Jones and Olson (1980). If the material was phosphodipeptides of the structure X-Ser(P) then removal of X by Edman degradation should generate phosphoserine. Figure 34 shows the result of this analysis. The control in this

Figure 33. High voltage paper electrophoresis of α-casein acid hydrolysate fractionated on a Dowex-1 anion exchange column

The material shown in figure 32, lane C was applied to an anion exchange column preequilibrated with ddH_2O. Stepwise increments in formic acid wash were collected as the individual fractions indicated and lyophilized. Samples were applied to the centre of a piece of Whatman 3M paper and subjected to electrophoresis (2000 volts, 30 minutes, pH 1.9) before ninhydrin staining of the electropherogram. (PS), phosphoserine marker; (2N), 2N formic acid wash; (0.3N), 0.3N formic acid wash; (0.13N), 0.13N formic acid wash. (OR), site of sample application.

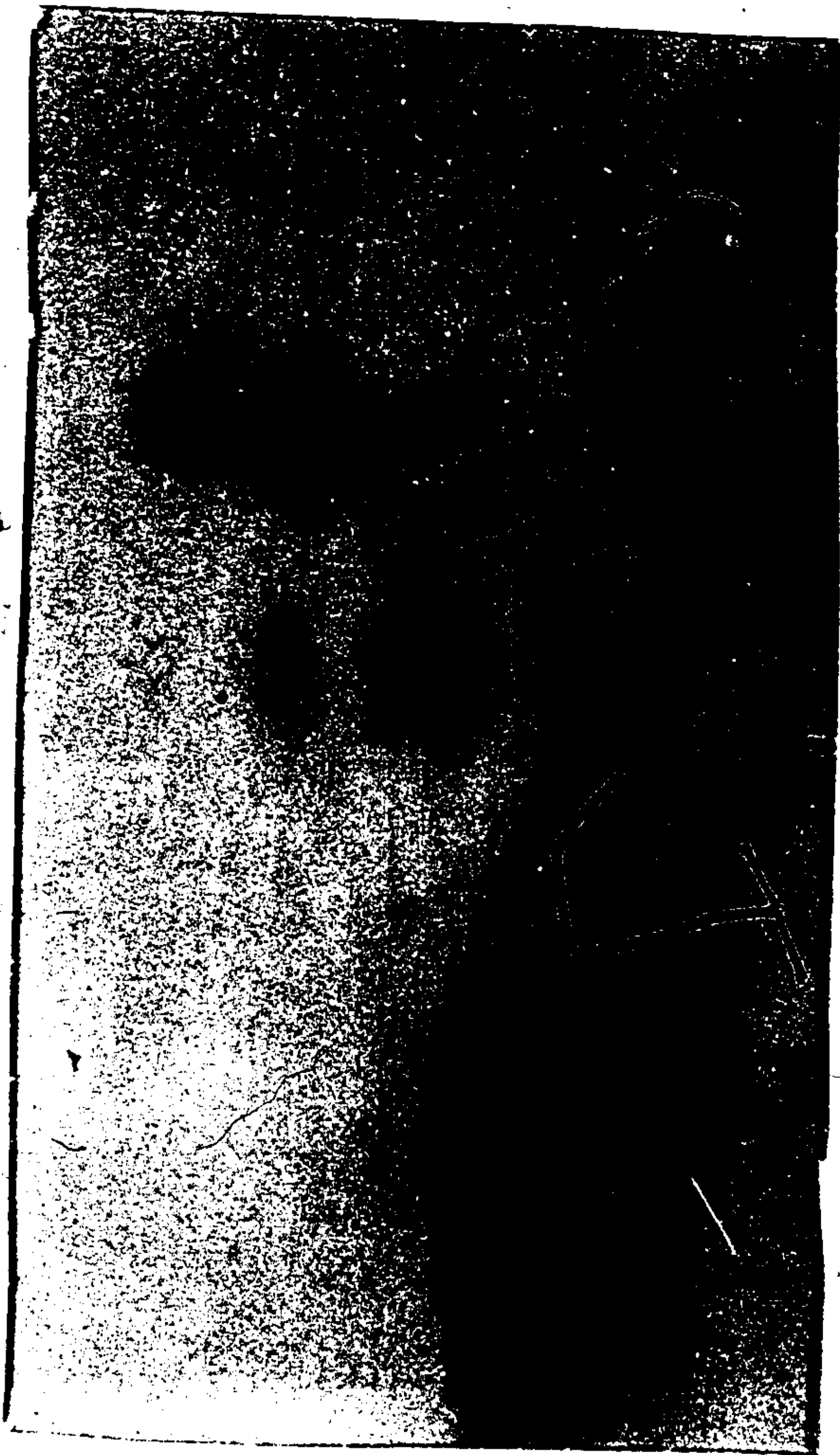
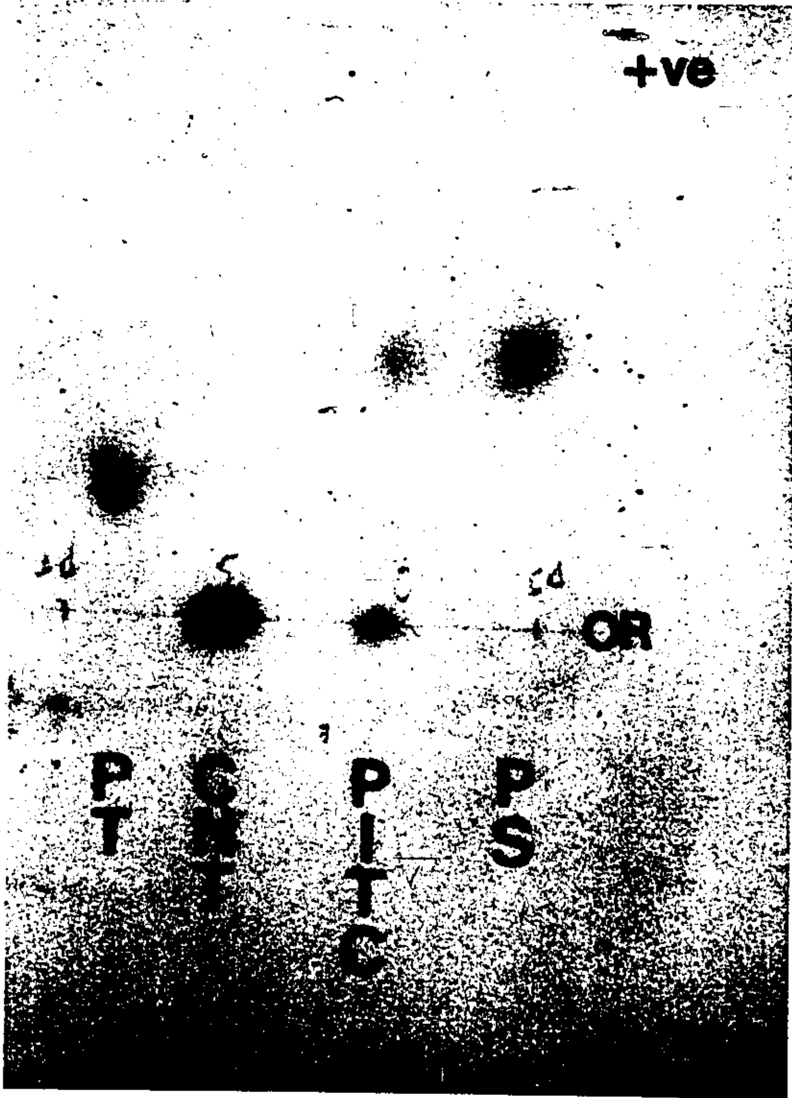

OR
P S
2N
.3N
.13N
P S

Figure 34. High voltage paper electrophoresis of phosphodipeptides after Edman degradation

The phosphodipeptides isolated in the 0.3N formic wash (figure 33) were mixed with 70% pyridine (CNT) or 70% pyridine plus phenylisothiocyanate (PITC) and then reacted under nitrogen as described in Materials and Methods. Following the coupling reaction both samples were exposed to anhydrous trifluoracetic acid to effect cleavage of the PTC amino terminal amino acid from the remainder of the peptide. Reaction by products and PTC derivatives were extracted with ethylacetate and the remaining peptide material was analysed by high voltage paper electrophoresis (pH 1.9, 2000 volts, 90 minutes). (PT), phosphothreonine marker; (CNT), phosphodipeptides plus solvent; (PITC), phosphodipeptides plus solvents and PITC; (PS), phosphoserine marker.


+ve
OR
PT
PITC
PS

case was incubated under identical conditions except that phenylisothiocyanate (PITC) was omitted. Evidently there is significant conversion of putative phosphodipeptide material into ninhydrin positive material which comigrates with phosphoserine. I believe that the material which remains at the origin following one cycle of sequential degradation represents phosphodipeptide which did not react with Edman reagent. Support for this notion can be found from numerous examples in the literature of the aberrant reactivity of phosphorylated amino acids and phosphodipeptides toward PITC. For instance, Jones and Olson (1980) have demonstrated that free phosphoserine and phosphothreonine are totally unreactive toward PITC. Naughton et al., (1960) report that in their hands, Edman degradation of phosphopeptides gives variable results, generating as well as predicted end products, inorganic phosphate and unchanged material. Recently, Mamrack et al., (1979) have shown that during automated sequential degradation of phosphorylated acidic proteins and peptides the release of PITC derivatized amino acids becomes asynchronous as phosphoserine or phosphothreonine is approached.

To perform nearest neighbour analysis the heterogeneous phosphodipeptide had to be further fractionated into unique species. Jones and Olson (1980)

used two dimensional paper electrophoresis to facilitate the separation of phosphodipeptide. However, in my hands, this procedure was inadequate. I found combined electrophoresis and chromatography on cellulose thin layers to be a more satisfactory method of resolution. Figure 35 is an example of a two dimensional cellulose thin layer analysis of phosphodipeptides derived from α-casein. Approximately eight ninhydrin positive spots of variable intensity were detected. This finding was consistent with the seven phosphodipeptides identified by Jones and Olson (1980) and the partial level of α-casein phosphorylation.

I have attempted to identify the amino terminal amino acid in each of the unique phosphodipeptide spots however due to poor recoveries from cellulose thin layers this work has been unsuccessful.

2.7 <u>Phosphodipeptide Analysis of NS Protein</u>

Several authors (Clinton and Huang, 1981; Hsu *et al.*, 1982) have identified serine and threonine as the sites of phosphorylation in Indiana NS protein produced in infected BHK cells. Figure 36 demonstrates that both of these residues are also labelled with ^{32}P-orthophosphate in NS protein produced from infected mouse (L) and human (KB) cells. Other products of limited acid hydrolysis of ^{32}P-labelled NS are inorganic phosphate

Figure 35. Two dimensional analysis of α-casein phosphodipeptides

Phosphodipeptides purified as described in the preceding figure were spotted onto cellulose thin layers (CEL 300) and subjected to electrophoresis (400 volts, 90 minutes, pH 3.5) and chromatography (isopropyl alcohol: formic acid: 0.01 M Na_3PO_4, 40:2:10). The resolved peptides were identified by ninhydrin staining.

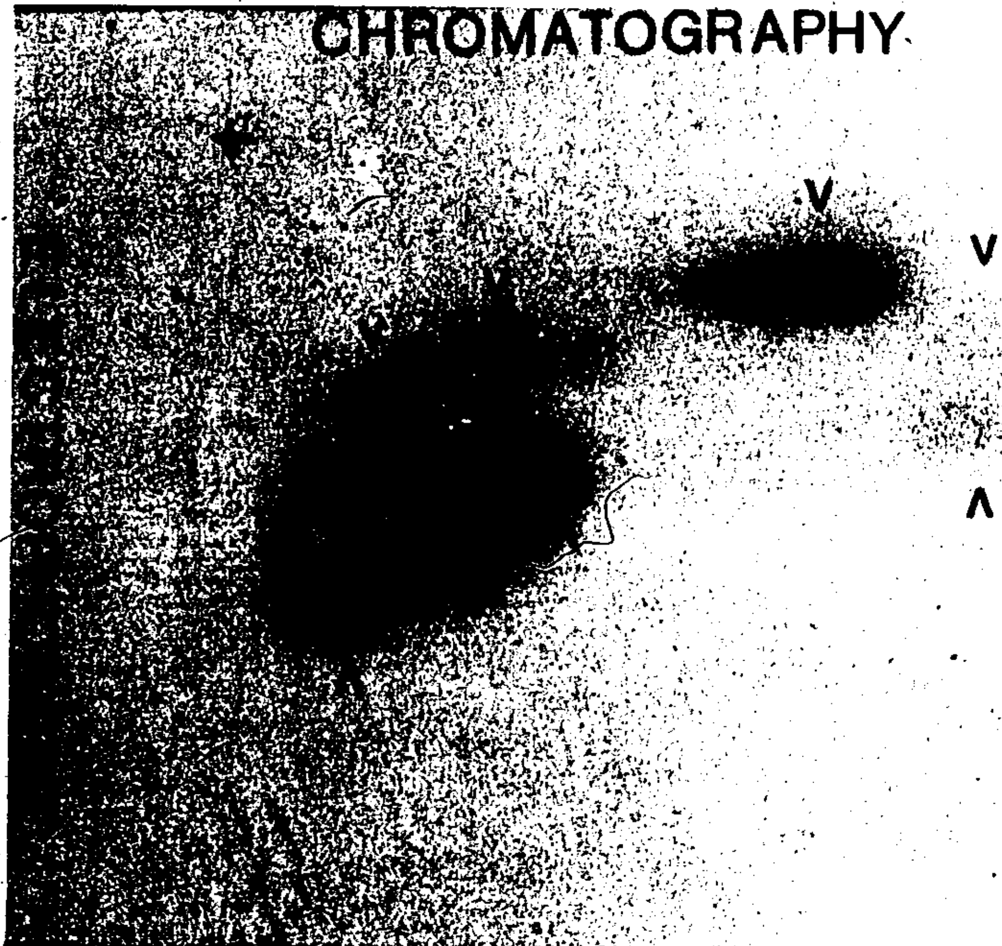
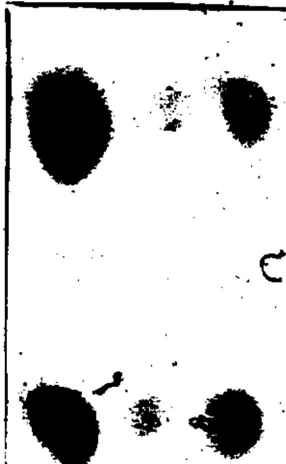
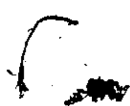

CHROMATOGRAPHY

Figure 36. High voltage paper electrophoresis of ^{32}P-labelled NS protein acid hydrolysates

^{32}P-labelled NS protein produced in BHK, L or KB cells was resolved by preparative SDS-PAGE and autoradiography all as described in Materials and Methods. The labelled NS bands were excised, eluted, TCA precipitated and acetone washed also as described in Materials and Methods. The purified NS proteins were subjected to acid hydrolysis (2NHCl, 110°C, 4 hours) under reduced nitrogen atmosphere. The hydrolysates were filtered through glass fibre filters lyophilized and then spotted onto Whatman 3M paper. Electrophoresis was carried out for ninety minutes at 2000 volts (pH 1.9). The resultant autoradiogram is presented in this figure. Phosphoserine and phosphothreonine were identified by ninhydrin staining.


P_i
PS
PT
O
PP
L
BHK
KB

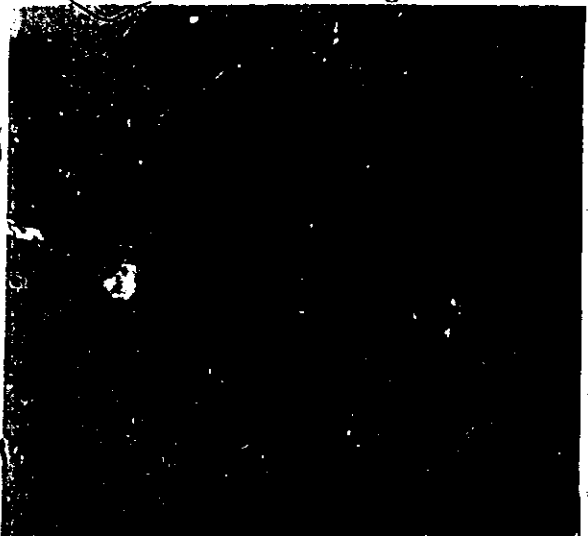


(Pi), positively charged phosphopeptides (PP) and material which remains at the origin (O) and presumed to be phosphodipeptides.

The purification procedure described in the previous section is a realistic approach for isolating pure phosphodipeptides when one can obtain milligram quantities of starting material. However when working with amounts of protein detectable by isotope labelling it becomes technically impractical to use this multistep protocol. I therefore adopted a three dimensional method of thin layer analysis to resolve the putative phosphodipeptides. The first dimension was electrophoresis at pH 1.9 which as shown in figures 32 and 33 separates phosphodipeptides from the other radioactive species. In particular positively charged phosphopeptides move rapidly toward the cathode and can be removed from the electropherogram simply by trimming off the lower edge of the cellulose sheet. The second dimension is another electrophoretic separation, this time at pH 3.5. The phosphodipeptides are now negatively charged (probably because the end COOH is no longer protonated) and begin to resolve into unique spots. The third dimension is chromatography using the conditions described in the legend to figure 37. The positions of phosphoserine and phosphothreonine are verified by inclusion of cold marker

Figure 37. Three dimensional analysis of acid hydrolysed Indiana NS protein

Indiana NS protein labelled with ^{32}P-orthophosphate was purified by preparative SDS-PAGE, eluted, TCA precipitated and acetone washed all as described in Materials and Methods. The purified protein was then subjected to acid hydrolysis (2N HCl, 4 hours, 110°C) under a reduced nitrogen atmosphere. The hydrolysate was filtered through glass fibre filters, lyophilized and resuspended in ddH_2O. A minimum volume (approximately 1 μl) was spotted onto CEL 300 cellulose thin layer and subjected to electrophoresis (90 minutes, 400 volts, pH 1.9) in the first dimension. Autoradiography was performed to locate the assorted labelled species and any positively charged material was removed by trimming the lower edge of the thin layer (position of cut indicated by the horizontal arrow in the extreme right hand panel). The second dimension (2D) was electrophoresis at pH 3.5 (for 90 minutes, 400 volts) and the final dimension (3D) was chromatography for 60 minutes in a isopropyl alcohol:formic acid 0.01M Na_3PO_4 buffer (40:2:10). (KB cell), ^{32}P-labelled NS protein produced in KB cells; (L cell), ^{32}P-labelled NS protein produced in L cells; (PS) phosphoserine; (PT) phosphothreonine; (OR) origin of sample application; (Pi) inorganic phosphate.


KB CELL

P_i
3D
2D
L CELL

P_i
PS
PT
OR
1D

phosphoamino acids and ninhydrin staining. Figure 37 shows the result of this three dimensional analysis using acid hydrolysed NS produced either in human (KB) or mouse (L) cells. Clearly these patterns are identical indicating that probably the same sites are phosphorylated in both preparations. Assuming that all phosphodipeptides have been resolved (although this is not necessarily true) I predict from this analysis there are a minimum of six phosphorylated sites in NS protein. Figure 37 also suggests that there is variability in the level of phosphorylation at different sites within the protein. A similar finding was obtained by Hsu et al., (1982) in their chymotryptic analysis of NS protein. I cannot however exclude the possibility that phosphodipeptides are differentially sensitive to acid hydrolysis and therefore the nature of the amino terminal amino acid may dictate the relative abundance of that species under particular hydrolysis conditions.

The phosphothreonine and phosphoserine residues were scraped from the thin layer shown in figure 37 and quantitated by scintillation counting. In both cases (i.e. KB and L cells) there was approximately four phosphoserine for every one phosphothreonine. Clinton et al., (1979) detected approximately three phosphoserines for every one phosphothreonine in their acid hydrolysates of ^{32}P-labelled NS protein.

2.8 Edman Degradation of (^{32}P) Labelled Phosphodipeptide

It was necessary to prove, as in the case of the α-casein derived phosphodipeptides, that the radioactive material which remained at the origin was of the structure X-Ser(P) or X-Thr(P). To this end, the labelled material which migrated near the origin in figure 36 was eluted with water and concentrated by lyophilization as described in Materials and Methods. The sample resuspended in 50% aqueous pyridine was divided in two, one half subjected to Edman degradation in the presence of PITC and the other half treated identically except that PITC was omitted. Following coupling and subsequent cleavage with TFA, all as described in Materials and Methods, both samples were resuspended in ddH_2O and then extracted with three cycles of water saturated ethyl acetate. The organic fractions were pooled and reduced to dryness under a nitrogen stream while the aqueous phases were concentrated by lyophilization. Previously Mamrack et al., (1979) had shown that amino terminal phosphoamino acids after reaction with PITC form unstable PTC derivatives which are extractable with organic solvents. In these experiments no radioactivity was detected in the organic phase indicating that putative dipeptides did not contain amino terminal phosphorylated residues.

The aqueous phase was analysed by high voltage

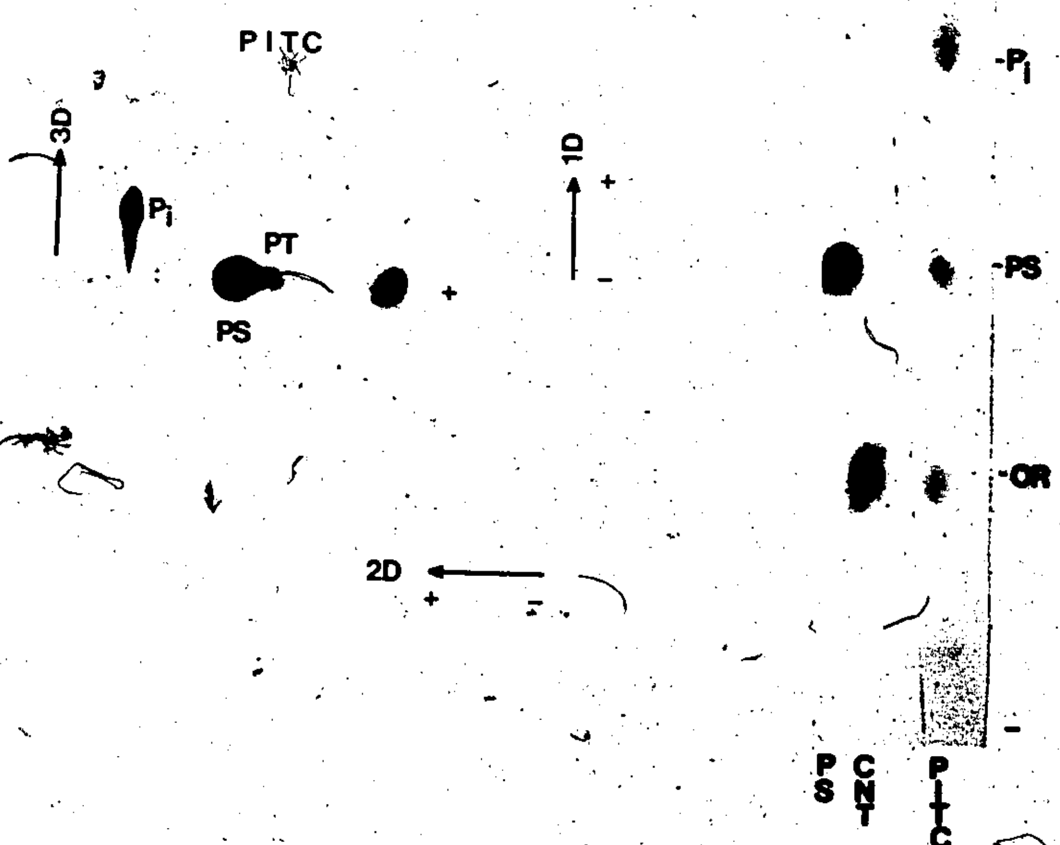
paper electrophoresis and the resultant autoradiogram is shown in figure 38. Radioactive phosphoserine eluted with water from a paper electropherogram was included as a marker phosphoamino acid in the one dimensional analysis. Clearly, in the control sample treatment of phosphodipeptides with all reagents but PITC does not alter their mobility on high voltage paper electrophoresis (figure 38, right hand panel, CNT). Inclusion of PITC in the reaction mix results in the generation of phosphoserine and inorganic phosphate. The identification of phosphoserine as an end product of one cycle of Edman degradation is consistent with the notion that the radioactive starting material is of the structure X-Ser(P). Inorganic phosphate, another by product of this reaction was not reported by Jones and Olson (1980) when they treated phosphodipeptides with Edman reagent. Other authors (Naughton et al., 1960; Mamrack et al., 1979) have however demonstrated that treatment of phosphopeptides with PITC results in nonspecific release of inorganic phosphate.

To determine the nature of the material which remained at the origin of electrophoresis following Edman degradation the PITC sample was subjected to three dimensional analysis as described in the previous section. In the left hand panel of figure 38 the autoradiogram of

Figure 38. Edman degradation of ^{32}P-labelled Indiana phosphodipeptides

In the right hand panel is shown the autoradiograph of the paper electropherograms of ^{32}P-labelled Indiana phosphodipeptides. Radioactive phosphodipeptides identified in figure 36 were eluted with ddH_2O and then concentrated by lyophilization. The dipeptides were reacted with Edman reagent and then analysed by high voltage paper electrophoresis all as described in Materials and Methods. (OR), point of sample application; (PS), radioactive phosphoserine eluted from figure 36; (CNT), radioactive dipeptides treated with all reactants except Edman reagent; (PITC), dipeptides reacted with phenylisothiocyanate; (Pi) inorganic phosphate.

In the left hand panel is shown the autoradiograph of a three dimensional peptide analysis. The sample indicated as PITC in the right hand panel was spotted onto a piece of cellulose thin layer (+ marks the point of application) and then subjected to three dimensional analysis as described in figure 37. (PT), phosphothreonine; (PS), phosphoserine; (Pi) inorganic phosphate.


PITC
3D
P_i
PT
PS
1D
2D
$-P_i$
-PS
-OR
PS
CNT
PITC

the three dimensional analysis is presented. Comparison of this figure with figure 37 reveals that no additional spots other than those previously attributed to phosphodipeptides are generated following Edman degradation. This finding is consistent with the idea (see section 2.6) that the material present at the origin following Edman degradation represents phosphodipeptides which did not undergo coupling under the conditions of this analysis.

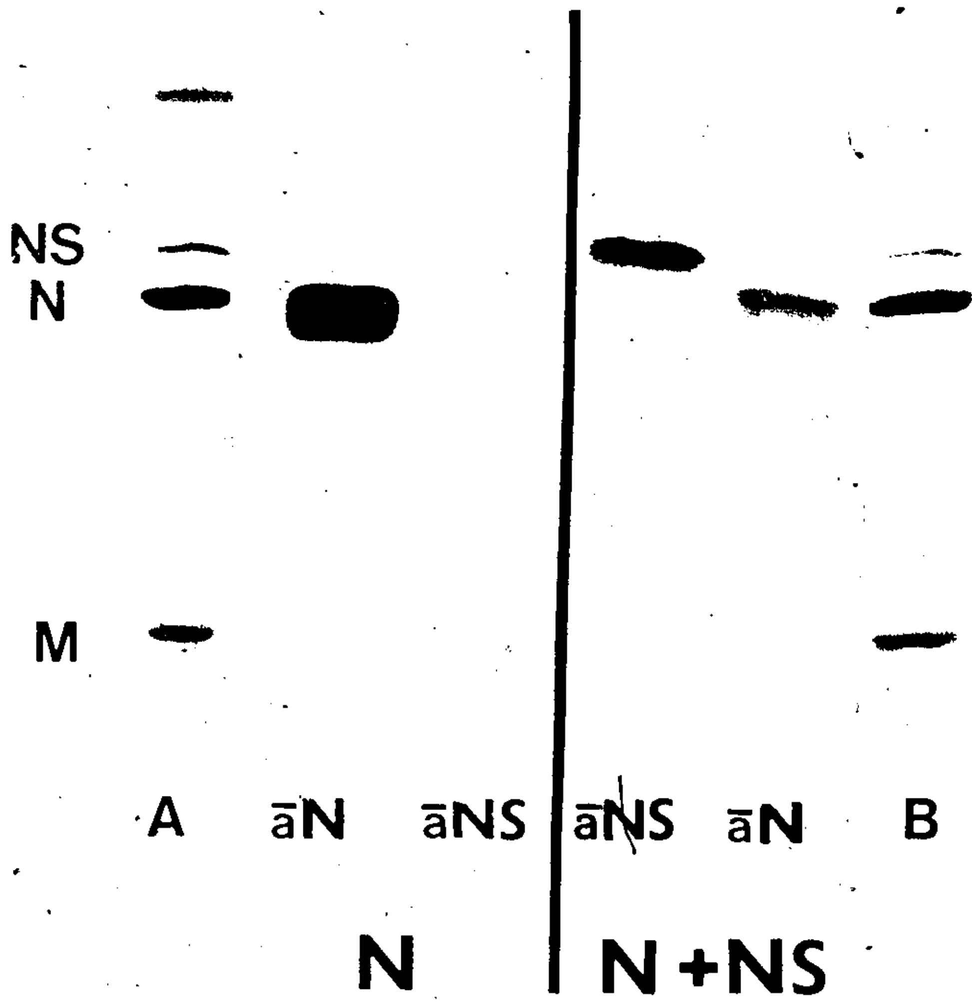
3. Protein Kinase Activity Associated with NS Protein

3.1 Characterization of Monospecific Antisera

Since the activity of NS protein is regulated by phosphorylation (see Introduction, section 7b) it seemed possible that the kinase responsible might be tightly complexed to its viral phosphoprotein substrate. I therefore tried to isolate the NS kinase by immunoprecipitation of protein complexes from infected cells and virions. To this end monospecific antisera were prepared against purified denatured viral proteins as described in Materials and Methods. The putative monospecific antibodies were tested for crossreactivity by immunoprecipitation of pure or mixed antigen preparations. N and NS proteins labelled *in vivo* with ^{35}S-methionine, were resolved on SDS polyacrylamide gels and then separately purified using the elution technique described in Materials and Methods. In figure 39 it can be seen that gel purified

Figure 39. Specificity of rabbit anti-NS and anti-N sera

Sera collected from rabbits injected with Indiana N protein or NS protein which had been purified from SDS-PAGE served as the source of anti-N (āN) or anti-NS (āNS) respectively. ^{35}S-methionine labelled N and NS proteins were prepared and purified on SDS-PAGE as described in Materials and Methods and the appropriate antiserum was reacted with the purified N protein (N) or with a mixture of purified N and purified NS protein (N + NS). The resulting precipitate was analysed on SDS-PAGE and autoradiography along with marker viral proteins (lanes A and B). The position of the viral proteins NS, N and M is indicated.


NS
N
M
A
āN
āNS
āNS
āN
B
N
N +NS

N protein can be immunoprecipitated with anti-N but not anti-NS serum. When a mixture of N and NS protein was probed with antisera, only the homologous protein was precipitated, (i.e. anti-NS precipitated only NS protein; anti-N precipitated only N protein). In similar experiments the anti-NS serum and anti-N serum showed no reactivity toward G or M proteins purified from acrylamide gels, figures 40 and 41.

Having shown that the antisera showed no crossreactivity when challenged with denatured antigens, I investigated the possible aggregation of viral proteins dissociated from purified virions in the following experiment. Virions labelled with ^{35}S-methionine were disrupted with high salt solubilizer (HSS) and separated into soluble and RNP fractions by centrifugation on 40% glycerol pads. The efficiency of fractionation is demonstrated in figure 42 by the fact that only a trace amount of N protein is present in the supernatant fraction and only trace amounts of the other proteins are present in the pellet fraction. Treatment of the supernatant fraction (diluted to 0.15 M NaCl) with anti-NS serum immunoprecipitated a complex containing L, NS, N and M proteins while anti-N serum co-precipitated N and M proteins. The two sera thus appear to discriminate between different protein aggregates in this mixture.

Figure 40. Reactivity of monospecific antisera with denatured viral proteins

^{35}S-methionine labelled Indiana proteins NS, M, G and N were separately purified by SDS-PAGE as described in Materials and Methods and then individually reacted with a putative monospecific antiserum. The resulting immunoprecipitate was analysed on SDS-PAGE and autoradiography. In this figure, the antigen (Ag) and antibody (Ab) involved in the reaction are indicated at the bottom of each lane. Infected cell extracts labelled with ^{35}S-methionine are included in the outside lanes to verify the positions of the purified proteins.

G

NS

N

M

Ag	N	N	G	G	NS+N	NS+N	M	M
Ab	αN	αNS	αG	αN	αNS	αN	αM	αN

Figure 41. Reactivity of monospecific antisera with denatured viral proteins

^{35}S-methionine labelled Indiana proteins NS, M, G and N were separately purified by SDS-PAGE as described in Materials and Methods and then individually reacted with a putative monospecific antiserum. The resulting immunoprecipitate was analysed on SDS-PAGE and autoradiography. In this figure, the antigen (Ag) and antibody (Ab) involved in the reaction are indicated at the bottom of each lane. Infected cell extracts labelled with ^{35}S-methionine are included in the outside lanes to verify the positions of the purified proteins.

I attempted to deplete N protein from the high salt solubilized supernatant by treatment with anti-N serum and removal of the immunoprecipitate. This approach was unsuccessful as clearly reprecipitation of the supernatant with anti-NS yielded the same assemblage of proteins (figure 42, āN + āNS).

I next used the sera to examine the natural aggregates of viral proteins present in the infected cell cytoplasm. As can be seen in figure 43 monospecific anti-NS serum immunoprecipitates a complex composed of L, NS and N proteins from infected cell extracts. Since in these experiments no attempt was made to separate nucleocapsid structures from cytoplasmic proteins, it seems likely that the immunoprecipitated complex represents NS protein bound to ribonucleoprotein (RNP) structures. Anti-N serum also precipitates a protein assemblage which can be distinguished from that precipitated by anti-NS in that anti-N serum co-precipitates M protein and other unidentified proteins from the unfractionated L cell extract. In these and all other experiments neither immune serum treatment of uninfected cells nor non-immune serum treatment of infected cells immunoprecipitated ^{35}S-methionine labelled material or significant kinase activity, (see figures 44 and 47).

Hsu *et al.*, (1979) have suggested that approximately

Figure 42. Antibody precipitation of disrupted VSV virions

Purified virions labelled during growth ^{35}S-methionine were disrupted with high salt solubilizer (HSS) and fractionated by centrifugation on a 40% pad into the supernatant and ribonucleoprotein fractions. The labelled proteins in each fraction, (HSS SUP) and (HSS RNP) respectively, were analysed on SDS-PAGE. The supernatant fraction was diluted to 0.15 M NaCl and reacted with either anti-N ($\bar{a}$N) or anti-NS ($\bar{a}$NS) serum and the precipitate analysed on SDS-PAGE. The material remaining after removal of the anti-N precipitate was tested first with protein A-sepharose beads and then the soluble material treated with anti-NS serum. The resulting precipitate was examined on SDS-PAGE ($\bar{a}$N + $\bar{a}$NS).

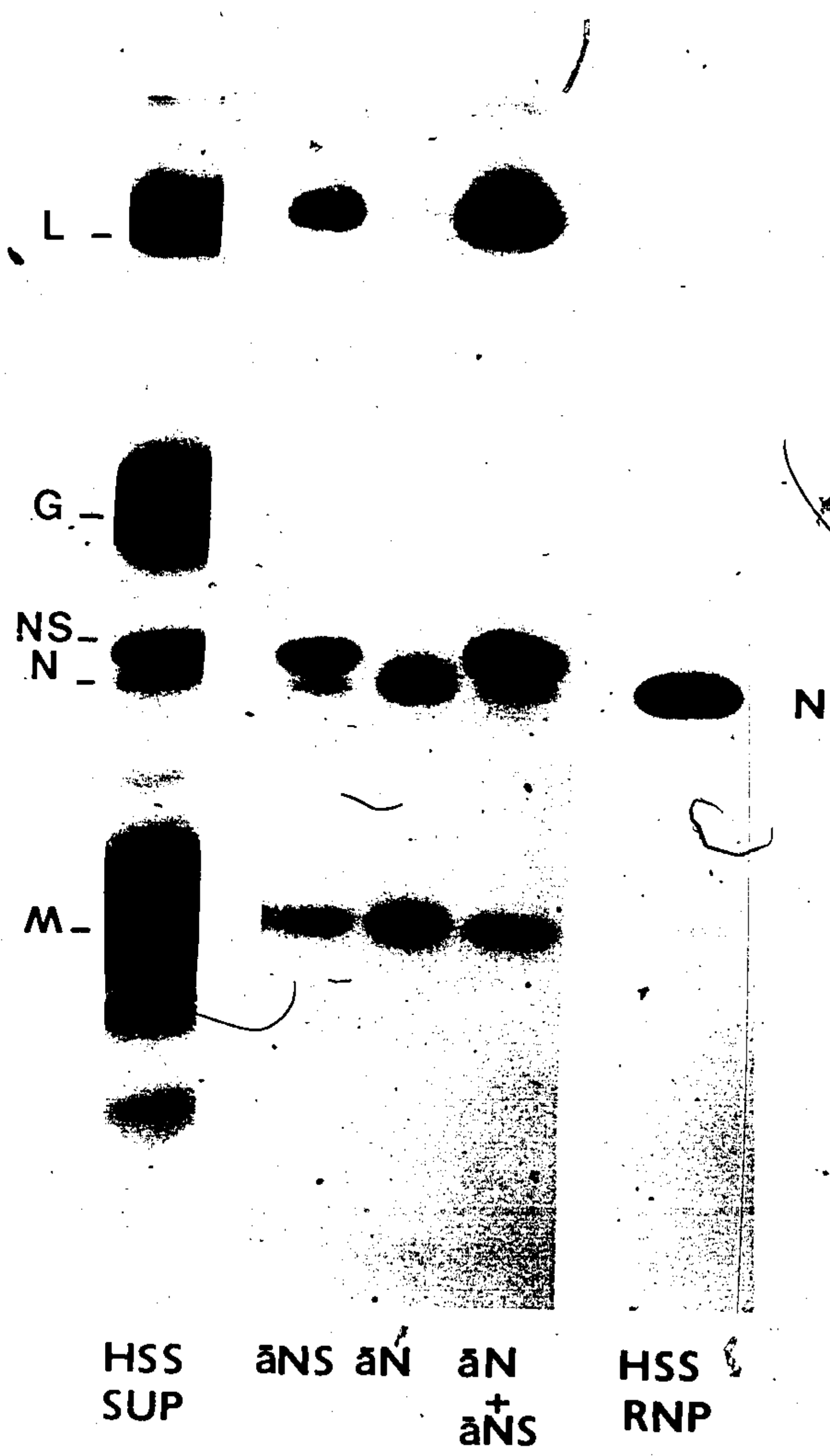

L
G
NS
N
M
N
HSS SUP
āNS
āN
āN + āNS
HSS RNP

Figure 43. <u>Antibody treatment of ^{35}S-methionine labelled infected cell extracts</u>

An Indiana infected cell monolayer was labelled with ^{35}S-methionine four hours post infection and then lysed for immunoprecipitation as described in Materials and Methods. Monospecific antisera were then used to immunoprecipitate the extracts all as described in Materials and Methods. (INF CELL), infected cell extract; ($\bar{a}$NS), anti-NS immunoprecipitate; ($\bar{a}$N), anti-N immunoprecipitate.

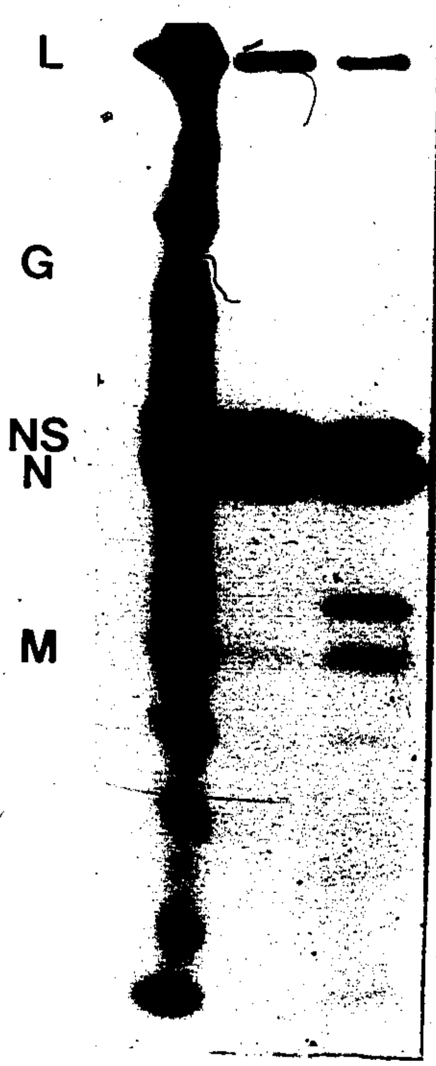

L
G
NS
N
M
INF CELL
āNS
āN

Figure 44. Antibody treatment of ^{35}S-methionine labelled infected and uninfected L cells

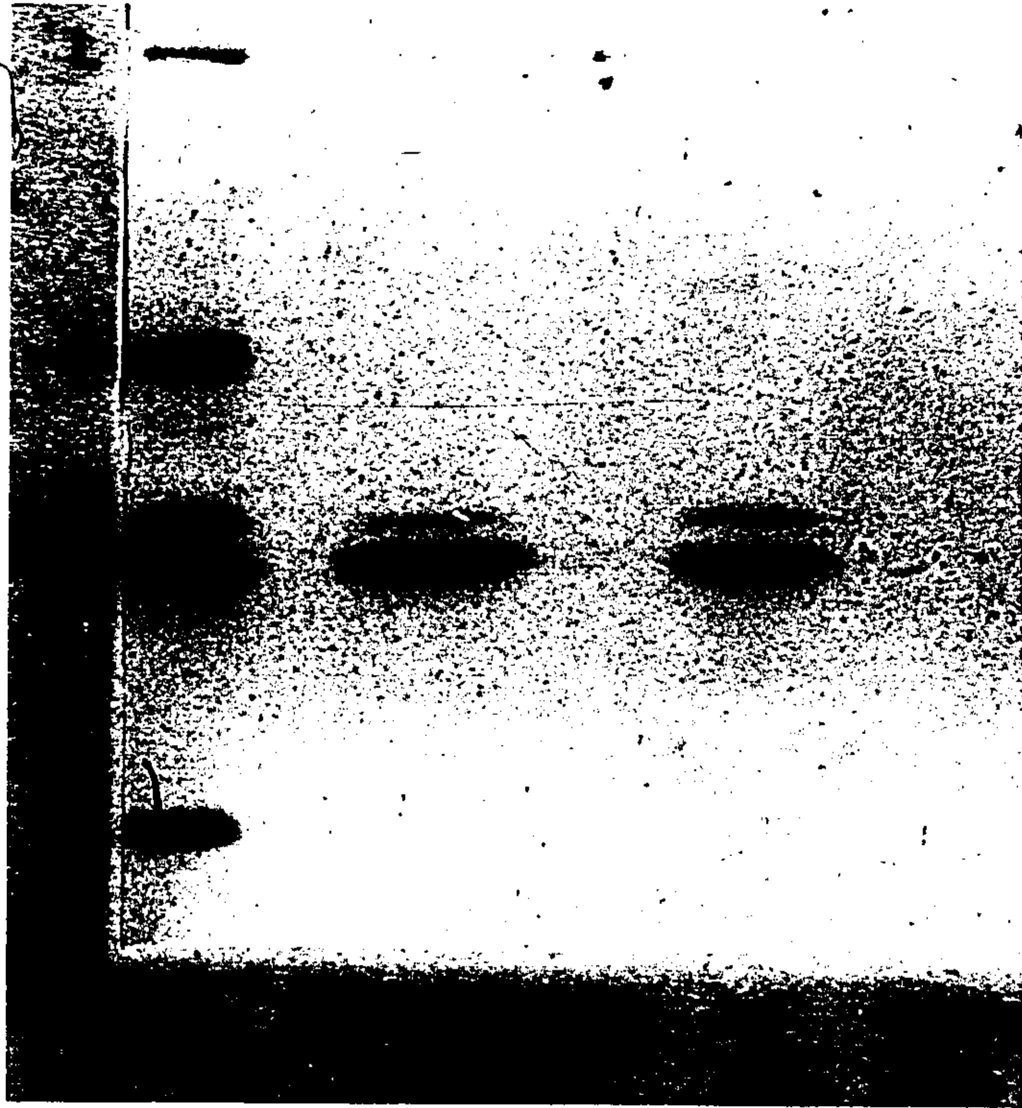
Anti-NS and anti-N sera raised against gel purified viral proteins were reacted with ^{35}S-methionine labelled infected or uninfected L cell extracts as described in Materials and Methods. The immunoprecipitates were then analysed on SDS-PAGE containing 0.13% bis methylene acrylamide.

(inf $\bar{a}$N), ^{35}S-methionine labelled infected L cell extract reacted with anti-N serum.

(mock $\bar{a}$N), ^{35}S-methionine labelled uninfected L cell extract reacted with anti-N serum.

(inf $\bar{a}$NS), ^{35}S-methionine labelled infected L cell extract reacted with anti-NS serum.

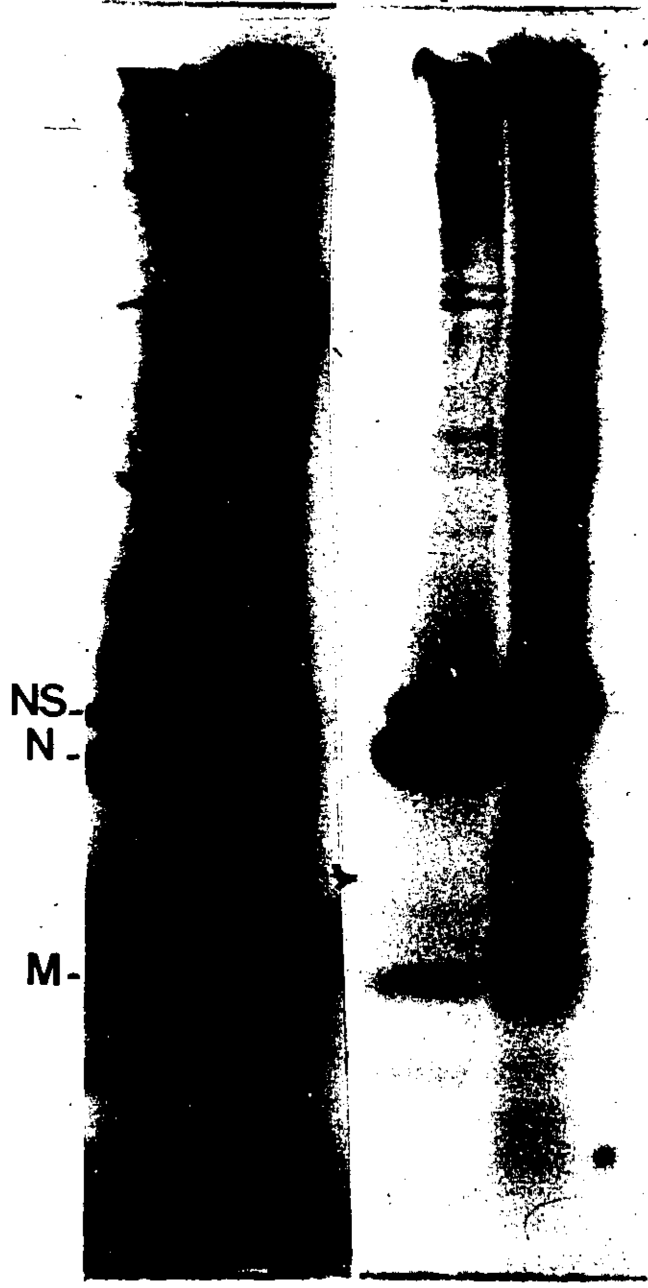
(mock $\bar{a}$NS), ^{35}S-methionine labelled uninfected L cell extract reacted with anti-NS serum.



75% of all NS protein synthesized during infection remains in a soluble cytoplasmic protein pool and not in nucleocapsid structures. To investigate the association of NS with other proteins in different cellular fractions two separate antigen sources were prepared. Infected cell extracts prepared in STM as described in Materials and Methods were layered over a 40% glycerol pad (in STM) and separated into the supernatant (material on top of the pad) and the pellet or RNP fraction. As seen in figure 45 both fractions contain NS, N and M protein although the ratio of N to NS is greater in the RNP than the SUP fraction. The material in each fraction was reacted with anti-NS serum. As can be seen in figure 46, (lane A) anti-NS serum immunoprecipitates a complex from the supernatant extract which differs in its N/NS ratio from complexes immunoprecipitated from the RNP fraction, (lane B). The RNP immunoprecipitates contain a high N:NS ratio characteristic of RNP (Emerson and Yu, 1975; Hsu et al., 1979) while immunoprecipitation of the supernatant fraction yields complexes with a N:NS ratio approaching one, consistent with the idea that these are not nucleocapsid structures. It has been my repeated finding in these and susequent experiments that NS protein cannot be immunoprecipitated from cytoplasmic extracts completely free of material migrating with N protein.

Figure 45. Fractionation of infected cell cytoplasmic extracts

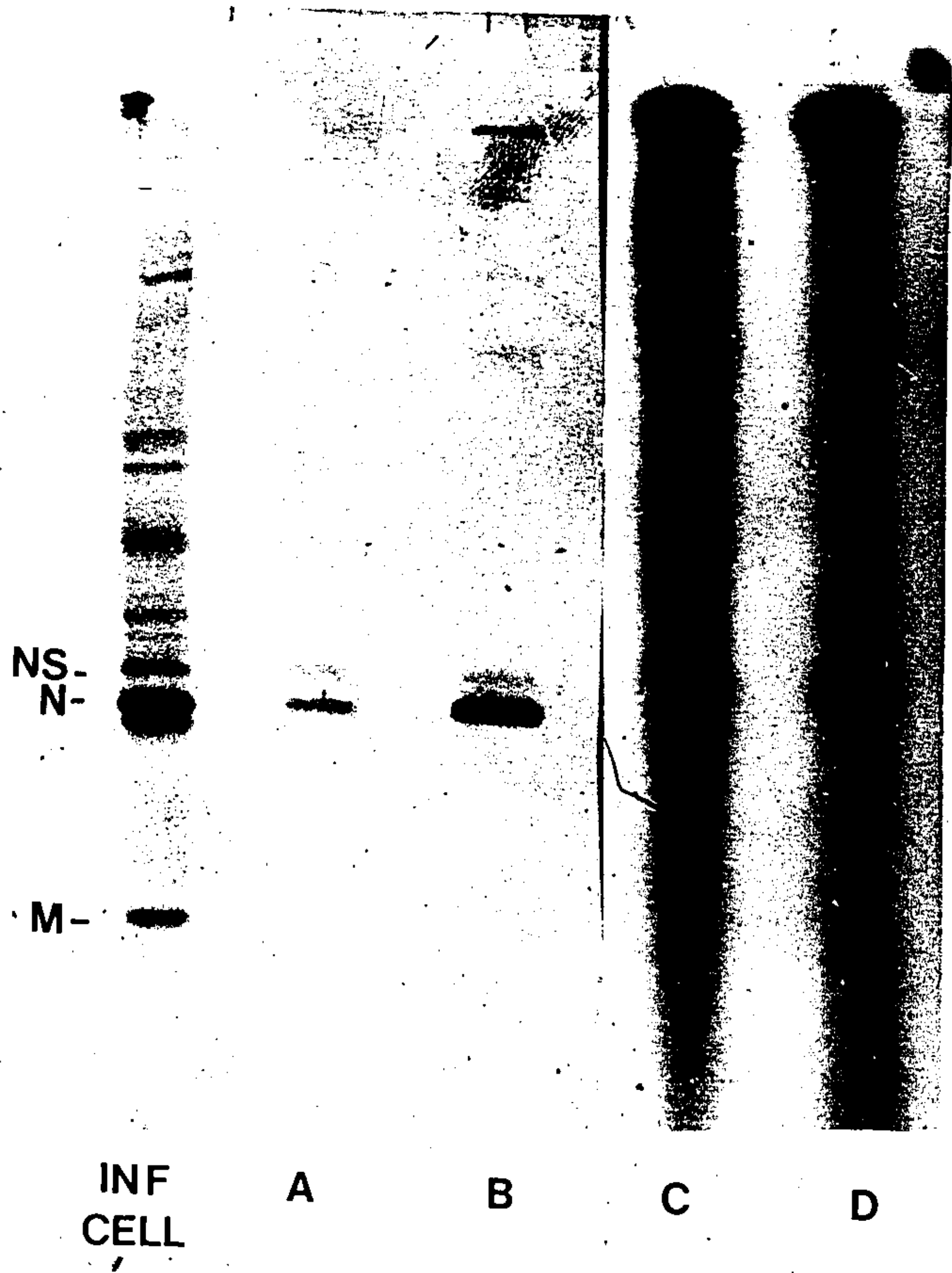
VSV-infected L cells labelled with ^{35}S-methionine or ^{32}P-orthophosphate were disrupted by homogenization in STM buffer and the cytoplasmic material fractionated by centrifugation on a 40% glycerol pad all as described in Methods. (SUP), the ^{35}S or ^{32}P-labelled material which remains on top of the 40% glycerol pad; (RNP), the ^{35}S or ^{32}P-labelled material which pellets through the 40% glycerol pad.



NS-
N-
M-
35S 32P
SUP
35S 32P
RNP

Figure 46. Immunoprecipitates from supernatant and pellet fractions of infected cells and their associated protein kinase activity

The ^{35}S-methionine labelled supernatant and pellet (RNP) fractions recovered from the experiment shown in figure 45 were antibody precipitated and then tested for kinase activity as described in Materials and Methods. (A), ^{35}S-labelled supernatant fraction reacted with anti-NS, (B), ^{35}S-labelled RNP fraction reacted with anti-NS; (C), the immunoprecipitate shown in lane A incubated with ^{32}P-γ-ATP; (D), the immunoprecipitate shown in lane B incubated with ^{32}P-γ-ATP. Lanes C and D were exposed through cardboard to eliminate exposure due to ^{35}S decay.


NS-
N-
M-
INF CELL
A
B
C
D

3.2 Kinase Activity Associated with Immunoprecipitates of Infected Cells

As demonstrated in the preceding section (3.1) anti-NS and anti-N serum immunoprecipitate complexes from unfractionated infected cells which contain both NS and N protein (see figures 43 and 44). Immunoprecipitates of this type were prepared from unlabelled cells and assayed for kinase activity by incubation with ^{32}P-γ-ATP as described in Materials and Methods. The reaction products were then analysed by SDS-PAGE with 0.26% bis in the resolving gel. Under these gel conditions NS migrates slightly faster than N protein. Figure 47 is a presentation of the autoradiogram of this gel and clearly kinase activity is associated with both anti-N and anti-NS immunoprecipitates. On the other hand, anti-N or anti-NS treatment of mock infected cells did not precipitate significant kinase activity. Furthermore, mixing of a non-viral antigen antibody complex (rabbit Ig : goat anti-rabbit Ig) with uninfected cells did not coprecipitate kinase activity.

The following experiment was designed to determine if the protein kinase activity was associated with soluble NS:N complexes as well as nucleocapsid structures. ^{35}S-methionine labelled NS protein, either free or bound to nucleocapsids, was prepared as outlined in the legend to figure 46. One

Figure 47. Kinase activity associated with anti-NS and anti-N immunoprecipitates

The monospecific antisera, anti-N and anti-NS were reacted with cytoplasmic extracts from unfractionated Indiana infected L cells. The resultant immunoprecipitates were incubated with ^{32}P-γ-ATP as described in Materials and Methods and then analysed on SDS-PAGE containing 0.26% bis methylene. Under these gel conditions NS runs below N protein as shown in the outside marker lane (^{35}S-methionine labelled infected cell extract). (m,āN), mock infected L cells reacted with anti-N serum and then incubated with ^{32}P-γ-ATP; (i,āN), infected L cells reacted with anti-N serum and then incubated with ^{32}P-γ-ATP; (m,āNS), mock infected L cells reacted with anti-NS serum and then incubated with ^{32}P-γ-ATP; (i,āNS), infected L cells reacted with anti-NS serum and then incubated with ^{32}P-γ-ATP;)m,AgAb), mock infected L cells reacted with non immune rabbit immunoglobulin, goat anti-rabbit immunoglobulin, and then incubated with ^{32}P-γ-ATP.

.26% bis
N
NS
M
m i m i m
āN āN āNS āNS AgAb

half of each immunoprecipitate was resuspended in sample buffer, while the remainder was incubated with ^{32}P-γ-ATP as described in Materials and Methods. All four samples were analysed by SDS gel electrophoresis and autoradiography (figure 46) under conditions which allowed me to identify ^{32}P-labelled material in the presence of ^{35}S-methionine labelled material. Clearly, protein kinase activity is associated with both forms of NS protein (free and RNP bound). Aliquots of these dually labelled samples were also analysed by electrophoresis on a 0.26% bis gel and autoradiography under conditions which did not discriminate between ^{32}P-labelled and ^{35}S-labelled material, (figure 48). In this analysis the heavy chain of immunoglobulin (HC, identified by Coomassie blue staining) runs slower than N protein and clearly is not phosphorylated in this assay.

3.3 Kinase Activity Phosphorylates NS at Serine Residues

The material which became labelled with ^{32}P-γ-ATP and comigrated with NS protein was eluted from the gel and acid hydrolysed as described in Materials and Methods. The hydrolysate was then spotted onto Whatman 1 MM paper and analysed at either pH 1.9 or pH 3.5 to assure separation of phosphoserine, phosphothreonine and phosphotyrosine. At pH 3.5, cold phosphotyrosine is separable from the other marker phosphorylated amino acids,

Figure 48. Products of *in vitro* kinase reaction analysed on a 0.26% bis acrylamide gel

The kinase reaction products described in figure 46 were analysed by SDS-PAGE in gels containing 0.26% bis acrylamide. Under these gel conditions NS runs below N protein. Autoradiography was performed by directly placing the X-ray film against the dried gel and as a result the ^{35}S-methionine labelled N protein is detected in the rnp lane. In this particular analysis non-equivalent amounts of rnp and sup sample were applied to the gel. (rnp), ^{35}S-methionine labelled anti-NS immunoprecipitate from the high speed ribonucleoprotein fraction after incubation with ^{32}P-γ-ATP; (sup), ^{35}S-methionine labelled anti-NS immunoprecipitate from the high speed supernatant after incubation with ^{32}P-γ-ATP.

.26% bis
-HC
N
NS
rnp sup

and as seen in figure 49 there is no ^{32}P-label associated with phosphotyrosine. To achieve good separation of phosphothreonine and phosphoserine the hydrolysate was rerun at pH 1.9. It is evident from this analysis that only serine is labelled during these *in vitro* kinase assays.

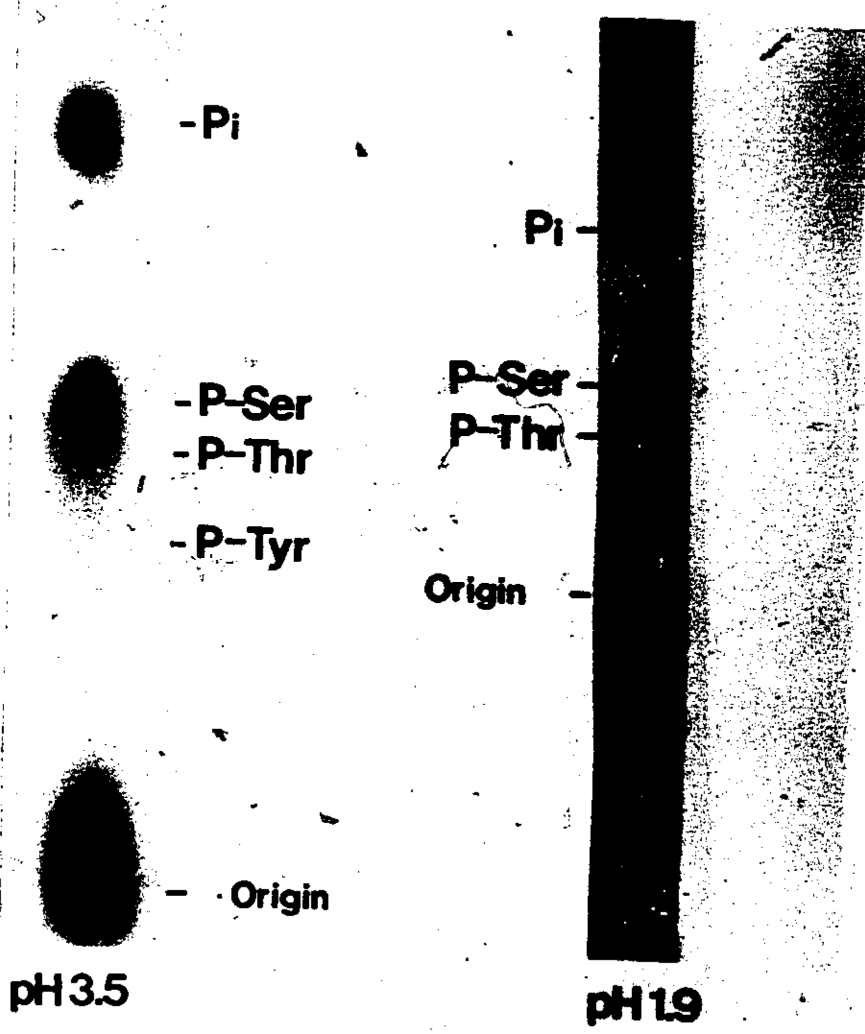
3.4 Phosphate Does Not Cycle Off in Immunoprecipitates

The following experiment was designed to determine if the sites phosphorylated *in vitro* by the NS associated kinase could also be dephosphorylated *in vitro*. That is, could cycling on and off of phosphate moieties be detected in these anti NS:NS immunoprecipitates. This may occur by one of two mechanisms; (1) the kinase could affect dephosphorylation as has been observed with the reversible autophosphorylation of cAMP dependent protein kinase from bovine cardiac muscle (Rubin and Rosen, 1975) (2) a phosphatase may be coprecipitated with the kinase and Ab:Ag complex.

Essentially an anti-NS immunoprecipitate from an infected cell extract was incubated with ^{32}P-γ-ATP as described in Materials and Methods. After ten minutes of incubation at 32°C the beads were pelleted and then resuspended in cold protein kinase buffer lacking ATP, repelleted and then split into two fractions. One aliquot was immediately boiled in SDS sample buffer to eliminate any further enzyme activity while the other was again

Figure 49. Identification of the amino acid phosphorylated in the protein kinase assay of anti-NS immunoprecipitate

The ^{32}P-labelled bands comigrating with the NS protein in figure 46 were eluted from the gel and acid hydrolysed as described in Materials and Methods. The hydrolysate was then spotted onto Whatman 1 MM paper and subjected to electrophoresis either at pH 1.9 or pH 3.5 for 1 hour at 2000 Volts. The ^{32}P-labelled material was detected by autoradiography and the cold phosphoamino acid markers were visualized by ninhydrin staining.



-Pi
-P-Ser
-P-Thr
-P-Tyr
Origin
pH 3.5
Pi
P-Ser
P-Thr
Origin
pH 1.9

incubated for 20 minutes at 32°C with kinase buffer containing unlabelled ATP. The reaction was terminated as described above and both samples were analysed by SDS-PAGE. In figure 50 it is evident that radioactive phosphates added *in vitro* by the NS associated kinase cannot be chased under the conditions of this assay. Although there appears to be a slight difference in intensity between the two bands when they were directly excised from the gel and counted there was an insignificant difference between the two (315 cpm for pulse; 295 cpm for chase).

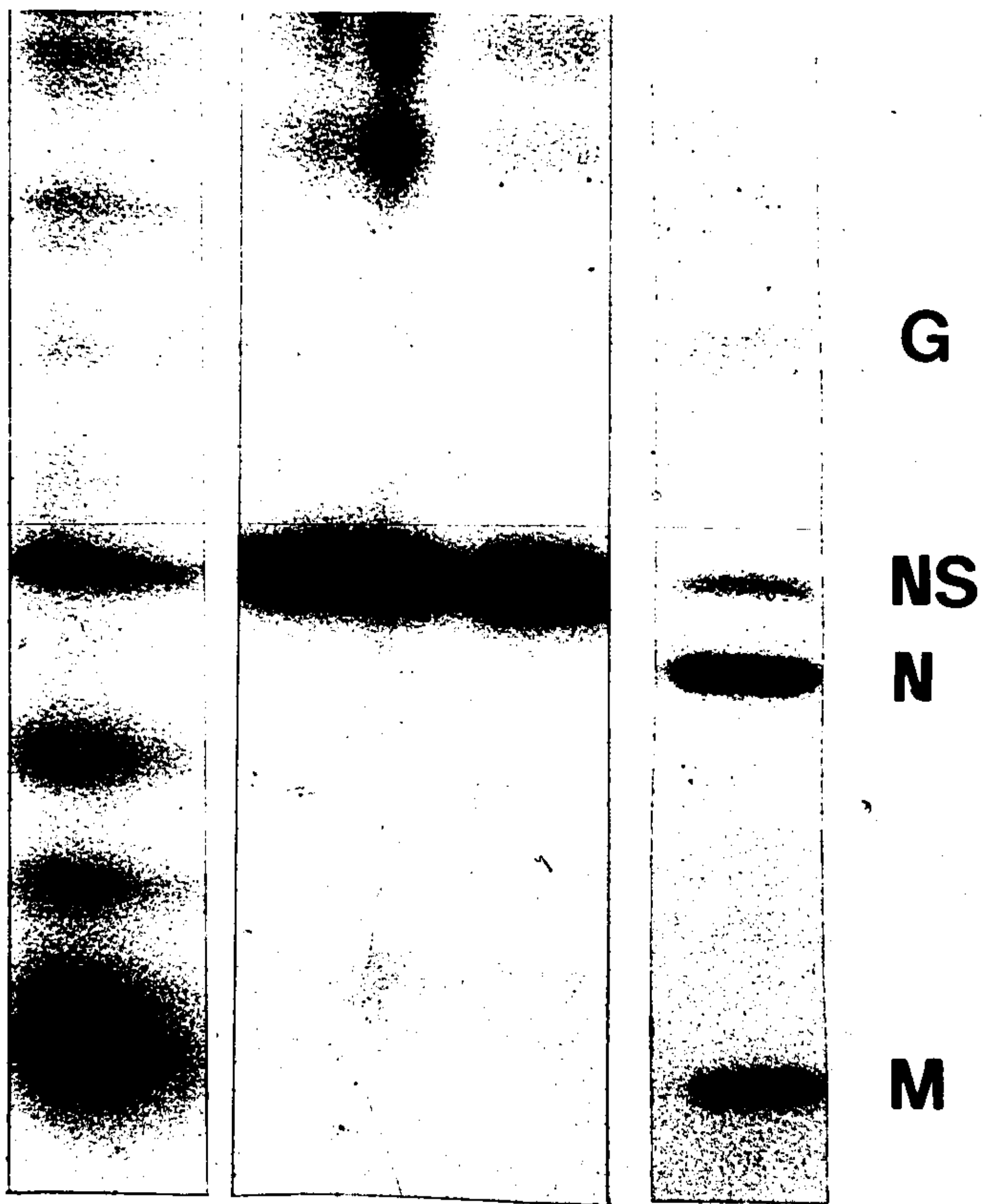
3.5 Kinase Activity of Immunoprecipitates from HSS-treated VSV Virions and Cytoplasmic Extracts

In vitro transcription extracts, dependent upon protein kinase activity, have been derived from disrupted VSV virions (Watanabe *et al.*, 1974; Witt and Summers, 1980). I therefore sought to determine if there is an association between virion NS and protein kinase activity. The experiments outlined in section 3.2 were essentially repeated, except that the antigen source was HSS supernatants from disrupted virions. The anti-NS immunoprecipitate identified in figure 42 was incubated with ^{32}P-γ-ATP and then analysed by SDS-gel electrophoresis as shown in figure 51.

In this and subsequent experiments, it appears

Figure 50. <u>In vitro</u> pulse-chase experiments with ^{32}P-γ-ATP

An anti-NS immunoprecipitate of an unfractionated infected cell extract was prepared as described in Materials and Methods. The immunoprecipitate was washed repeatedly with RIPA buffer and then protein kinase buffer before incubation with ^{32}P-γ-ATP as described in the preceding figures. Following a pulse period of ten minutes at 32°C the immunoprecipitate was again collected by centrifugation and washed with cold protein kinase buffer lacking ATP. The sample was then divided in half, one aliquot boiled in sample buffer and the other chased in protein kinase buffer containing cold ATP for twenty minutes. The samples were then analysed by SDS-PAGE (0.13% bis). (^{32}P), ^{32}P-labelled infected L cell extract; ($\bar{a}$NS), anti-NS immunoprecipitate pulsed with ^{32}P-γ-ATP; ($\bar{a}$NS + ATP) anti-NS immunoprecipitate pulsed with ^{32}P-γ-ATP and chased with unlabelled ATP; (^{35}S), ^{35}S-methionine labelled infected L cell extract.

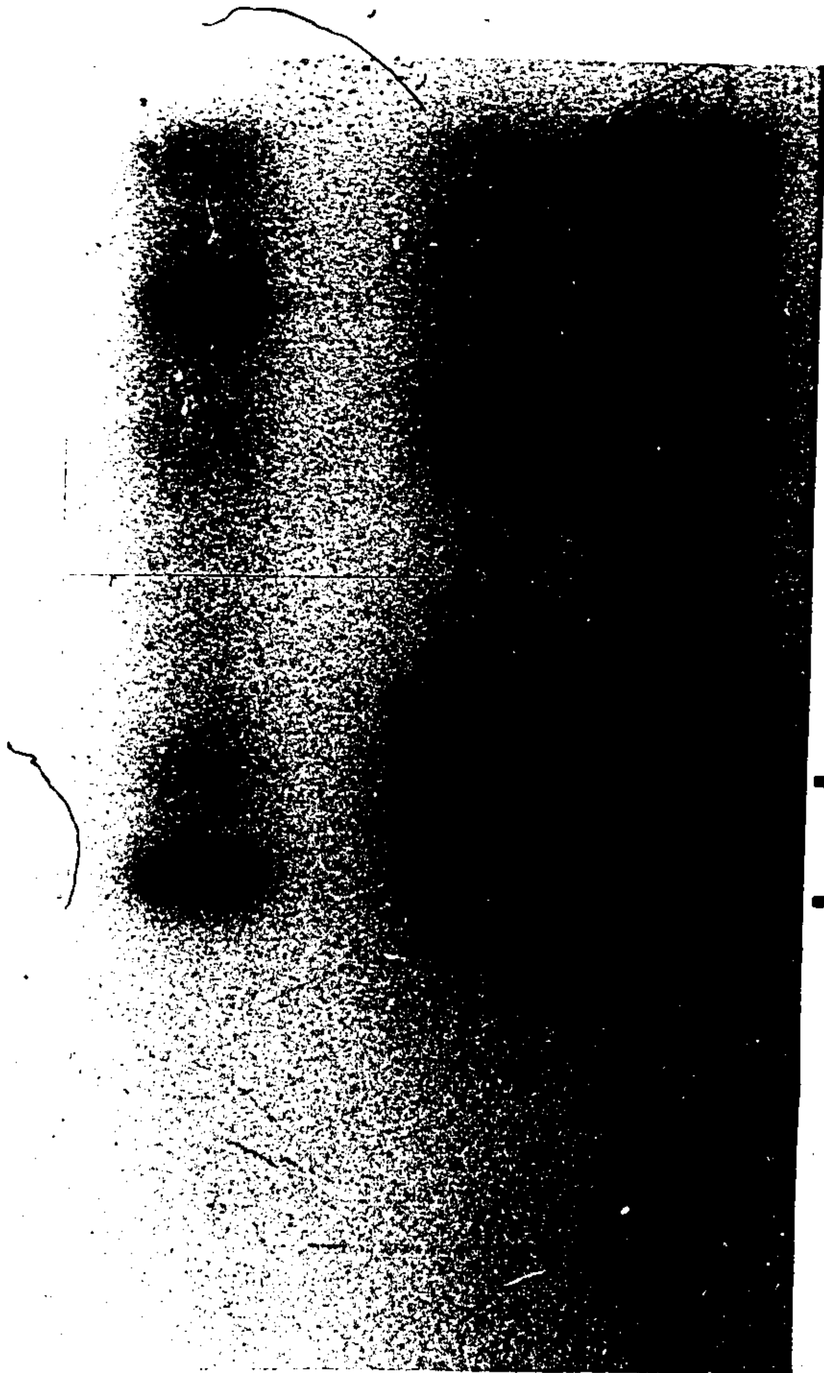

G
NS
N
M
32P
āNS
āNS
+
ATP
35S

as if HSS treatment of protein extracts results in altered kinase specificity. It is evident in figure 51 that not only is there phosphorylation of NS protein, but as well phosphorylation of material comigrating with the heavy chain of immunoglobulin.

Clinton et al., (1982), have demonstrated that the endogenous sarc protein of BHK cells is incorporated into mature virions. I have repeated this finding using anti-sarc serum (gift of Dr. P. Branton) to immunoprecipitate the HSS supernatant derived from virus grown in L cells (figure 51, lane C). Clearly, there is phosphorylation in these immunoprecipitates of anti-sarc heavy chain (identified by Coomassie blue staining) providing evidence for the presence of sarc protein in VSV virions. Further experiments suggest a possible association between sarc and NS protein. Protein complexes immunoprecipitated with anti-NS were washed and incubated with anti-sarc serum. After further washing with RIPA buffer ^{32}P-γ-ATP was added under standard protein kinase assay conditions. Since sarc activity is assayed by the ability of this kinase to phosphorylate heavy chain of its own specific immunoglobulin (Clinton _et al_., 1982), I was looking for increased phosphorylation of heavy chain in precipitates treated with both antisera (i.e. anti-NS and anti-sarc) as compared to anti-NS treatment alone. In figure 51

Figure 51. Protein kinase activity identified in anti-NS immunoprecipitate of virion proteins having sarc-type activity

Proteins precipitated by anti-NS serum from the supernatant fraction of HSS dissociated virions, as shown in figure 42 were incubated with ^{32}P-γ-ATP as described in Materials and Methods were analysed on SDS-PAGE containing 0.26% methylene bisacrylamide. At this concentration of crosslinker the NS protein migrates faster than immunoglobulin heavy chain (HC) as indicated. (A), anti-NS immunoprecipitate incubated with ^{32}P-γ-ATP; (B), anti-NS immunoprecipitate washed with RIPA buffer, reacted with anti-sarc antibody, washed again with RIPA and then incubated with ^{32}P-γ-ATP; (C), the HSS supernatant reacted with anti-sarc and then incubated with ^{32}P-γ-ATP.

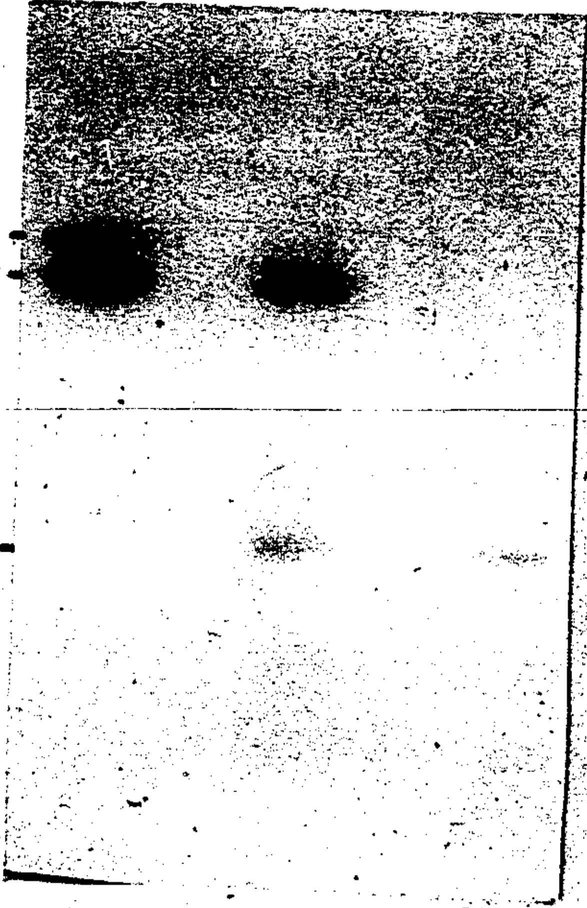

HC
NS
A
B
C

it can be seen that this was in fact my finding (compare lanes A and B).

To see if this phenomenon was restricted to HSS extracts derived from virions, I repeated the HSS treatment on cytoplasmic extracts of infected cells. ^{35}S-methionine labelled supernatants derived from the experiment described in figure 46, were mixed with an equal volume of 2 x HSS, incubated for one hour at 5°C and then subjected to centrifugation at 125,000 x g for 120 minutes on a 40% glycerol pad. The material remaining on top of the pad was diluted to 0.15 M NaCl and then treated with antisera as indicated in figure 52. The anti-N serum did not pull down detectable NS protein and therefore served as a useful control in the following kinase experiments. Replicates of the samples shown in figure 52 were either directly incubated with ^{32}P-γ-ATP or reacted with anti-sarc serum, washed and then incubated with ^{32}P-γ-ATP. As was the case with HSS disrupted virions, anti-NS serum precipitates from HSS treated cytoplasmic extracts contained a kinase activity which labels both heavy chain and NS protein figure 53. Additionally there appears to be an association of sarc activity with these precipitates as indicated by increased phosphorylation of heavy chain in lane C. The anti-N serum precipitates similar amounts of ^{35}S-methionine labelled immune complex as anti-NS serum (figure 52) yet

Figure 52. Antibody precipitates of HSS treated infected cell extracts

The supernatant fraction from figure 46 was incubated with HSS and subjected to centrifugation on a glycerol pad as described in Materials and Methods. The material which remained on top of the glycerol pad was made 0.15 M NaCl by dilution with ddH_2O and then reacted with antisera. ($\bar{a}$NS), HSS supernatant reacted with anti-NS serum; ($\bar{a}$N), HSS supernatant reacted with anti-N serum; (aM) HSS supernatant reacted with anti-M.


NS
N
M

shows insignificant phosphorylation of heavy chain in either singly or doubly treated precipitates (figure 53, lanes D and E). This result is consistent with the notion that the association of sarc protein with anti-NS immunoprecipitates is not merely fortuitous entrapment of sarc activity but may be functionally significant. Anti-M serum (prepared in identical fashion as the N and NS sera) also pulls down kinase activity which phosphorylates M protein. An association of sarc with anti-M precipitates is suggested by increased labelling of heavy chain after anti-sarc treatment of washed anti-M immunoprecipitates (figure 53, lanes F and G).

Figure 53. <u>Kinase activity associated with immunoprecipitates of HSS treated infected cell extracts</u>

Immunoprecipitates identical to those shown in figure 50 were either incubated directly with ^{32}P-γ-ATP or treated with anti-sarc, washed and then incubated with ^{32}P-γ-ATP. After completion of the protein kinase assay the samples were analysed by SDS-PAGE (0.23% crosslinker) and autoradiography. (a), ^{35}S-methionine labelled infected cell extract; (b), anti-NS immunoprecipitate from figure 50 incubated with ^{32}P-γ-ATP; (c), anti-NS immunoprecipitate treated with anti-sarc immunoprecipitate from figure 50 incubated with ^{32}P-γ-ATP; (d), anti-N immunoprecipitate incubated with ^{32}P-γ-ATP; (e), anti-N immunoprecipitate treated with anti-sarc and incubated with ^{32}P-γ-ATP; (f), anti-M immunoprecipitate incubated with ^{32}P-γ-ATP; (g), anti-M immunoprecipitate treated with anti-sarc and incubated with ^{32}P-γ-ATP.

NS N–

–HC

M–

a b c d e f g

DISCUSSION

The discussion of the work described here falls into three sections. The first section deals with the multiple forms of NS protein present in Piry infected cells. The individual characteristics of each species have been summarized and compared to the multiple NS forms reported in other VSV serotypes. The second section deals with structural studies carried out on Indiana NS protein and in particular how these findings relate to the predicted amino acid sequence of Gallione et al., (1981). In the final section the specificity of monospecific antibodies and the kinases they precipitate will be discussed.

1. Multiple NS Species in Piry Infected Cells

The first section of this work clearly demonstrates the existence of multiple NS forms in Piry infected cells and virions.

NS_v appears to be the predominant species in infected cells and virions (figures 1 and 3). NS_i, a metabolic precursor to NS_v is found at highest concentrations in the infected cell and is apparently absent or a minor component of the virion (figure 1).

Xp is probably a third member of the Piry NS family.

Three lines of evidence support this idea; (1) Xp can be detected in infected but not uninfected ^{32}P-labelled cells (figure 8); (2) Xp is present in mature sucrose gradient purified virions (figure 9); (3) the fragment pattern resulting from partial NCS cleavage of ^{32}P-labelled material closely resembles that of ^{32}P-labelled NS_v protein (figure 10). Furthermore DeTina, (1980) has demonstrated that ^{32}P-labelled Xp is coprecipitated with NS_v from infected cell extracts using Piry anti-NS serum (this serum was prepared in parallel with the monospecific Indiana anti-NS serum).

The two species NS_i and NS_v appear to be related by a post-translational mechanism which causes an apparent increase in the molecular weight of NS_i (figure 4). It is not evident from this work how Xp might fit into the NS maturation scheme. Although it is possible to deduce a definite relationship between NS_i and NS_v using short pulses of ^{35}S-methionine (figure 3) the comigration of N protein and Xp (figure 8) makes it difficult to determine if Xp is a precursor or a product.

Multiple NS species resolvable by SDS-PAGE have been identified in other rhabdovirus systems. Lesnaw _et al._, (1979) have identified two NS species in New Jersey infected cells. The major species has a lower electrophoretic mobility than N protein while a second NS protein

(designated as X by these authors) comigrates with N protein on SDS-PAGE. As in the case of Piry Xp the New Jersey X protein was demonstrated to be related to the major NS species by partial cleavage mapping of ^{32}P-labelled material. Furthermore, analogous to Piry Xp the New Jersey X protein can be a component of the mature virion. Under particular gel conditions the New Jersey X protein can be resolved away from N protein and therefore Lesnaw et al., (1979) were able to determine the relative amount of radioactive phosphate present in both X and NS proteins. Their findings suggest that either X is more heavily phosphorylated than NS or that it is derived from NS protein by proteolytic cleavage. Either one of these possibilities could also explain the presence of Xp in Piry infected cell extracts.

Clinton et al., (1978 and 1979) demonstrated the existence of two NS forms in cells infected by the San Juan strain of Indiana virus. These two species differed in their degree of phosphorylation by approximately 10% and could be interconverted in vitro presumably by the action of kinases and phosphatases. This finding and others (Kingsford and Emerson, 1980) suggested to me that Piry NS_i and NS_v may differ quantitatively in their extent of phosphorylation. The results of the theophylline experiments do not support this hypothesis (figure 11).

Despite extensive inhibition of kinase activity in infected cells at higher theophylline concentrations the relative ratio of ^{35}S-methionine labelled NS_i to NS_v remained the same. As mentioned in the Results, cordycepin had the identical effect at corresponding drug concentrations. These experiments suggest that NS_i and NS_v may not differ quantitatively in terms of phosphorylation.

There may exist however, qualitative phosphorylation differences between the two NS species. That is, if phosphorylation affects the conformation of NS protein as some authors have suggested, (Hsu et al., 1982; Sinacore and Lucas-Lenard, 1982) then changes in secondary structure due to different sites of phosphorylation may be reflected in the mobility difference between NS_i and NS_v.

1.1 Isoelectric Focussing of Piry NS

The results of isoelectric focussing (IEF) (figure 16) and nonequilibrium pH gradient electrophoresis (NEPHGE) (figure 18) neither support nor refute the possibility that NS_i and NS_v differ quantitatively in their extent of phosphorylation. Although the two species showed no mobility differences in NEPHGE consistent with the idea that they do not differ in net overall charge, this may simply reflect the inability of the employed system to resolve single charge differences at very low pH values.

The results of conventional IEF and NEPHGE both suggest that Piry NS is a very acidic protein. I have calculated the theoretical isoelectric point of NS protein using the primary sequence data for the San Juan strain of Indiana virus (Gallione et al., 1981 and see Appendix III). A pH value of 4.26 was determined to be the isoelectric point of fully denatured, unphosphorylated NS protein and post-translational addition of phosphate groups would lower this value further. Although direct comparisons between Piry and Indiana NS proteins cannot be made since their primary sequences undoubtedly differ, (compare tryptic maps figures 2 and 29; NCS maps figures 10 and 25) the isoelectric point prediction for phosphorylated Piry NS of approximately pH 3 (figure 18) is not inconsistent with the theoretical calculation presented above.

On the other hand, Maack and Penhoet (1980) found New Jersey NS protein to focus between pH 4.0 and 5.0 in conventional IEF systems. Hsu and Kingsbury (1980) originally reported that Indiana NS resolves between pH 4.0 and 5.0 however have recently observed with a new lot of ampholines NS protein focussing between pH 6.8 and 7.2.

The range in pI values reported for Piry, Indiana and New Jersey NS proteins may in part reflect primary sequence differences between the three strains. A similar polymorphism in pI values for non-structural protein 1 (NS1)

has been reported for several strains of influenza A virus (Petri *et al*., 1982). Other factors which may affect the experimental determination of the pI of NS protein include ananolous binding of NS protein to ampholines from different sources as suggested by Hsu and Kingsbury (1982) or incomplete denaturation of NS protein preventing participation of all charged groups in the determination of the protein's overall isoelectric point.

1.2 Lack of Detectable Compartmentalization of Piry NS Protein

The two NS species identified by Clinton *et al*., (1978 and 1979) differed not only in their degree of phosphorylation but also in their ability to bind to ribonucleoprotein cores from infected cells. They found that the more heavily phosphorylated NS species were incapable of binding to RNP cores. In the experiments reported here with Piry virus NS_i and NS_v appear to be capable of binding to RNP cores isolated from cytoplasmic extracts exactly as described by Clinton *et al*., (1979) (figure 1). In fact fractionation of whole infected cells on sucrose gradients did not reveal differential compartmentalization of either species (figure 7), suggesting that the structural difference between NS_i and NS_v is not reflected in their respective abilities to bind to cellular or viral components.

1.3 Possible Acetylation of Piry NS Protein

The only post-translational modification described in the literature for NS protein has been phosphorylation (Sokol and Clark, 1973). I was unable to discern any phosphorylation differences between NS_i and NS_v as described above and therefore investigated the possibility that previously undetected processing of NS protein may occur. In particular, the possibility of acetylation was investigated. The finding that ^3H-acetate can be incorporated *in vitro* into a band comigrating with Piry NS_v is consistent with the idea of NS acetylation (figure 12). Since only a very small amount of labelled material could be generated in this fashion it was not possible to prove that the ^3H-labelled protein was in fact NS_v or that the ^3H-label was added by an acetylation pathway. Further suggestive evidence that NS protein may be acetylated at least at the amino terminus was provided by the finding that whole SDS-PAGE purified Indiana NS protein is resistant to automated sequencing.

The conversion of NS_i to NS_v can be inhibited *in vitro* by the addition of an acetyl CoA scavenging system (figure 14). This finding taken together with the above observation was suggestive that NS_i and NS_v did differ in their acetylation status. However citrate, an end product of the scavenging system, can also prevent

the conversion of NS_i to NS_v. Therefore although it still remains possible that NS_i and NS_v differ by acetylation an equally likely possibility is that citrate as an end product of the scavenging system is inhibiting some other enzymatic pathway which normally converts NS_i to NS_v.

2. Structural Analysis of Indiana NS Protein

2.1 Does NS Protein Exist as a Dimer During SDS-PAGE?

As pointed out in the Introduction, the mobility of NS on SDS polyacrylamide gels indicates that the protein should have a molecular weight of 39-50K Daltons (Knipe *et al.*, 1975). On the other hand, estimates, based on the size of NS mRNA indicate that the final unprocessed product cannot exceed a molecular weight of 26K Daltons.

Using dually labelled NS, N and M proteins an independent estimate was derived for the actual molecular weight of NS protein synthesized *in vitro*. The value of 20K Daltons (Table 1) is relatively close to the actual size of NS protein (25K Daltons; Gallione *et al.*, 1981) but is inconsistent with its mobility in the SDS-PAGE system employed in this work (48-54K Daltons).

As recently suggested by Gallione *et al.*, (1981) the discrepancy between the size of the NS mRNA and the protein's electrophoretic mobility may have arisen because NS migrates as a dimer in SDS-PAGE. If this were the case intermolecular bonds between NS monomers would have to

be resistant to heat, SDS and reducing agents. Since the polypeptide is also a phosphoprotein the possibility of an intermolecular phosphodiester linkage was investigated. Snake venom phosphodiesterase clearly had an effect on the mobility of NS protein converting it to a species with an apparent molecular weight of approximately 19.5K Daltons (figures 20 and 22). Unfortunately this enzyme preparation also removed from the native protein both its radioactive phosphate groups and its ^{35}S-labelled amino terminal methionine residue. Furthermore different commercial preparations of SVPD produced a variety of digestion products (see figure 21). These observations considered as a whole probably indicate that the effect of SVPD on NS protein is mediated through a contaminating protease. Still, it must be noted that if this is true, at least in the case of the Worthington enzyme preparation, the protease appears to show specificity toward NS protein.

Clearly these experiments do not answer the question of whether NS migrates as a dimer or monomer in SDS-PAGE. The results of NCS cleavage of end-labelled NS protein may however resolve this issue. As shown in figure 25, cleavage of end-labelled Indiana NS protein produces four labelled fragments. This is exactly the number of fragments one would expect to generate in an end-labelled monomer containing three cleavage sites (see Appendix II).

Gallione et al., (1981) predict from their nucleotide sequence data that NS contains three tryptophan residues which are the sites of cleavage by NCS (see Appendix I). If NS actually existed as a covalently bridged dimer then partial cleavage should generate no fewer than seven end labelled fragments, some of these migrating faster than the predicted monomer molecular weight of 25K Daltons (see Appendix II). I conclude then that anomalous mobility of NS protein is not due to dimerization but rather is probably due to a deficiency of SDS binding as suggested by Gallione et al., (1981).

2.2 NS Phosphorylation Sites Are Predominantly Found on the Amino Terminal NCS Fragment of NS Protein

N-chlorosuccinimide was used to generate partial cleavage maps of three differentially labelled preparations of Indiana NS protein. Comparison of the maps shown in figure 25 revealed that locations on the amino terminal side of all tryptophan sites represented the majority of phosphorylated residues in cytoplasmic Indiana NS protein. This pattern of labelling was observed not only in mouse L cells but also in NS protein produced and labelled in KB cells (figure 27). Furthermore Piry NS gave a similar pattern of labelling, that is only fragments which contain the amino terminus were labelled with ^{32}P-orthophosphate (figures 10 and 26). The consistent pattern of labelling

in two different host cells and between two different serotypes suggests that this site of phosphorylation may be functionally significant to NS protein. Cytoplasmic NS was the only form used in these partial cleavage experiments and may indicate that the sites identified in this work are comparable to the primary cluster of phosphorylation sites described by Hsu et al., (1982) in cytoplasmic NS1.

The primary cluster described by Hsu et al., (1982) contained at least six phosphoserines and five phosphothreonines. Based on the nucleotide sequence data of Gallione et al., (1981) seven serine residues and four threonine residues are located on the amino terminal side of all NS tryptophan sites. These data suggest that by default the secondary cluster described by Hsu et al., (1982) must be located on the carboxy terminal side of the primary cluster.

2.3 A Large Tryptic Fragment is Phosphorylated in NS Protein

Tryptic hydrolysis of ^{35}S-methionine labelled Indiana NS protein generated three well defined tryptic peptides and one of these peptides contained the amino terminal methionine (figures 19 and 29). Both of these findings correspond exactly with the amino acid sequence prediction for NS protein (Gallione et al., 1981). Parallel digestion of ^{32}P-labelled NS protein produced

only a single labelled spot with low mobility in thin layer electrophoresis (figure 30). A similar finding was reported by Hsu et al., (1982) with Indiana NS protein while Maack and Penhoêt (1980) resolved two tryptic phosphopeptides derived from New Jersey NS protein.

At least some of the ^{32}P-labelled tryptic digest was resolvable as a single large fragment by SDS-PAGE (figure 31). As mentioned in the Results small tryptic phosphopeptides with low mobility on thin layer electrophoresis would probably not be detected on a SDS gel.

The large fragment was not labelled with ^{35}S-methionine and appeared to have an estimated molecular weight of 5.5K Daltons. Examination of the nucleotide sequence data of Gallione et al., (1981) (see Appendix I) reveals that Indiana NS contains a large tryptic fragment starting at Ser_{35} and ending at Lys_{110}. Although there exists a lysine at position fifty, trypsin probably does not cleave here since residue fifty-one is a proline (Boyer, 1971).

The tryptic peptide contains six serines, three threonines, no methionines and all the potential sites of phosphorylation in this fragment are located on the amino terminal side of all NS tryptophan residues.

These observations considered as a whole suggest that a majority of NS sites of phosphorylation are localized in one large tryptic fragment.

2.4 Phosphodipeptide Analysis of NS Protein

The major labelled products of acid hydrolysed ^{32}P-labelled NS protein include inorganic phosphate, phosphoserine, phosphothreonine and phosphopeptides (figure 36). Clinton and Huang (1981) made similar observations using ^{32}P-labelled NS produced in BHK cells.

The phosphopeptides isolated under the conditions of hydrolysis and purification described in Materials and Methods can be further subdivided into two groups by high voltage paper electrophoresis at pH 1.9.

The first group is positively charged and migrates rapidly toward the negative terminal while the second group is composed of neutral or poorly charged phosphopeptides which remain at the origin or smear slightly toward the negative electrode (figure 36).

The second group of phosphopeptides was identified in an acid hydrolysate of non-histone nuclear phosphoproteins by Jones and Olson (1980). These authors demonstrated that this group was composed of a heterogeneous population of phosphodipeptides all of the structure X-Ser(P) or X-Thr(P) where the identity of X was variable. Therefore simply by identifying the X component of each phosphodipeptide these authors were able to perform nearest neighbour analysis on the sites of phosphorylation in non-histone nuclear protein extracts.

In this work results are presented which demonstrate that this technique can be adapted to analyse the number and potentially the sites of phosphorylation in proteins detectable only by radioactive isotope labelling. A three dimensional system of analysis (figure 37) has been developed which permits isolation and resolution of a heterogeneous population of phosphodipeptides with a minimum amount of sample manipulation. When three dimensional analysis was applied to NS protein produced in either human KB or mouse L cells identical patterns of phosphorylation were observed (figure 37).

Six unique phosphodipeptide spots were identified in both preparations suggesting that cytoplasmic NS is phosphorylated at a minimum of six positions. Inspection of the amino acid sequence predictions for NS protein (see Appendix I) revealed that there exists 11 potential sites of phosphorylation located on the amino terminal side of NS tryptophan residues however two of these are Lys-Ser and therefore the maximum number of resolvable unique phosphodipeptides in a cytoplasmic NS acid hydrolysate should be ten.

Hsu and Kingsbury (1982) identified eleven unique sites of phosphorylation using chymotryptic analysis of cytoplasmic NS protein. Only six sites of phosphorylation were identified in this work suggesting that either;

(1) the three dimensional system employed is not resolving all of the unique phosphodipeptides present in NS or (2) different degrees of labelling are being observed in the two studies.

The phosphodipeptide maps presented in figure 37 suggest that there exists intermolecular variation in the extent of phosphorylation observed in the cytoplasmic population of NS polypeptides. Hsu and Kingsbury (1982) reported similar observations and suggested that the efficiency of phosphorylation or turnover of phosphate moieties may be variable at different locations in the native protein.

The technique of phosphodipeptide analysis when used in conjunction with primary sequence data can be a very powerful tool for localizing sites of phosphorylation within a protein.

At present the major limitations for using this procedure with radioactive amounts of protein are that; (1) it is not possible to directly identify the X component of the phosphodipeptide and (2) it is difficult to guarantee that all unique dipeptides have been resolved. Both of these problems could be overcome however if it were possible to obtain a complete set of cold marker phosphodipeptides. One approach to this problem would be to purify phosphodipeptides from α-casein or other phosphoproteins

which are available in gram quantities, using the protocol described in the Results section of this thesis. I believe that a more direct and unequivocal method would be to separately chemically synthesize each of the potential phosphodipeptides and then use these as authentic marker dipeptides for analysis of radioactive acid hydrolysates.

3. Protein Kinase Associated with Viral Protein Immunoprecipitates

3.1 Preparation of Monospecific Antibodies

The antisera prepared by injection of rabbits with gel purified viral antigens appears to be monospecific at least with respect to denatured viral proteins (figures 39, 40 and 41). Both anti-N and anti-NS serum appear to immunoprecipitate protein complexes from infected cell extracts and disrupted virions. Interestingly, the sera appear to discriminate between different types of protein complexes from these two sources. In particular, the anti-N serum pulls down aggregates from disrupted virions which contain N and M proteins while anti-NS coprecipitates L, NS and small amounts of N and M proteins (figure 42). On the other hand the two sera when challenged with unfractionated infected cell extracts pull down aggregates which appear virtually identical except that anti-N immunoprecipitates contain small amounts of M and another unidentified protein (figure 43). Finally,

immunoprecipitation of high speed supernatants from infected cell extracts demonstrated that anti-N pulls down N-M aggregates while anti-NS coprecipitates N-NS complexes, (figure 52).

Based on the known activities of these various proteins <u>in vivo</u> it is possible to speculate on the functional significance of these assorted complexes. For instance, Newcomb and Brown (1981) have demonstrated that M protein is responsible for maintaining viral nucleocapsids in the condensed form found in native virions probably through M-N interactions. Therefore it may be that the M-N associations identified by immunoprecipitation reflect real <u>in vivo</u> affinities that these two proteins have for each other.

Anti-NS invariably immunoprecipitates both N and NS proteins and in particular from high speed soluble cytoplasmic fractions (figures 46 and 52). Similarily monospecific anti-NS serum produced by Imblum and Wagner (1975) showed coprecipitation of N and NS protein from a high speed soluble cytoplasmic extract. In light of the fact that NS can interact directly and specifically with ribonucleoprotein (Isaac and Keene, 1982) it may be that these soluble NS-N complexes represent intermediates in the assembly of ribonucleoprotein complexes.

3.2 Coprecipitation of Kinase with Viral Proteins

The data presented in the Results section demonstrates the coprecipitation of NS protein and kinase activity using monospecific antiserum. The kinase activity precipitated is associated with NS either in the soluble or RNP bound form.

In all experiments performed with native protein complexes (i.e. 0.15 M NaCl treatment) there is no phosphorylation of Ig heavy chain. In contrast, HSS treatment (0.72 M NaCl) of virus or cellular extracts results in precipitation of a kinase which phosphorylates NS protein and Ig heavy chain. There may be several explanations for this phenomenon. After HSS treatment, as viral proteins reassociate at low salt concentrations (0.15 M NaCl), a nonspecific protein kinase may be trapped and fortuitously immunoprecipitated. Although I cannot rule out this possibility, it would be necessary to postulate that the contaminating kinase cannot be coprecipitated in anti-N protein complexes (figure 53). Alternatively HSS treatment may in some fashion alter the protein kinase activity (e.g. irreversible removal of a regulatory factor) and in this way broaden its spectrum of substrates.

As shown previously (Clinton et al., 1982) a sarc activity can be immunoprecipitated from virions.

I have further shown (figures 51 and 53) that sarc activity is present in anti-NS-NS protein complexes, suggesting a possible association of sarc and NS protein. A comparable activity is not coprecipitated with anti-N serum.

Perhaps of even more significance is the finding that anti-M serum also coprecipitated sarc activity. M protein has been identified as an endogenous substrate for tyrosine specific kinase so the suggested close association of M protein with sarc may represent a real functional relationship. Interestingly, M protein coprecipitated by anti-N serum is not phosphorylated in this <u>in vitro</u> assay, which may imply that only a specific class of M protein is associated with kinase activity.

The kinase coprecipitated in these experiments appear to phosphorylate only serine residues in NS protein (figure 49) which sets it apart from the bulk of kinase activity found in purified virions (Clinton and Huang, 1981).

In very recent work Sinacore and Lucas-Lenard (1982) suggest that the virion bound kinases of VSV are not necessary for primary transcription in vitro and show that dephosphorylated NS cannot be reactivated by virion kinases. Hsu and Kingsbury (1982) demonstrated that the sites phosphorylated <u>in vitro</u> by the virion kinase are distinct from those modified in the infected cell and also

demonstrate that dephosphorylated NS cannot be reactivated by virion kinases. Thus the relevance of virion bound kinases to the activity of NS protein is still open to question.

Similarily none of the experiments described here prove that the kinase associated with NS in immunoprecipitates is relevant to the activity of NS *in vivo*. However using the dephosphorylation system developed by Hsu *et al.*, (1982) and kinase(s) purified by immunoprecipitation this question would appear to be resolvable. If these kinases should prove relevant to NS protein activity then immunoprecipitation of kinase substrate complexes may be a useful initial step in the identification and purification of intracellular kinases involved in the viral infectious process.

SUMMARY

In the course of carrying out this work over the last few years I have on many occasions read Pasteur's quote on the cover of Journal of Virology: "It is characteristic of Science and Progress that they continually open new fields to our vision". I believe that the observations reported here do in fact "open new fields" to the vision of those interested in the study of molecular biology of phosphoproteins and in particular NS protein.

For instance, this work is to my knowledge the first reported attempt to physically map the sites of phosphorylation in NS protein. Furthermore, it clearly demonstrates the applicability of the partial cleavage mapping technique originally developed by Brown and Prevec (1982) for localization of post-translational protein modifications.

The observations of Hsu _et al._, (1982) and those reported here indicate that NS undergoes multisite phosphorylation. Are all of these post-translational events relevant to the activity of NS protein? I believe that with the cumulative data about NS phosphorylation sites (Gallione _et al._, 1981; Hsu _et al._, 1982; this thesis) and currently available DNA recombinant technology it should be possible to address this question directly. That is, it may be possible to prepare a cDNA clone of the NS gene for expression in mammalian cells. This clone could then be mutagenized _in vitro_ to selectively eliminate known phosphorylation sites and then its activity could be tested in reconstituted transcriptional or replication assays.

The phosphodipeptide analysis (Jones and Olson, 1980) of NS protein is again to my knowledge the first reported application of this technique to a purified protein available in amounts detectable only by radioactive

isotope labelling. The next logical step in refining this technique will be to develop a complete bank of marker phosphodipeptides (e.g. from phosphoprotein hydrolysates or by chemical synthesis). I believe that with a complete set of marker dipeptides this technique could become a very powerful tool for the localization of phosphorylation sites especially where there is a limited amount of material available for analysis.

The studies with monospecific antibody are informative from two view points. First of all they corroborate previously established (e.g. N:NS) or postulated (e.g. N:NM) viral protein interactions and secondly suggest a new approach toward the isolation of the NS protein kinase by immunoprecipitation of its substrate. In this respect it would also be worthwhile to use the monospecific sera to investigate possible associations between viral proteins and other cellular macromolecules (i.e. RNA or protein).

A number of experiments presented here indirectly suggest that as well as phosphorylation NS protein may be post-translationally acetylated. Although further studies will be required to either prove or disprove this contention these findings do point out the necessity to test NS for additional post-translational modifications. Thus although NS is heavily phosphorylated and its degree of phosphorylation seems critical to its activity, it may be that other less obvious modifications may be involved in the regulation of NS activity.

APPENDIX I

NH$_2$ MET ASP ASN LEU THR LYS VAL ARG GLU TYR LEU LYS SER
TYR SER ARG LEU ASP GLN ALA VAL GLY GLU ILE ASP GLU ILE
GLU ALA GLN ARG ALA GLU LYS SER ASN TYR GLU LEU PHE GLN
GLU ASP GLY VAL GLU GLU HIS THR LYS PRO SER TYR PHE GLN
ALA ALA ASP ASP SER ASP THR GLU SER GLU PRO GLU ILE GLU
ASP ASN GLN GLY LEU TYR ALA GLN ASP PRO GLU ALA GLU GLN
VAL GLU GLY PHE ILE GLN GLY PRO LEU ASP ASP TYR ALA ASP
GLU GLU VAL ASP VAL VAL PHE THR SER ASP TRP LYS GLN PRO
GLU LEU GLU SER ASP GLU HIS GLY LYS THR LEU ARG LEU THR
SER PRO GLU GLY LEU SER GLY GLU GLN LYS SER GLN TRP LEU
SER THR ILE LYS ALA VAL VAL GLN SER ALA LYS TYR TRP ASN
LEU ALA GLU GYS THR PHE GLU ALA SER GLY GLU GLY VAL ILE
MET LYS GLU ARG GLN ILE THR PRO ASP VAL TYR LYS VAL THR
PRO VAL MET ASN THR HIS PRO PHE PRO ILE ARG SER SER ILE
ARG GYS LEU VAL SER LEU LYS ASP ILE HIS ASP PHE PRO THR
GLN GLU SER LYS SER SER ALA SER HIS HIS ILE PHE GLY COOH

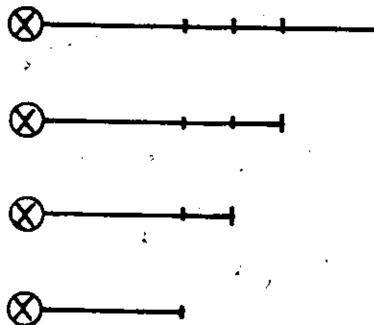
Shown above is the complete amino acid sequence for NS protein of Indiana virus, San Juan strain as predicted from the nucleotide sequence data of Gallione et al., (1981). The three methionine containing tryptic peptides and the large internal tryptic peptide are

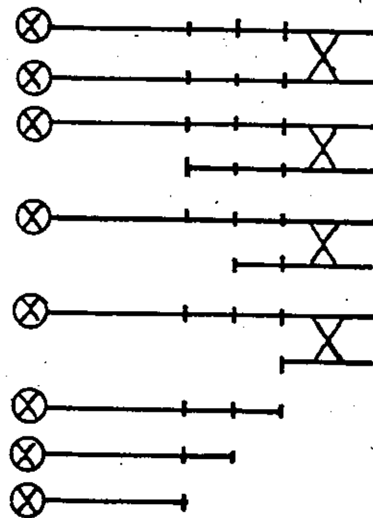
underlined. Although this large internal tryptic fragment is depicted in this figure as having a serine residue at its amino terminus, there is an additional potential internal trypsin sensitive site Lys-Pro. As pointed out in the discussion, cumulative evidence suggests that trypsin rarely cleaves between Lys-Pro or Arg-Pro residues.

Notice in the large tryptic fragment the preponderence of acidic residues (Asp Glu) which Gallione *et al.*, (1981) have suggested may influence the mobility of NS protein in SDS-PAGE.

Tryptophan residues, the site of chemical cleavage by NCS are indicated by underlining with two strokes.

APPENDIX II





The schematic diagrams presented above represent two possible models for the structure of NS protein and its partial cleavage products. Only molecules which retain their amino terminus (⊗) are depicted. Sites of NCS cleavage (i.e. tryptophan residues) are indicated by vertical lines.

Essentially the argument is that if molecules are labelled only at their amino terminus (i.e. with N-formyl-^{35}S-methionine) and then subjected to partial cleavage one will detect by autoradiography only those fragments which

retain their amino terminus. Thus a monomer with three cleavage sites will produce only four labelled products. On the other hand a dimer formed by an intermolecular bridge will generate greater than four products. In the example presented here seven different labelled molecules are depicted, however this is a minimum number. As many as eleven partial fragments all containing the amino terminus may be generated from a dimer depending upon where the crosslink (X) is positioned. Furthermore the dimer model suggests that some of the labelled fragments should be less than the monomer in molecular weight. Clearly, the monomer model and not the dimer model can satisfactorily explain the partial cleavage data obtained for NS protein (see figure 25).

APPENDIX III

Theoretical Isoelectric Point Determination of Indiana NS Protein

To determine the theoretical isoelectric point (pI) of Indiana NS protein I used the method of pI determination developed by Harley (1979). This author deduced that the net charge of a protein at any pH could be determined by using the equation for net charge (N):

$$N = -\, n(A_i)/(1+10^{(pK_a(A_i^-)-pH)}) + n(B_j)/(1+10^{(pH-pK_a(B_j))})$$

where $n(A_i)$ and $n(B_j)$ are the number of acidic and basic residues respectively in the protein. Table 3 lists the number and nature of charged groups in Indiana NS protein and their assigned pK_a values. This information was then used to solve equation (1) shown above and a plot of net charge versus pH was prepared (figure 54). The pI for NS was then determined by inspection of figure 54 and found to be approximately 4.26.

Table 3 Charged Residues and Their pK_a Values for Indiana NS

Charged Residue	Number of Residues (a)	pK_a (b)
Arginine	7	12.5
Histidine	6	6.0
Lysine	13	10.5
Aspartic	18	3.9
Glutamic	28	4.3
N-terminal amino group	1	8.7
C-terminal carboxyl group	1	3.0

(a) number of residues determined from the amino acid sequence of Gallione *et al.*, (1981).

(b) pK_a values of free amino acid side groups (Lehninger, 1976).

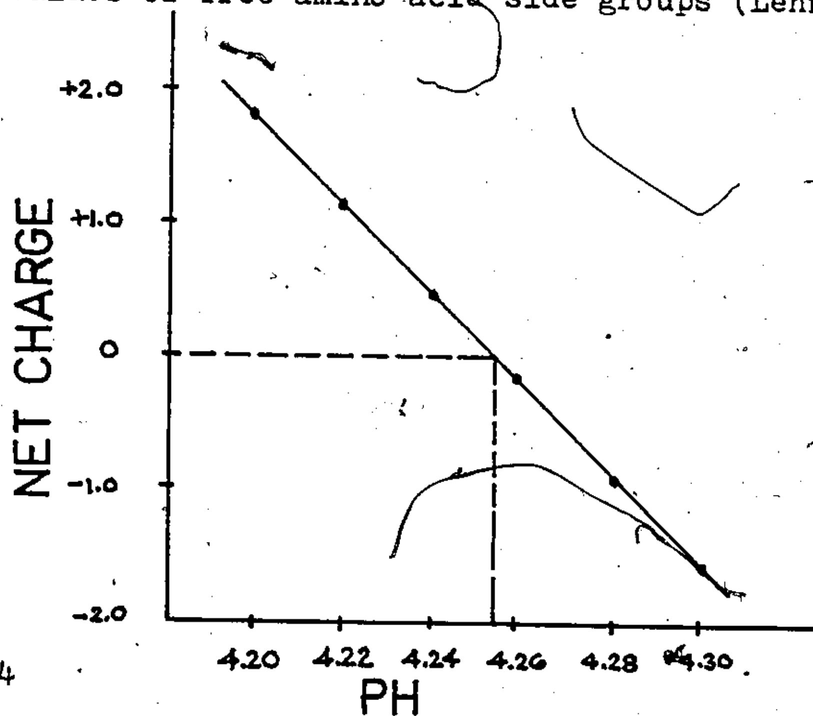


Figure 54

Calculated Net Charge of Indiana NS Protein as a Function of pH

The net charge of NS protein was determined at

several pH values using equation (1). The pH value at which the net charge is zero (dotted line) indicates the theoretical pI of the protein.

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