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Analysis of Polypeptides Associated with
Tipula Iridescent Virus Particles.

by

David.C.Watson.

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of

Master of Science

Department of Biology

Carleton University

Ottawa, Ontario

March 1993

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**Analysis of Polypeptides Associated with
Tipula Iridescent Virus Particles.**

submitted by David. C. Watson
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis Supervisors

Chair, Department of Biology

Carleton University

April 1993

Abstract

Tipula iridescent virus (TIV) is an icosahedral cytoplasmic deoxyribovirus which infects a wide range of insect species. Previous estimates based upon one dimensional polyacrylamide gel electrophoresis suggested that virus particles contain 25-30 associated polypeptides ranging in molecular mass from 17.5kDa to 300kDa. However, comprehensive mapping of TIV associated polypeptides reported in this thesis, using 2-D polyacrylamide gel electrophoresis in combination with high sensitivity silver staining, indicated that the total number of virion associated polypeptides is 67-70. This polypeptide mapping strategy was used to further identify 10 putative outer shell or surface polypeptides and 11 glycosylated polypeptides 'tagged' by ^{125}I radioiodination and a glycan specific staining technique, respectively. Comparative disruption conditions used for TIV particles suggest that the putative capsid polypeptide is associated into covalently bound pentameric oligomers. Further use of an *in situ* microsequencing method indicated that at least 16 of the more abundant polypeptides were N-terminally blocked. However, the amino terminal sequence of one 32kDa polypeptide displayed a sequence similar to that found in 'zinc finger' DNA binding motifs which is suggestive of this protein being associated with the core of the virus particle.

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DEDICATION.

For Dad.

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LIST OF ABBREVIATIONS.

ASFV	African swine fever virus.
BPL	β -propiolactone.
CAPS	3-Cyclohexylamino-1-propanesulphonic acid.
cDNA	Complementary DNA.
Ci	Curie.
CIV	Chilo iridescent virus.
ConA	Concanavalin A.
DNA	Deoxyribonucleic acid.
dsDNA	Double stranded DNA.
EMBL	European molecular biology laboratory.
Fab	Immunoglobulin antigen binding fragment.
FASTA	Pearson search algorithm
FLDV	Fish lymphocystis disease virus.
FV3	Frog virus 3
HPLC	High performance liquid chromatography.
ICDV	Icosahedral cytoplasmic deoxyribovirus.
IEF	Isoelectric focussing.
IgG	Immunoglobulin.
kb	Kilobase.
kDa	Kilodalton
LPO	Lactoperoxidase.
MIV	Mosquito iridescent virus.
M_m	Molecular mass.
M_r	Relative molecular weight.
NRC	National Research Council.
1-D	One dimensional.
PAGE	Polyacrylamide gel electrophoresis.
PAS	Periodic acid-Schiff.
PE-Cys	Pyridylethylated Cysteine.
PITC	Phenylisothiocyanate.
PTH	Phenylthiohydantoin.
PVDF	Polyvinylidene difluoride.

RNA	Ribonucleic acid.
SDS	Sodium dodecyl sulphate.
2-D	Two dimensional.
UV	Ultraviolet.
VPC	Viroplasmic centre.

Summary

Tipula iridescent virus (TIV) is an icosahedral cytoplasmic deoxyribovirus which infects a wide range of insect species. TIV is type 1 of 33 insect iridescent viruses which contain dsDNA and replicate in cytoplasmic inclusion bodies or viroplasmic centers (VPC's).

Since its discovery in 1954, substantial amounts of information on the gross structural properties of TIV has accumulated. While these studies have been extremely informative with regards to viral architecture and icosahedral design, it is only recently that molecular information to explain the mode of replication, pathogenesis and the regulation of gene expression has been forthcoming. The major capsid protein is the only polypeptide associated with TIV which has been studied and characterized in any detail to date and knowledge of the characteristics of other TIV associated polypeptides is sadly lacking. Previous studies on polypeptides associated with TIV suggested that virus particles contain 28 polypeptides ranging in molecular mass from 17.5-300kDa with a total estimated molecular mass of 1.72×10^6 daltons. These estimates were based upon electrophoretic analysis in one dimensional SDS-polyacrylamide gels (SDS-PAGE) which resolves polypeptides on the basis of their size. The development of two-dimensional (2-D) gel electrophoretic techniques to resolve proteins by separating in one dimension according to charge by isoelectric focussing (IEF) and then in a second dimension by conventional SDS-PAGE, has produced a powerful tool to explore complex mixtures of proteins. Combined with high sensitivity silver staining, the results show that 2-D electrophoretic analysis of TIV associated polypeptides is vastly superior to conventional one dimensional analysis and allows 67-70 polypeptides to be identified. This same technique has been applied to identify 10 structural polypeptides located or partially exposed on the surface of TIV particles following

surface labelling of purified particles with ^{125}I . Studies regarding post-translational modifications of TIV-associated polypeptides have been inconclusive, although reports have appeared in the literature identifying modifications such as phosphorylation occurring in other iridoviruses. Of the 28 polypeptides visualized in one-dimensional gels, none appeared to be glycosylated as judged by staining using the Periodic acid-Schiff reaction. However, sensitive glycan specific staining techniques demonstrated the presence of 11-17 putative glycosylated polypeptides. The availability of microsequencing techniques for 2-D separated polypeptides, electroblotted onto immobilizing supports, has greatly enhanced obtaining amino acid sequence data on picomole quantities of polypeptides purified to homogeneity by gel electrophoresis. By applying this technique to TIV associated polypeptides, a partial amino acid sequence was obtained from a 32kDa polypeptide which displays a sequence similar to that which is found in 'zinc finger' DNA binding motifs.

Introduction

1.1 Iridoviruses; background and classification.

Insects can act as hosts for a variety of viruses some of which are also capable of infecting plants and animals other than insects. Those viruses which multiply exclusively in insects can be further subdivided into four broad groups: granulosis and polyhedrosis viruses (1), cytoplasmic polyhedrosis viruses (2) and entomopox viruses (3), and a heterogeneous group including picorna, birna, parvo and iridescent viruses (4). The iridescent virus sub-group is the largest with more than thirty viruses provisionally recognized (5,6).

The first iridovirus isolated from the larvae of the European crane fly, *Tipula paludosa* (diptera) was discovered in the field in Shropshire, England in 1954 and brief descriptions were made by Xeros (7) and Smith (8) of this type 1 iridescent virus. The cytoplasmic disease caused by the virus was reported to be primarily in the larval fat body which appeared purple through the wetted skin. After high speed, differential centrifugation of disrupted infected fat bodies, the virus particles were collected as pellets which displayed a blue iridescence in reflected light and an orange coloration in transmitted light similar to that seen in the infected tissue. Consequently the virus was later named *Tipula* iridescent virus (TIV) (9).

A number of vertebrate and invertebrate viruses with properties similar to TIV have since been isolated and grouped within the family Iridoviridae. These viruses have been aptly called icosahedral cytoplasmic deoxyriboviruses or ICDV's (10) in accordance with their site of replication in the cytoplasm of infected cells, their possession of a dsDNA genome and their icosahedral shape.

Until recently the Iridoviridae family was subdivided into five genera referred to as iridovirus (small iridescent viruses), chloriridovirus (large iridescent viruses),

Ranavirus and lymphocystivirus, and the African swine fever virus (11). The most intensively studied members of this family are the frog virus 3 (FV3) (12), the African swine fever virus (ASFV) (13), which has recently been classed into another family, and TIV. Other prominent family members include fish lymphocystis disease virus (FLDV) (14) and insect chiloiridescent virus (CIV) (15). The members of the iridoviridae family share several common structural characteristics. Iridescent viruses are very large which raises the question as to exactly why they are the remarkably stable entities that they are. One explanation for this is that they contain an internal lipid membrane and based upon electron microscopy of thin sectioned virus particles, Stoltz (16) suggested that iridescent viruses such as the mosquito iridescent virus (MIV) and TIV possess such a membrane. Furthermore it has been demonstrated that there is sufficient lipid present in virus particles to form a continuous lipid bilayer at an internal site (17).

1.2.1 TIV and iridovirus structure.

Some of our basic understanding of the structure of icosahedral viruses has come from the pioneering studies carried out with TIV (18). Early studies inferred that the site of TIV multiplication was in the cytoplasmic regions of fat-body cells since it was here that the virus was found to be most heavily concentrated. Since then other studies demonstrated that TIV contained DNA as its genetic material and that TIV particles were not simple spheres but irregular in shape resembling a polyhedral design (7). The large size of iridescent viruses made them obvious samples for pioneering studies in electron microscopy, and in 1955 Smith used micrographs of negatively stained TIV to show its hexagonal shape for the first time (8). Subsequently, this hexagonal shape was seen several times in thin sections of infected tissue (19, 20).

Advances in electron microscope technology made tremendous strides in the 1960's, and in 1970 Wrigley published a study on the subunit structure of the TIV particle (21). When TIV was treated with a nasal decongestant, Afrin, whose mode of action on virus was not understood, a structure was seen suggesting an icosahedral shape consisting of approximately 1472 subunits per particle. The value of 1472 subunits per virus particle was subsequently confirmed in 1977 by Manyakov (22) who produced similar electron micrographs to those of Wrigley although different procedures were used to break up TIV into its structural sub-components.

Using a combination of methods such as negative staining, freeze etching and small angle neutron and X-ray scattering, a detailed structural model of the FV3 virion has been described (17, 23-26). It consists of two outer icosahedral shells and two inner spherical shells. Approximately 80% of the mass of the virus is confined to a set of three concentric shells with the innermost of these shells comprising a nucleoprotein core containing the viral DNA and its associated proteins. The nucleoprotein core of FV3 can be released from the shell by treatment of viral suspensions with the non-ionic detergent NP40 (27) or by treatment with chymotrypsin as described for the R strain of the mosquito iridescent virus (MIV) (28), and it has been demonstrated that the viral DNA within the core is attached to the coat protein to form a nucleosomal like structure (29). Enclosing the nucleoprotein core is an intermediate lipid bilayer of approximately 4nm thickness surrounding which is a dense protein shell containing about 50% of the virus mass. Finally, beyond this dense protein shell another highly hydrated protein shell was detected.

On the surface of some insect iridoviruses such as MIV and CIV, the presence of a fringe has been documented (16,23). Available evidence suggests that this fringe is probably microfibrillar and that each subunit bears a single fibril (15).

These fibrillar fringes have also been observed on other Iridoviridae members such as the fish lymphocystis disease virus (FLDV) but seem to be absent from others such as FV3 (12). It has been suggested that these fibres cause the interplanar spacings in crystals of the virus (30).

1.2.2 TIV heterogeneity.

When crude preparations of TIV are subjected to sucrose density gradient centrifugation usually two major bands are detected (31-35). The main band, which is the most dense, has been shown by electron microscopy (36) to be homogenous in composition, consisting of intact virions. Immediately above this band is a minor one referred to as the top component. This band is heterogeneous in composition and is enriched in less dense particles. Above this band, and near the meniscus of the gradient, is a band which consists mainly of empty capsids. Ultrastructurally, the virus particles of the top band differ from virions in the other bands in two respects. Firstly, they are permeable to negative stains and are often disrupted during negative staining. Secondly they stain poorly suggesting that they contain little DNA or nucleoprotein. Like virions of the main band, the icosahedral form of virions obtained from the top component fraction is retained on fixation (23). Biochemical studies have further confirmed that top component particles contain less DNA than do virions of the main band. However, the total amount of lipid associated with these two fractions is about the same as determined by lipid analysis performed using the sulphophosphovanillin colorimetric method (35). Comparative polyacrylamide gel profiles of SDS solubilized polypeptides of the main band and top component particles, appear to be identical. However, quantitative antigenic differences have been observed using antisera raised against virus and top component particles (35).

1.3.1 Pathogenesis.

TIV infects a wide range of insect species in at least three different orders namely Lepidoptera, Diptera and Coleoptera (37). The process of pathogenesis of iridescent viruses is not particularly well understood. When administered orally, these viruses display low levels of infectivity and are more likely transmitted in nature through wounds, parasites, the respiratory tract, cannibalism or direct contact (38-40). The primary site of replication of TIV is not known although hemocytes and fat body cells have been suggested (26,37) as initial sites from which virus can spread throughout the larva. Systemic infection results in rapid multiplication of TIV in muscles, head, legs and wing buds of the larva and the infection renders the larvae flaccid after 7-10 days (26).

Knowledge of the cell pathology of iridescent viruses has come mainly from *in vitro* studies by the infection of insect cells in culture. The major focal point of replication and the assembly of progeny virions is the cytoplasm although it is possible that the nucleus is involved as seen in the replication of FV3 (41). TIV particles initially adsorb to the plasma membrane of cells in culture and subsequently enter the cells by a form of endocytosis to become engulfed into lysosomes (36,42-44). Within the lysosomes the particles disintegrate presumably by an uncoating process (42). This process is followed by the formation of viroplasmic centers (VPC's) within the cytoplasm. Over the course of infection, VPC's increase in size and number. It is not known how the uncoated viral particle is released from the lysosome to establish VPC's in the cytoplasm. They are made up of a peripheral, cytoplasmic, particulate-free matrix and a central, dense particulate-rich matrix. The central matrix is enriched in ribosomes and DNA. Progeny virions are localized in the cytoplasmic portion of the viroplasmic center (42), the likely site of their assembly. Progeny virions are subsequently released from the cells by either budding off at the plasma membrane or cell lysis. The latter

appears to be the more common mode of release, and dispersion of virus occurs through the hemolymph or cell culture medium *in vitro* (30).

1.3.2 Host cell changes.

The infection of cells by TIV has several profound effects amongst which are an increase in the size of the nucleus, a decrease in the rate of cellular DNA, RNA and protein synthesis (36,45,46) and the formation of multinucleate cells due to cell fusion (42). Lee and Brownrigg (36) have shown that UV inactivated TIV is as efficient as live virus in causing a reduction in nucleic acid synthesis based on the failure of nuclei of infected cells to incorporate ^3H -thymidine. However, virus previously treated with the protein inhibitor β -propiolactone (BPL) had no effect on nucleic acid synthesis which suggests that possibly a protein component of the invading virus subverts nucleic acid metabolism similar to what has been observed with FV3 and poxvirus (47,48). BPL has been reported to inactivate many viruses in different taxonomic groups. The efficacy of BPL lies in the stability of the lactone form. However, in solution the lactone ring opens at both the alkyl and acyl bonds making it highly reactive with all of the radicals associated with proteins (49). Virus inactivation is probably a result of BPL binding to viral proteins. Little is known about specific changes in host enzyme responses following TIV infection. However, Jahagirdar and Seligy have shown that trehalase, a key enzyme in insect development and metabolism dramatically decreases in activity following infection of cells with TIV (50).

1.4 TIV genome.

The TIV genome is a complex entity and has been shown to consist of up to four DNA components (51), two major components L and S and at least two minor components of S detected after agarose gel electrophoresis of total TIV DNA and

staining the DNA with the intercalative dye ethidium bromide. Detailed analysis of the S fraction revealed that it has at least three components referred to as S1, S2 and S3. S1 represents about 90% of S while S2 and S3 are very minor components. All of the S components are related to the L moiety in some way with S1 and S2 being the most similar. S3 may be a subset of S2 and it has been speculated that either all three components are derivatives of L, and possibly deletion products, or S2 and S3 are derived from S1. S1 DNA possesses sequence which is common to about 1kbp of the L moiety (51). It is not known whether the S1 DNA sequence is included in L DNA; S DNA does not contain any cellular DNA sequences of *Galleria mellonella* which is the host generally used to propagate TIV (51). Analysis of DNA from a putative TIV sample obtained from Ireland, indicated that there was no S1 or S present. However, the L DNA of this sample also showed some differences in restriction endonuclease sites compared to TIV used at NRC (52).

The larger TIV L DNA is believed to be double stranded and linear and in size about 175-225 kbp. Summations of fragments obtained using different restriction endonucleases indicate that the size estimate of TIV is within the range of previous estimates which indicated the TIV genome to be 197-235 kbp (31). The analysis of DNA from TIV particles fractionated on sucrose density gradients, revealed that the top component fraction corresponding to partially filled virions contained less DNA relative to S1. These data suggested that S1 DNA may be exclusively or preferentially packaged into partially filled capsids of this fraction. It is possible that S1 is a product of normal or defectively assembled viral particles rather than a degradation product formed during the purification procedure. Studies involving measurement of transcription products of TIV expressed in infected cells by use of radiolabelled cDNA prepared from infected cell mRNA, revealed that both L and S1 DNA hybridized to the cDNA (52, 53). This would suggest that transcribed

sequences of L are present on S1 DNA, although it is not known if the latter serves directly as a transcription template. None of the TIV genes that have been isolated so far, including the capsid gene, have been found to hybridize to S1 DNA (53, 54).

1.5.1 Iridescent virus proteins.

The ability of a virus to multiply is dependent upon the synthesis and function of virus gene products, i.e the proteins. Viruses differ considerably in the number of genes that they contain. However, all viruses encode proteins for carrying out replication of the viral genome, packaging of the genome into virus particles and alteration of the structure and / or function of the infected cell. Strategies that viruses employ to ensure the execution of these functions vary. Sometimes viral proteins merely assist host proteins that replicate the virus genome e.g. papovavirus and papillomavirus. In most cases it is the virus proteins that replicate its genome but all viruses utilize at least some host proteins to accomplish this process. However, in all instances viral proteins ultimately package the genome into virions even though host proteins may interact with viral genomes before or during the biogenesis of the viral particle.

Within the Iridoviridae family, earlier studies have shown that the small iridescent viruses such as FV3 and CIV generally contain in excess of twenty polypeptides (55-58) whereas large iridescent viruses, such as MIV, have been reported as possessing only nine polypeptides (59). Comparisons of the proteins associated with the most intensively studied members of the Iridoviridae family, FV3, CIV and TIV, reveals several similarities amongst them i.e. numbers of polypeptides associated with the virus particles and their respective molecular weight ranges (Table 1.). Twenty six polypeptides, ranging in size from 10-230 kDa, have been observed in CIV following their solubilization under denaturing

Table 1.

Virus	Number of virion associated polypeptides	Mol. mass Range	Ref.
CIV	26-30	10-230 kDa	(58)
FV3	29	10-235 kDa	(68)
FLDV	33	4-220 kDa	(60)
ASFV	24	10-243 kDa	(61)
TIV	25-30	8.5-240 kDa	(34)

Table 1. A comparison of the polypeptides associated with the five most intensively studied iridescent viruses. CIV (Chilo iridescent virus), FV3 (Frog virus 3), FLDV (fish lymphocystis disease virus), ASFV (African swine fever virus), TIV (Tipula iridescent virus).

conditions in SDS-urea. By means of two-dimensional electrophoresis at least thirty polypeptides were observed with the major protein having an approximate molecular weight of 65kDa (58). Furthermore, this major polypeptide was located on the outside of the virus particle as demonstrated by surface labelling by radioiodination. Analysis of fish lymphocystis disease virus proteins by polyacrylamide gel electrophoresis under denaturing conditions, followed by subsequent staining with Coomassie blue, has revealed the presence of approximately 33 viral polypeptides. The number of polypeptides is probably underestimated and the molecular weights range from 4-220 kDa (60). A number of enzymatic activities has been found in FLDV but, however, there was no evidence of an RNA polymerase (60). The African swine fever virus (ASFV), which is no longer classified as a member of the iridescent virus family, has been estimated to contain approximately twenty-four proteins whose relative molecular weights range from 10-243 kDa (61). Experiments using radioactive ^3H fucose, ^{14}C glucosamine or ^{32}P -phosphate indicated that some ASF viral proteins were glycosylated and phosphorylated (61,62). ASFV has been shown to contain a protein kinase and also a DNA-dependent RNA polymerase which *in vitro* synthesizes RNA complementary to the viral DNA (63).

Studies on TIV have indicated that TIV virions contain approximately 28 polypeptides with a total estimated molecular mass of 1.72×10^6 daltons (34). More recent studies involving the use of protein binding reagents such as silver and Coomassie blue, and radioactive labelling with ^{35}S -Methionine, confirms the presence of between 25-30 polypeptides in medium to abundant amounts (52). Theoretically, with 200 kbp of DNA and an average gene size of 2-3 kb, TIV L DNA alone could encode more than 70-100 mid-sized protein products depending on gene density along the two strands. Therefore, the 25-30 gene products seen in TIV virions are perhaps the minimum needed for the final assembled virion.

Presumably other gene products are involved at various stages of TIV infection: transcription of early, mid and late genes, the shutting down of host macromolecular synthesis, replication of TIV DNA, assembly of VPC's and virions, and cell lysis.

The location of most iridescent virus proteins is not known with the exception of the major polypeptide which likely makes up most of the surface of the virus particle (64). Some proteins are DNA binding proteins and it is to be expected that these proteins are associated with the viral core (65). The location of several FV3 structural polypeptides has been established by stripping the different layers of the virion by controlled degradations using Brij, NP40 and pronase. The degradation products were separated on sucrose gradients and characterized by electron microscopy and SDS gel electrophoresis (66). Utilizing this technique the authors proposed the radial distribution of some of the FV3 constituent polypeptides to be within the viral envelope, in the capsomeres and others beneath the icosahedral capsid probably associated with the lipids as lipoproteins or perhaps as glycoproteins. By the utilization of two-dimensional gel electrophoresis, 29 viral structural proteins and 61 nonstructural proteins have been reported for FV3 (67). The FV3 virion is also reported to contain at least six enzymatic activities (68), two of which are found in the viral cores (69). One, a protein kinase of 44 kDa, has been purified to homogeneity (70).

1.5.2 Iridescent virus protein heterogeneity.

The heterogeneity of proteins found in TIV virions can be attributed to several factors. Each type of protein can potentially exist in a variety of forms owing to post-translational modifications of certain side chains. The modulation of charge, hydrogen-bonding capabilities and shape of the protein by these reversible covalent modifications may be important for protein function. For example, in the case of

the core associated proteins, post-translational modifications by phosphorylation, methylation, acetylation and ADP-ribosylation (71-76) may be important in packaging the DNA and regulating its availability for replication and transcription as in the case of host chromatin. Other forms of post-translational modifications commonly seen, involve varying degrees of glycosylation, not only in terms of specific residues being modified, but also of variations in oligosaccharide chain length and type. Heterogeneity in the protein complement of TIV virions may also arise owing to proteolysis and the inclusion of cellular proteins during packaging of the virion. In this regard, it has been shown that vaccinia virus will fortuitously package chloramphenicol acetyl transferase, a bacterial enzyme produced by the virus during its propagation in mammalian cells (77). Enzymes such as protein kinase, nucleotide phosphohydrolase, and RNA polymerase activity have all been reported as being associated with iridescent virus particles (55, 78-80). However, none of these enzymes has been shown to be virus encoded and may, therefore, be accidentally packaged during virus particle assembly.

1.6 Thesis objectives.

The analyses of viral proteins and their expression is one approach which can be used to gain insight into the intricacies of virion structure and the complexities of virion assembly. To date no iridovirus has been critically examined with respect to its protein composition. The molecular biology of TIV has been extensively investigated (51-54) and the acquisition of data regarding proteins associated with TIV will serve not only to complement existing data but also potentially to aid in its interpretation. The only polypeptide associated with TIV which has been studied and characterized in detail is the 48kDa capsid protein, in as much as the capsid gene has been cloned, sequenced (and putative amino acid sequence predicted), characterized and expressed in insect and bacterial cells (54). This thesis examines

the complexity of polypeptides associated with TIV particles and experiments have been designed to characterize individual polypeptides. Several approaches are described which were used in an effort to characterize TIV associated polypeptides.

1.6.1 Sub-unit composition of TIV polypeptides.

Polypeptides associated with TIV have traditionally been analyzed in polyacrylamide gels under denaturing conditions incorporating the use of mercaptoethanol as a reductant. This technique naturally excludes information with regards to oligomeric forms of viral proteins and their existence within the virus particle as polypeptide chains cross-linked by disulphide bridges. As a starting point for an investigation of the sub-unit composition of oligomeric TIV associated polypeptides, a comparative analysis of polypeptides following solubilization in SDS with and without 2-mercaptoethanol is described.

1.6.2 2-D polyacrylamide gel electrophoresis.

Previous estimates of the number of polypeptides associated with TIV have been based upon their separation and visualization in one dimensional SDS-polyacrylamide gels. Polypeptide chains in general bind similar amounts of SDS (~1.4 g SDS/g of polypeptide) (81) and become highly negatively charged by the addition of strongly acidic sulphonic acid groups. Thus the charge of the native protein is masked and proteins show a relatively constant charge to mass ratio. SDS induces conformational changes in polypeptides, although the absolute configuration in SDS is not known with certainty, and polypeptide chains saturated with SDS have been shown to migrate upon electrophoresis in polyacrylamide gels according to their molecular weight (82). The sizes of the polypeptide chains are subsequently determined by comparing their electrophoretic mobilities to the mobilities of well characterized standard proteins of known molecular weights. In

this thesis TIV associated polypeptides were analyzed by two-dimensional electrophoresis utilizing separations in the first dimension according to charge by isoelectric focussing, and in the second dimension upon a size basis by SDS-PAGE. 2-D gel electrophoresis produces separation patterns far superior in terms of the number of proteins which can be separated and identified and is, therefore, more advantageous when dealing with complex mixtures. When visualizing proteins in polyacrylamide gels, silver staining methods are reported to be about 100-200 times more sensitive than those based on Coomassie Brilliant Blue. Ochs et al. (83) have reported that of six silver staining methods studied, the best ones could resolve as little as 0.5 ng protein / mm² polyacrylamide gel. In examining the component polypeptides associated with TIV particles, and to ensure that those polypeptides in trace quantities were included in the evaluation, gels were silver stained to ensure maximal levels of sensitivity.

1.6.3 Amino acid sequence analysis.

Having mapped and catalogued TIV associated polypeptides, the question naturally arises as to individual protein function(s). To gain insight into a protein's function it must first be characterized. As an initial step in the detailed characterization of a polypeptide, a knowledge of even limited primary structural data can be of immense value. With the availability of efficient methods for the synthesis of oligodeoxynucleotides and DNA cloning, the most direct route to the isolation of specific cDNA or genomic DNA is the use of probes designed from partial amino acid sequences obtained from a target protein by direct protein sequencing methods. Limited protein amino acid sequence data, therefore, has several potentially useful applications such as gene isolation, mapping and DNA (and by inference protein) sequence determination. The latter in turn opens up other avenues for exploration such as protein structural analysis and the identification of

structural motifs, specific sites of post-translational modifications and as a basis for preparing antibodies specific for the protein of interest. One and two dimensional electrophoresis and subsequent electrotransfer to a suitable immobilizing support afford a rapid and efficient method of preparing polypeptides in an homogenous state for primary structural sequence analysis. Using this technique, N-terminal sequence analyses of TIV associated polypeptides is described.

1.6.4 Identification of surface structural polypeptides.

Further clues to a protein's function(s) can be obtained from its topographical location since the two are indirectly related. With the exception of the capsid protein, which is presumed to be located on the surface of the virion, no information is available on the locations of other TIV polypeptides. Polypeptides located on the surface of intact structures such as virions, can be identified by the use of specific probes. The iodination of cell surface proteins is well established, as is the fact that under defined conditions only surface proteins are labelled (84-87). Utilizing this technique the radioiodination of TIV surface polypeptides was investigated and is described in this thesis.

1.6.5 Glycoproteins.

Earlier studies on TIV by Krell and Lee (34) have suggested that none of the TIV virion polypeptides are glycosylated. This observation was based on the staining of polyacrylamide gels specifically for glycoproteins using the periodic acid-Schiff's (PAS) method of Bolognesi and Bauer (88). Using this method a faint pink staining was observed for six bands but this was interpreted as probably being non-specific and that the polypeptides in these bands were not glycosylated. A slightly more intense staining of a seventh band corresponding to a molecular mass of approximately 17.5kDa was suggested to be owing to its higher

concentration and was also considered to be non-specific. These authors did not make use of other staining techniques such as plant lectin concanavalin A (Con A). In this procedure Con A is coupled to horseradish peroxidase and colour development of the complex occurs with addition of diaminobenzidine. This exhibits a higher level of sensitivity (50X) than the PAS staining technique. In this thesis, Con A binding was used to re-evaluate the glycoprotein content of TIV particles following their separation on polyacrylamide gels and subsequent transfer to polyvinylidene (PVDF) membrane.

1.6.6 Empty capsid shells.

When crude preparations of TIV, propagated in *G. mellonella* larvae, are subjected to linear sucrose density gradient centrifugation, the fractionation of up to three viral bands generally results. These consist of a main band corresponding to full capsids, a less dense band corresponding to partially filled virions and a band consisting of empty capsids near the meniscus of the gradient. The occurrence of empty capsid shells and incomplete particles is a well recognized phenomenon and has been observed with various types of viruses, e.g. influenza viruses (89). The interpretation of the significance of these empty or partially empty capsids is debatable and it is not known whether they represent intermediates which have been blocked at a more advanced stage in the reproductive process or whether they are aberrant forms altogether. An analysis of the proteins of these intermediate virus particles has been made and compared with that obtained from full capsid components of the main viral band. This provides possible clues with respect to protein heterogeneity and virion location.

Materials and Methods

Materials.

Na^{125}I for protein iodination and ^{35}S ulphur "labelling reagent" (see section 2.2.2) were purchased in 1mCi and 500 μCi quantities respectively from Amersham Canada Limited, Oakville, Ontario. The enzyme lactoperoxidase (EC 1.11.1.7) was obtained from Bio-Rad Laboratories as an immobilized preparation coupled to a solid phase support (Enzymobeads). Chloramine-T was purchased from the Aldrich Chemical Company Inc., Milwaukee, WI 53233, U.S.A. The reagents used for electrophoresis were of electrophoresis purity grade and were purchased from Bio-Rad Laboratories. With the exception of SDS and Triton X-100, all the reagents were used without further purification. SDS was further purified by crystallization from ethanol-water as described in section 2.3.1 and the Triton X-100 was deionized as described in section 2.3.3.

The visualization of proteins following electrophoresis employed staining techniques using Coomassie brilliant blue, silver and 3,3'-diaminobenzidine. Coomassie brilliant blue was obtained from Bio-Rad Laboratories and 3,3'-diaminobenzidine from the Aldrich Chemical Company Inc. Silver staining employed analytical grade silver nitrate, high purity Clelands reagent (dithiothreitol) which was obtained from Calbiochem-Behring Corp., San Diego, CA, U.S.A. and glutaraldehyde which was purchased as a 50% solution from the Aldrich Chemical Company.

Reagents and solvents, used in automated gas-phase sequencing and for on line HPLC identification of PTH amino acid derivatives and ProBlottTM PVDF membrane, were obtained from Applied Biosystems Inc., Foster City, CA, U.S.A.

Tri-n-butylphosphine and 4-vinylpyridine for S-pyridylethylation were obtained from the Aldrich Chemical Company. Similarly, 6N HCL, 5% phenylisothiocyanate in heptane and other reagents and solvents employed in automated amino acid analysis were obtained from Applied Biosystems. All other reagents and solvents used (e.g. buffers) were prepared using Baker analytical reagent grade chemicals and BDH HPLC grade solvents. Solutions were made up as w/v, except for methanol and acetic acid which were v/v, unless otherwise indicated.

Methods.

2.1 Virus purification.

Purification of TIV from infected larvae was based on the procedure originally described by Youngusband and Lee (90) with modifications. Infected *Galleria mellonella* larvae, previously frozen at -80°C, were powdered and mascerated in 0.01M sodium phosphate buffer, pH 7.2. The mascerated larval suspension was centrifuged at 2,000xg for 5 minutes in a benchtop clinical centrifuge. The supernatant was poured off and subjected to high speed centrifugation at 35,000xg for 30 minutes using a Sorval SA 600 rotor in an RC-5 ultracentrifuge. The supernatant from the high speed centrifugation was discarded and the pellet was gently dispersed in 1ml of 0.01M sodium phosphate buffer pH 7.2. The suspended pellet was layered onto a linear, 10-40% (w/v) sucrose density gradient in 0.01M sodium phosphate buffer pH 7.2 and centrifuged at 15,000 rpm for 30 minutes in a SW-27 Beckman rotor at 4°C in a L3-50 Beckman ultracentrifuge. Following centrifugation, the opaque main viral band was removed, diluted 1:4 with 0.01M sodium phosphate buffer pH 7.2 and centrifuged in a SW-27 Beckman rotor at 4°C at 25,000 rpm for 1 hr. The viral pellet was resuspended in 1 ml of

phosphate buffer pH 7.2, layered onto a second identical sucrose gradient and centrifuged at 15,000 rpm for 30 min. The viral band was again diluted 1:4 with sodium phosphate buffer pH 7.2, centrifuged at 35,000xg for 1 hr, resuspended in buffer containing Garamycin at a concentration of 50µg per ml and stored at -70°C.

For the fractionation of ^{125}I or ^{35}S labelled TIV (see section 2.2) the following was done. Radiolabelled virus particle suspensions were layered onto 30ml 10-45% sucrose gradients in 0.01M sodium borate buffer pH 7.5. The gradients, in Beckman Ultraclear 25 x 89mm centrifuge tubes, were centrifuged at 15,000 rpm for 30 min in a SW-27 Beckman rotor at 4°C using a model L3-50 Beckman ultracentrifuge. Following centrifugation the tubes were removed from the rotor and clamped stationary in a vertical position. The base of the tube was punctured with an 18 gauge hypodermic needle connected to a short length of intramedic tubing and sixty 0.5ml fractions collected. A 20µl aliquot of each fraction was removed and counted in 2.5ml of Aquasol liquid scintillant (New England Nuclear) using an LKB model 1217 Rackbeta liquid scintillation counter. Appropriate fractions were pooled into lots and dialysed extensively in 1,000 mol.wt. cut-off dialysis membrane against 0.01M Na_2HPO_4 buffer pH 7.5 at 4°C. Dialysed fractions were transferred to Eppendorf tubes, centrifuged at 15,000 x g for 30 min at 4°C and the virus particle pellets resuspended in 50µl of 0.01M Na_2HPO_4 buffer pH 7.5 and stored at -70°C.

2.2 Radioisotope labelling of viral proteins.

Radioisotope labelling of viral proteins was performed using ^{125}I in the presence of lactoperoxidase or chloramine T and with ^{35}S using a "general purpose labelling reagent". Virus suspensions used in the labelling experiments were all previously purified by sucrose density gradient centrifugation as described above.

2.2.1 ^{125}I labelling of viral polypeptides.

Two methods of iodine-labelling utilizing ^{125}I labelled sodium iodide were used. The first method utilized the enzyme lactoperoxidase to radiolabel the surface proteins as described by Stanley and Haslam (91) and Marchalonis (87). To provide the most gentle possible conditions for enzymatic iodination, immobilized preparations of lactoperoxidase and glucose oxidase (Enzymobeads) were used (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Upon addition of glucose to a suspension of the Enzymobeads, Na^{125}I and protein, the immobilized glucose oxidase continuously generates a small but steady amount of hydrogen peroxide. The lactoperoxidase in turn catalyzes the peroxide oxidation of labelled iodide to iodine. The labelled iodine then reacts with the protein to produce radioiodinated protein (92-97). The specific activity of the [^{125}I]-sodium iodide was determined immediately prior to use using the decay curve supplied by the manufacturer. Detailed components of the reaction were as follows:

In a 1.5ml Eppendorf tube, a 20 μl aliquot of purified virus suspension was mixed with 50 μl of rehydrated Enzymobead reagent. To the suspension, 10 μCi of Na^{125}I in 1 μl of 0.001N sodium hydroxide, 25 μl of a 1% β -D-glucose solution and 7.6 μl of 100 mM Na_2HPO_4 pH 7.5 buffer were added. The reaction mixture was gently vortexed and allowed to incubate at room temperature for 30 minutes. To stop the reaction, the reaction mixture was centrifuged at 1,000 x g for 5 minutes to pellet the Enzymobeads and the supernatant was transferred by pipette to a clean 1.5 ml Eppendorf tube. Virus particles were pelleted by centrifuging at 15,000 x g for 30 minutes in an Eppendorf model 5414 benchtop centrifuge and the supernatant discarded. The pelleted virus particles were washed twice by resuspension in 50 μl of 10 mM Na_2HPO_4 pH 7.5 buffer, centrifuged at 15,000 x g for 30 minutes and the supernatants discarded.

The second method used chloramine-T to selectively iodinate the proteins of TIV. This reaction contained 30 μ l of virus suspension, mixed with 10 μ Ci of Na^{125}I , followed by 15 μ l of chloramine-T solution (5mg/ml). After 90 seconds the reaction was terminated by the addition of 30 μ l of a 10mg/ml solution of sodium metabisulfite. Both the lactoperoxidase and chloramine-T iodinated virus particles were centrifuged on 10%-40% sucrose gradients in 0.01M sodium borate buffer pH 7.5, as described in section 2.1, to eliminate any disrupted virus particles. The gradient banded chloramine-T and lactoperoxidase labelled virus particles were extensively dialysed against 0.01M Na_2HPO_4 pH 7.5 to remove any remaining traces of free ^{125}I and sucrose prior to electrophoresis. Dialyzed virus particles were pelleted at 15,000 x g for 30 min, resuspended in 30 μ l 0.01M Na_2HPO_4 pH 7.5 and stored at -70°C .

2.2.2 ^{35}S labelling of viral polypeptides.

Virus associated polypeptides were also radiolabelled with ^{35}S using a general purpose ^{35}S -labelling reagent (^{35}S -LR), t-Butoxycarbonyl-L-(^{35}S) methionine N-hydroxysuccinimidyl ester (Amersham Canada limited). This reagent reacts with amino groups via an active ester reaction and takes place under very mild conditions. The specific activity of ^{35}S -LR was determined immediately prior to use using the ^{35}S decay curve supplied by the manufacturer. The reaction sequence was as follows:

First, 10 μ Ci of ^{35}S -LR in 10 μ l of toluene was pipetted into a 1.5 ml Eppendorf tube, dried under a gentle stream of argon and cooled on ice. To this was added a 30 μ l suspension of purified TIV in 0.01M Na_2HPO_4 pH 7.5 and the mixture was gently vortexed. The reaction mixture was incubated on ice and the reaction terminated by the addition of 100 μ l of 0.2 M glycine in 0.01M Na_2HPO_4 pH 7.5. The radiolabelled virus particles were recovered by centrifugation at

15,000 x g for 30 minutes, washed twice in 50 μ l of 0.01M Na_2HPO_4 pH 7.5 and stored at -70°C .

2.3 Polyacrylamide gel electrophoresis.

Polypeptides associated with TIV particles were analyzed by both one and two dimensional electrophoresis in polyacrylamide gels according to methods described in section 2.3.2 and 2.3.3.

2.3.1. SDS purification.

Bio-Rad electrophoresis grade SDS was purified by crystallization from ethanol-water prior to use in SDS-PAGE according to the method of Hunkapillar (98). 100g of SDS was gradually added to 450 ml of ethanol with stirring and heated to 55°C . Distilled water at 75°C was added until all of the SDS had dissolved (~175 ml). To this was added 10g of activated charcoal. After stirring the solution for 10 min, it was filtered through a Buchner funnel with a Whatman No.5 filter paper to remove the charcoal. The filtrate was stored at 4°C for 24 hr to initiate crystallization and at -20°C for a further 24 hr. The crystalline SDS was collected on a coarse-frit sintered-glass funnel and washed with 900 ml of ethanol previously chilled to -20°C . The SDS was then recrystallized a second time using the same procedure but without the charcoal treatment and the purified SDS dried under vacuum overnight at room temperature.

2.3.2 One dimensional electrophoresis.

One dimensional electrophoresis was performed according to the method of Laemmli (99) on 5.5cm x 8cm x 0.5mm gels containing 12.5% acrylamide with a 0.5cm 4% acrylamide stacking gel prepared from a stock solution of 30% acrylamide and 0.8% N,N'-bis-methylene acrylamide. Virus particles were

dissociated by heating at 95°C for 3 min in sample application buffer (0.0625M Tris-HCL (pH 6.8), 2% SDS, 10% glycerol (v/v), 5% 2-mercaptoethanol (v/v), 0.001% bromophenol blue) and 5µl aliquots were loaded onto the gels under electrode buffer (0.025M Tris, 0.192M glycine, 0.1% SDS pH 8.3). Electrophoresis was carried out at 200V constant voltage until the bromophenol blue marker dye had reached the bottom of the gel (approx. 45 min.). The gels were removed and fixed in 50% methanol / 10% acetic acid for 30 minutes at room temperature prior to silver staining. Alternatively, following fixation of the gels in methanol / acetic acid solution, the gels were incubated in 2% (v/v) glycerol solution and dried using a Bio-Rad model 224 slab gel dryer. Dried gels were subsequently autoradiographed at -70°C using Dupont Cronex film (E.I. Dupont Inc., Wilmington, DE, U.S.A.) with an intensifying screen.

2.3.3 Two dimensional electrophoresis.

Two dimensional gel electrophoresis was performed according to the procedure of O'Farrell (84,85) with some minor modifications. The first dimension isoelectric focussing gel (IEF) had the following composition: 4% acrylamide, 9.2M urea, 2% ampholytes (from a 40% stock solution containing ampholytes in the pH range 3-10) and 2% (v/v) Triton X-100 diluted from a 10% (v/v) stock solution deionized overnight with AG501-X8 ion exchange resin (Bio-Rad Laboratories, Richmond, CA, U.S.A). Analytical IEF was performed in disposable 200 µl glass capillary tubes (1mm ID x 7 cm). Glass capillary tubes were inserted in an upright position into a casting tube (7cm x 1cm), the bottom of which was sealed with several layers of Parafilm laboratory film. The gel solution (5mls) was prepared, degassed for 15 min on a water aspirator and TEMED (N,N,N',N'-tetramethylethylenediamine) and ammonium persulfate polymerizing agents added. The gel solution was pipetted into the casting tube, so as to allow the

capillary tubes to fill from the bottom, to a height of 6 cm. After polymerization (approx. 2hr), the Parafilm was gently removed from the bottom of the casting tube and the capillary tubes were carefully pushed out of the casting tube from the top, care being taken not to introduce air bubbles into the bottom of the capillaries. The outer surface of the capillaries was wiped clean to remove excess acrylamide and assembled in the tube gel IEF microchamber (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The bottom of the tubes was rinsed with anolyte buffer (10 mM H_3PO_4) and the upper surface of the gel rinsed with catholyte buffer (20 mM NaOH). The gels were pre-electrophoresed at 300V for 1hr and 400V for 3hr. Following pre-electrophoresis, the upper and lower chamber buffers were discarded and replaced with freshly degassed buffers.

Virus particles were disrupted by heating at 95°C for 3 min in 5 μl of sample buffer (0.2% SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.0625M Tris-HCL pH 6.8), cooled to room temperature and 30 μl of first dimension sample buffer added (9.5M urea, 2% (v/v) Triton X-100, 5% (v/v) 2-mercaptoethanol, 2% 3-10 ampholytes). The sample was applied under catholyte buffer using a 50 μl Hamilton syringe and overlayed with 20 μl of sample overlay buffer (9M urea, 2% 3-10 Ampholytes, 0.001% bromophenol blue). The proteins were focussed at 400V for 1hr, 600V for 18hr followed by 750V for 2hr. Following isoelectric focussing, the tube gels were extruded onto strips of Parafilm laboratory film by the gentle application of pressure on one end of the tube using a 1ml disposable syringe filled with second dimension electrode buffer. The gels were wetted in 150 μl of SDS electrode buffer and equilibrated at room temperature for 10mins. The tube gels were straightened out on the Parafilm strips and gently slid with the aid of a spatula between the glass plates of a second dimension slab gel consisting of a 5.5cm x 8.0cm x 1mm 12.5% polyacrylamide separating gel and a 0.25 cm x 8.0cm x 1mm 4% polyacrylamide stacking gel. The second dimension was run at

200V constant voltage until the bromophenol blue marker dye in the standard well reached the bottom of the gel. The gels were removed from between the plates and fixed in 50% methanol /10% acetic acid for 30min at room temperature. Protein spots were subsequently visualized by silver staining.

2.3.4 Two-dimensional SDS-PAGE.

Two-dimensional SDS-PAGE was used to detect sub-units of oligomeric TIV associated polypeptides. TIV particles were dissociated in sample application buffer (0.0625M Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue) lacking 2-mercaptoethanol by heating at 95°C for 3 minutes. A 5µl aliquot was loaded onto a 1mm (ID) x 6cm 12.5% acrylamide tube gel, containing a 0.5 cm 4% acrylamide stacking gel, under electrode buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3). The TIV polypeptides were electrophoresed through the gel at 250V constant voltage until the bromophenol blue marker dye was approximately 1cm from the bottom of the gel. The tube gel was extruded into a petri dish and soaked in buffer containing 0.5M Tris-HCL pH 6.8, 0.4% SDS and 5% 2-mercaptoethanol for 30 minutes at room temperature in order to dissociate the disulphide bonds of contained protein. The tube gel was slid between the glass plates of a 5.5 cm x 8.0 cm x 1mm 12.5% acrylamide separating gel and a 0.25 cm x 8.0 cm x 1mm 4% acrylamide stacking gel. A 5µl sample of TIV particles dissociated under reducing conditions was applied under second dimension electrophoresis buffer to the standard well of the gel and the second dimension was run at 200V constant voltage until the bromophenol blue marker dye in the standard well reached the bottom of the gel. Following electrophoresis the gel was fixed in 50% methanol / 10% acetic acid for 30 minutes and protein spots subsequently visualized by silver staining.

2.4 Silver staining of SDS/PAGE gels

Polyacrylamide gels were stained with silver according to the method of Morrissey (102). Following electrophoresis, gels were prefixed in 50% methanol /10% acetic acid for 30 minutes followed by 5% methanol / 7% acetic acid for 30 minutes. The gels were fixed in 10% glutaraldehyde for 30 minutes and subsequently washed extensively in Milli-Q water (4 x 30min plus overnight). After the complete removal of glutaraldehyde, the gels were incubated for 30 minutes with dithiothreitol solution (5µg/ml) followed by treatment with 0.1% AgNO₃ solution for 30 minutes. Prior to development, the gels were rapidly rinsed with Milli-Q water (~1-2 min) and then soaked in developer (50µl of 37% formaldehyde in 100ml 3% sodium carbonate) with gentle agitation until the desired level of staining was attained. Staining was stopped by the addition of 20 ml 2.3 M citric acid directly to the developer and agitating for 5 minutes. Stained gels were washed in two changes of Milli-Q water and stored at room temperature in 2% v/v glycerol solution.

2.5.1 Molecular mass determination of polypeptides by SDS/PAGE.

Calibration curves relating log₁₀ molecular mass and relative mobility were drawn from data obtained by electrophoresing proteins of known molecular mass in 12.5% acrylamide gels. SDS-PAGE protein standards were purchased from Novex Ltd., San diego, CA. 92121, U.S.A. The protein standards ranging in molecular mass from 6kDa to 200kDa were as follows: rabbit muscle myosin (200kDa), *E.coli* β-galactosidase (116.3kDa), rabbit muscle phosphorylase b (97.4kDa), bovine serum albumin (66.3kDa), glutamate dehydrogenase from bovine liver (55.4kDa), lactate dehydrogenase from porcine muscle (36.5kDa), bovine erythrocyte carbonic anhydrase (31kDa), soybean trypsin inhibitor

(21.5kDa), chicken egg white lysozyme (14.4kDa), and aprotinin from bovine lung (6kDa).

The contents of one vial of protein standards, containing 200µg of each of the above proteins, were dissolved in 200µl of SDS sample buffer (0.0625M Tris-HCL pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue). The vial was placed in a heating block at 95°C for 4 minutes, cooled to room temperature and 10µl of the diluted standard pipetted out into a 0.5ml Eppendorf tube and further diluted by the addition of 190 µl of SDS sample buffer. 3µl aliquots (150 ng of each protein) of the diluted protein standards were electrophoresed through 12.5% acrylamide gels, as described above, alongside samples of purified, dissociated TIV particles. The gels were silver stained and the $\log_{10}$ molecular mass of the virus particle polypeptides determined from their mobilities relative to the protein standards by interpolation from the calibration curve.

2.5.2 Molecular mass determination of radiolabelled polypeptides.

The apparent molecular masses of virus associated polypeptides which were radiolabelled and subsequently detected by autoradiography, were determined from their mobilities relative to ^{14}C -labelled protein standards. ^{14}C -labelled proteins ranging in molecular mass from 200kDa to 14.3kDa and consisting of myosin heavy chain (200kDa), phosphorylase b (97.4kDa), bovine serum albumin (66.3kDa), ovalbumin (43kDa), carbonic anhydrase (29kDa), β -lactoglobulin (18.4kDa) and lysozyme (14.3kDa) were obtained from Bethesda Research Laboratories, Gaithersburg, MD 20877, U.S.A.. Typically 0.05 µCi of ^{14}C -labelled protein standards were electrophoresed simultaneously with samples of radiolabelled virion-associated polypeptides. The molecular masses of the radiolabelled TIV associated polypeptides were subsequently determined by

interpolation from the calibration curve derived from mobility versus $\log_{10}$ molecular mass of the ^{14}C -labelled standards.

2.6.1 Automated sequence analysis.

Polypeptide samples for sequence analysis were prepared by 2-D gel electrophoresis and subsequently transferred to PVDF membrane. Virus particles were dissociated in electrophoresis sample buffer as described in section 2.3.3 and loaded onto the first dimension IEF tube gels and focussed as described above. Following equilibration in Laemmli buffer, the tube gels were applied to and electrophoresed through a pre-aged (2 days at 40°C) 10cm x 10 cm x 0.5mm thick 12.5% separating gel as described by Laemmli (99) using a Bio-Rad Mini-Protean II cell (Bio-Rad Laboratories, Richmond, CA, U.S.A.) After electrophoresis the gel was soaked in transfer buffer (10mM CAPS (3-Cyclohexylamino-1-propanesulphonic acid) pH 11.0 in 10% methanol) and electroblotted onto ProBlotTM PVDF membrane at 50V constant voltage for 1 hr according to the method of Masudaira and LeGendre (103,104). The membrane was removed from the transfer unit and washed extensively with Milli-Q water (Millipore Corp., Bedford, MA, U.S.A.) prior to staining. The washed membrane was immersed in methanol for a few seconds and then stained with 0.05% Coomassie brilliant blue in 50% methanol / 10% acetic acid for one minute at room temperature. Following destaining in 50% methanol / 10% acetic acid for 3-5 minutes at room temperature, the stained membrane was air dried. Protein spots were excised with a clean razor blade and stored in Eppendorf tubes at -20°C until use.

The stained PVDF membrane / protein spots were cut into approximately 1mm squares and carefully aligned around the periphery in the recess of the upper cartridge block of the sequencer reaction chamber, care being taken to avoid occluding the reagent / solvent inlet port. The membrane pieces were held stationary in the cartridge block by insertion into the recess of a glass fibre disc

containing 0.1mg of biobrene (Applied Biosystems, Foster City, CA, U.S.A.). Automated gas phase sequencing was performed on an Applied Biosystems 475A protein sequencing system incorporating a model 470A gas-phase sequencer equipped with an on-line model 120A PTH analyser under the control of a model 900A control/data analysis module.

2.6.2 *In situ* alkylation of Cysteine residues.

Of the 20 naturally occurring amino acids, cysteine (Cys) is the most commonly derivatized for analytical purposes since it is chemically labile. During the Edman degradation unmodified Cys produces inconsistent results unless the reactive thiol group is properly "neutralized" by appropriate chemical modification. S-alkylation of Cys residues with 4-vinylpyridine is a simple and selective procedure and the PTH-PE-Cys derivative is well resolved from other PTH amino acid derivatives. The S-pyridylethylation of proteins was performed following their transfer to PVDF membrane prior to sequencing. Membrane containing protein to be sequenced was aligned in the recess of the upper cartridge block of the sequencer reaction chamber as described in section 2.6.1. The membrane was wetted with a reduction/alkylation cocktail consisting of 2 μ l tri-n-butylphosphine, 5 μ l 4-vinylpyridine and 100 μ l of sequencer grade acetonitrile and the reaction cartridge immediately reassembled. Advantage was taken of the controlled programmable environment available in the cartridge assembly of the sequencer for the pyridylethylation reaction and the program shown in table 2 was executed. Following completion of the program, sequencing was initiated using a normal sequencing cycle.

2.6.3 Data base protein sequence alignment.

Protein sequences of TIV polypeptides, determined as described in section 2.6.1, were used to screen the Genbank and modified EMBL data bases using the Genetics computer group sequence analysis software package version 7.2 (105).

Table 2. Automated sequencer program used for pyridylethylation reaction of TIV polypeptides.

Step	Function	Time (secs).	Elapsed time.
1	Prep R2	4	0 min 4 sec
2	Deliver R2	60	1 min 4 sec
3	Pause	3000	51 min 4 sec
4	Argon dry	300	56 min 4 sec
5	Deliver S3	300	61 min 4 sec
6	Argon dry	120	63 min 4 sec
7	Deliver R2	60	64 min 4 sec
8	Argon dry	120	66 min 4 sec
9	Deliver S2	300	71 min 4 sec
10	Argon dry	120	73 min 4 sec

R2; 12.5% trimethylamine in water.

S2; Ethyl acetate

S3; Butyl chloride

2.7 Amino acid composition analysis.

Amino acid composition analysis of TIV associated polypeptides, was performed using a fully automated Applied Biosystems 420A amino acid analysis system (Applied Biosystems Inc., Foster City, CA, U.S.A.). Virus particle associated polypeptides were separated by 2-D PAGE techniques as described in section 2.3.3. Immediately following separation in the second dimension using SDS-PAGE, the gel was transferred to electroblotting buffer (10 mM CAPS pH 11 in 10% methanol) and incubated at room temperature for a period of 10 mins with gentle agitation. The separated polypeptides were subsequently electroblotted onto PVDF membrane at 50V for 1 hr and the membrane stained in 0.05% Coomassie brilliant blue and destained as described in section 2.6. Visualized protein spots were excised from the membrane and stored at -20°C in Eppendorf tubes. The PVDF-immobilized, stained protein spots were placed onto the sintered glass discs of the sample application slides and subjected to automated on-line gas-phase hydrolysis using 6N HCL at 155°C for 75 minutes. Following hydrolysis the samples were reacted with 5% PITC in heptane for automated derivatization of hydrolyzate amino acids to the corresponding PTC amino acids. The PTC amino acids were analyzed by HPLC on a 2.1mm x 25cm C18 reverse phase column (Applied Biosystems Inc.), thermostatted at 35°C, by gradient elution with 70% acetonitrile in 32mM sodium acetate pH 6.4 at a flow rate of 300 µl per minute. The combined cystine and cysteine content of polypeptides was determined after oxidation to cysteic acid with performic acid (106). Protein spots excised from PVDF membrane were placed inside of 500 µl Eppendorf tubes whose caps had been punctured with an 18 gauge hypodermic needle. The Eppendorf tubes were placed in a desiccator containing 10ml of performic acid, evacuated on a water aspirator for 10 minutes and exposed to performic acid vapour for 4 hours at room temperature. Following oxidation the membrane pieces were transferred to a

second desiccator, dried under vacuum for 1 hour and subjected to automated amino acid analysis.

2.8 Lectin staining of glycoprotein.

Polypeptides associated with TIV were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane as described in sections 2.6 and 2.7. Immediately following the electrotransfer of separated polypeptides, the membrane was rinsed in Milli-Q water and then soaked in a 2% solution of polyvinylpyrrolidone-360 (PVP-360) in phosphate buffered saline pH 7.4 for 45 minutes at room temperature. The membrane was then transferred to and incubated in biotinylated concanavalin A solution (10 μ g / ml in 2% PVP-360 solution) for 1 hr at room temperature followed by extensive washing in 25 mM Tris / HCL in 0.5M NaCl pH 7.5 containing 0.1% Triton X-100 (TNT buffer). The washed PVDF membrane was incubated with avidin-biotinylated horseradish peroxidase complex (Vector laboratories Inc., Burlingame, CA. 94010, U.S.A.) in phosphate buffered saline for 45 minutes at room temperature followed by extensive washing (4 x 10 min) with TNT buffer. Glycoproteins were visualized by staining with 3,3'-diaminobenzidine- H_2O_2 reagent (5 mg of 3,3'-diaminobenzidine in 30 ml of 25 mM Tris-HCL / 0.5M NaCl pH 7.5 buffer containing 10 μ l of 30% H_2O_2) at room temperature for ~1 minute. The staining reaction was stopped by washing the membrane extensively in Milli-Q water and drying the developed membrane by placing it between two sheets of Whatman 3MM filter paper.

2.9 Computing laser densitometry of polyacrylamide gels.

Quantitative analysis of TIV polypeptides in polyacrylamide gels was performed by laser scanning densitometry using a Molecular Dynamics model 300A computing laser densitometer (Molecular Dynamics Inc., Sunnyvale, CA 94086

U.S.A). Scans were obtained either from photographic prints of stained gels or from X-ray film autoradiographs of gels containing radiolabelled polypeptides.

Results.

3.1. Characterization of TIV associated polypeptides by SDS-PAGE.

Gel electrophoresis is extremely useful as an analytical tool for the separation and quantification of protein species from complex mixtures. The initial characterization of TIV associated polypeptides was therefore performed using SDS-PAGE.

3.1.1. Optimization of the separation of TIV associated polypeptides by one-dimensional SDS-PAGE.

Shapiro, Vinuela and Maizel reported that the separation of proteins by PAGE in the presence of the anionic detergent SDS is dependent upon the molecular masses of their polypeptide chains (107). The migration of protein molecules through a polyacrylamide matrix, therefore, is also dependent upon the pore size of the gel itself. To obtain optimal separation of both high and low molecular mass TIV associated polypeptides, and to ensure that very low molecular mass polypeptides were not lost during attempts to resolve high molecular mass species, a preliminary experiment was conducted using gels consisting of 10%, 12.5% and 15% acrylamide concentrations (Figure 1).

Polypeptides separated in gels containing a monomer, i.e acrylamide plus N,N'-methylenebisacrylamide, concentration of 10% (Figure 1A) gave well-defined and well-resolved banding patterns of polypeptides ranging in molecular mass from 211kDa to 15kDa. However, polypeptides with molecular masses less than 15kDa were lost due to elution from the bottom of the gel. In gels containing acrylamide concentrations of 12.5% or greater (Figures 1B and 1C) all low

Figure 1. SDS-PAGE of TIV associated polypeptides in gels containing different concentrations of acrylamide. A. 10% acrylamide; B. 12.5% acrylamide; C. 15% acrylamide. Electrophoresis was performed at 200V constant voltage (20V/cm) for 45 minutes at room temperature (22°C). Following electrophoresis, the gels were removed from the apparatus and silver stained. Lane 1 of each gel contains 2µg of molecular weight markers and lane 2 10µg of TIV particles disrupted at 95°C in SDS sample application buffer.

molecular mass polypeptides were retained in the gel. However, in 15% gels there was a loss in resolution among the high molecular mass species, particularly in excess of 60kDa. The use of monomer acrylamide concentrations of 12.5% (Figure 1B) allowed the retention of low molecular mass polypeptides without adversely affecting the resolution of the higher molecular mass species. All subsequent separations of TIV associated polypeptides were therefore performed in 12.5% acrylamide gels. Gradient gel electrophoresis which utilizes an acrylamide concentration gradient was not used as this method required special equipment for producing the gradient and there were problems in forming reliable and reproducible gradients.

3.1.2. TIV protein detection in one-dimensional SDS-PAGE.

The visualization of proteins in polyacrylamide gels can be achieved by either dye or silver staining methods. However, dye staining methods for proteins in gels are not always as sensitive as silver staining methods. The latter are reported to be about 100-200 times more sensitive than those based on the most commonly used dye, Coomassie brilliant blue (83). This was found to be true for TIV polypeptides.

As shown in Figure 2, the silver staining method reported by Morrisay (102) visualized 50-56 polypeptides and only 25-28 by the Coomassie brilliant blue method. Reduction of protein before electrophoresis had a significant effect, since under non-reducing conditions staining with Coomassie brilliant blue resulted in only 16 polypeptide bands being visualized, while in contrast silver staining revealed a total of 31 polypeptides. Given these results all subsequent experiments were performed using silver stain.

Figure 2. A comparison of staining methods for the detection of TIV associated polypeptides separated by SDS-PAGE. 10 μ g aliquots of TIV particles were disrupted in SDS at 95°C and electrophoresed through 12.5% SDS-polyacrylamide gels at 20V/cm for 45 minutes at room temperature (22°C). The gels were fixed in methanol/acetic acid and stained using either silver (A) or Coomassie brilliant blue (B). Respective samples were run under identical conditions both non-reducing (Lane 1) and reducing (Lane 2).

3.1.3. Unit complex of TIV polypeptides in the SDS soluble fraction of the virus.

A comparison of the electrophoretic pattern of TIV polypeptides under reducing and non-reducing conditions indicates that some of the viral polypeptides can be polymerized by their sulphydryl residues. To detect structural virus proteins linked by disulphide bonds, TIV was solubilized and electrophoresed under non-reducing conditions in the first dimension and under reducing conditions in the second dimension. Under these conditions viral polypeptides originally linked by disulphide bonds were expected to separate into subunits.

The first dimension tube gel, in which the SDS soluble fraction of TIV under non-reducing conditions was electrophoresed, was immersed in the standard sample lysis buffer containing 5% 2-mercaptoethanol, incubated at room temperature for 15 minutes and then subjected to a second dimension SDS-PAGE (Figure 3). Bands containing intermolecularly disulphide bonded polypeptides should theoretically fall off the diagonal line while those consisting of single chain polypeptides should remain on the diagonal. Several bands in the first dimension gel were found to be composed of complexed polypeptides. For example, a complex with a molecular mass of 167kDa after electrophoresis in the first dimension, following equilibration in buffer containing 2-mercaptoethanol and subsequent SDS-PAGE is seen to be an oligomer consisting of polypeptides with molecular masses of 71kDa, 49kDa and 40kDa with a relative copy ratio of 1:1:1. Similarly a complex migrating with an approximate molecular mass of 151kDa under non-reducing conditions is an oligomeric complex consisting of polypeptides with apparent molecular masses of 73kDa, 40kDa and 39kDa, also in a stoichiometric 1:1:1 ratio.

Figure 3. Relationship between TIV polypeptide size and intermolecular disulphide bridges. TIV polypeptides were electrophoresed by two-dimensional SDS-PAGE under (1) non-reducing conditions and (2) reducing conditions as described in section 2.3.4 under Materials and Methods. Three, possibly four, spots (indicated by arrows) appear above the diagonal line of polypeptides and these are discussed in section 3.1.3. For reference, an SDS-PAGE profile of TIV associated polypeptides under reducing conditions was run in the standard well of the gel in the second dimension.

It is evident that not all of the oligomeric complexes are dissociated into their constituent polypeptides upon exposure to reducing agent following electrophoresis under non-reducing conditions. Evidence that these complexes are in part maintained by disulphide bonds is apparent from the electrophoretic separations of TIV particles dissociated under both reducing and non-reducing conditions (Figure 2). However, in this case the TIV particles were dissociated by heating at 95°C for 3 minutes in 2% SDS in either the presence or absence of 2-mercaptoethanol. However, equilibration of the tube gel, following electrophoresis in the first dimension, in buffer containing 2% SDS/2-mercaptoethanol was performed at room temperature and not at an elevated temperature as described above. Under these conditions certain of the oligomeric species may be able to maintain their subunit association by means of non-covalent hydrogen bonds and hydrophobic interactions which are resistant to SDS at room temperature but more labile at higher temperatures. However, this does not appear to be the case with respect to the 167kDa and the 151kDa oligomeric complex which readily dissociates into subunits upon exposure to 2-mercaptoethanol. With these oligomeric complexes, heating at 95°C under non-reducing conditions prior to the first dimension electrophoresis, probably destroys the structure of the complex even though the covalent disulphide bonds remain intact. Subsequent exposure to reducing agent at room temperature prior to the second dimension electrophoresis results in the cleavage of disulphide bonds giving rise to the monomeric subunits of the complex.

Other forms of complexes were also noted. Three spots, possibly four, indicated by arrows in Figure 3, appear to migrate above the diagonal line of polypeptides. This indicates that these polypeptide species migrated in a lower molecular size in the first dimension and behaved as larger molecules in the second dimension. This suggests that these polypeptides were probably compact

forms held together by intramolecular disulphide bond(s). Upon reduction of the disulphide bond(s), the polypeptides were unfolded and thus migrate as larger molecules in the second dimension. Therefore, the increase in apparent molecular weight in the reduced state may be due to a change in molecular conformation to a more random form.

3.1.4 Calibration of TIV polypeptides according to molecular mass.

In this thesis, TIV polypeptides were numbered and catalogued on the basis of their apparent molecular masses, calculated by interpolation from a standard calibration curve shown in Figure 4. The term relative molecular weight or M_r , is avoided since it is dimensionless (M_r is the ratio of the mass of the molecule to one twelfth of the mass of the nuclide ^{12}C). However, molecular mass, M_m , is the mass of one molecule of a substance, expressed in daltons or atomic mass units, which is defined as one twelfth the mass of one atom of ^{12}C . The calibration curve was constructed using the relative mobilities of proteins of known molecular mass shown in lane 3 of Figure 5. The M_m of TIV polypeptides was determined under both reducing and non-reducing conditions which permitted molecular mass determinations of intermolecular disulphide-bonded oligomers.

Table 3 summarizes the molecular masses for TIV polypeptides under reducing and non-reducing conditions. The numerical value of a given TIV polypeptide was based on its relative size beginning with the largest. Hence those polypeptides visualized under non-reducing conditions were numbered 1-31 and those under reducing conditions 1-56. The combined molecular masses of all polypeptides based on non-reducing conditions was estimated to be 2.52×10^6 daltons. This value is in good agreement with the value of 2.57×10^6 daltons obtained for the total molecular mass of polypeptides determined under reducing conditions. A greater error exists in estimating a high molecular mass

Figure 4. Relationship of electrophoretic mobility and molecular mass of known polypeptides. Mobility was determined relative to the migration of bromophenol blue marker dye through a 12.5% acrylamide, 0.34% bisacrylamide slab gel at 20V/cm for 45 minutes at 22°C. The molecular masses of proteins (data provided by the manufacturer, see section 2.5.1 of Materials and Methods) were plotted as $\log_{10}$.

Points: 1, Bromophenol blue (173); 2, bovine lung aprotinin (6kDa); 3, chicken egg white lysozyme (14.4kDa); 4, soybean trypsin inhibitor (21.5kDa); 5, bovine erythrocyte carbonic anhydrase (29kDa); 6, porcine muscle lactate dehydrogenase (36.5kDa); 7, bovine liver glutamic dehydrogenase (55.4kDa); 8, bovine serum albumin (66.3kDa); 9, rabbit muscle phosphorylase b (97.4kDa); 10, E.coli β -galactosidase (116.3kDa); 11, rabbit muscle myosin heavy chain (200 kDa).

Figure 5. One dimensional SDS-PAGE of TIV associated polypeptides under reducing and non-reducing conditions used for polypeptide molecular mass determination. TIV particles were disrupted in SDS sample buffer in the absence of 2-mercaptoethanol (lane 1) and in buffer containing 5% 2-mercaptoethanol (lane 2). Both samples were electrophoresed in parallel through a 12.5% acrylamide gel at 20V/cm for 45 minutes at 22°C with standard molecular mass markers (lane 3). Following electrophoresis the gel was fixed in methanol/acetic acid and protein bands subsequently visualized by silver staining. Polypeptide bands were numbered in descending order with respect to molecular mass. A summary of three such experiments is given in Table 3.

Table 3. Numerical identification of TIV associated polypeptides and their estimated molecular masses. The numerical value assigned to each polypeptide is based on its relative size beginning with the largest. The molecular masses of polypeptides were determined under both non-reducing conditions (Table 3A) and reducing conditions (Table 3B). They were calculated from their mobility relative to standard molecular mass markers and y interpolation from a calibration curve constructed from the relative mobilities of molecular mass markers (Fig.4). The average molecular mass of individual polypeptides was calculated from a total of three determinations. Component polypeptides of the 167kDa and 151kDa oligomeric complexes (Table 3A) identified as described in section 3.1.3, are indicated by the superscripts ¹ and ² in Table 3B.

Table 3A. Molecular mass determinations of TIV polypeptides under non-reducing conditions.

Virion Polypeptide	Molecular mass determination			Standard error of the mean (%)	Average M _m
	(1)	(2)	(3)		
1.	295kDa	310kDa	301kDa	2.6	302kDa
2.	274kDa	290kDa	282kDa	2.8	282kDa
3.	228kDa	236kDa	232kDa	1.7	232kDa
4.	205kDa	209kDa	219kDa	3.7	211kDa
5.	163kDa	168kDa	170kDa	2.3	167kDa ¹
6.	148kDa	156kDa	149kDa	3.6	151kDa ²
7.	122kDa	127kDa	129kDa	3.1	126kDa
8.	110kDa	113kDa	113kDa	1.8	112kDa
9.	98kDa	101kDa	104kDa	3.0	101kDa
10.	90kDa	89kDa	94kDa	3.3	91kDa
11.	83kDa	84kDa	88kDa	3.6	85kDa
12.	81kDa	80kDa	76kDa	3.7	79kDa
13.	61kDa	60kDa	62kDa	1.6	61kDa
14.	51kDa	54kDa	54kDa	3.8	53kDa
15.	50kDa	50kDa	53kDa	4.0	51kDa
16.	48kDa	48kDa	51kDa	4.1	49kDa
17.	47kDa	48kDa	47kDa	2.1	47kDa
18.	46kDa	46kDa	46kDa	-	46kDa
19.	40kDa	41kDa	40kDa	1.2	41kDa
20.	39kDa	40kDa	39kDa	1.3	39kDa
21.	32kDa	31kDa	32kDa	1.6	32kDa
22.	29kDa	31kDa	30kDa	3.4	30kDa
23.	25kDa	25kDa	26kDa	2.0	25kDa
24.	22kDa	23.kDa	23kDa	1.7	23kDa
25.	19kDa	20kDa	20kDa	2.5	20kDa
26.	17kDa	18kDa	18kDa	2.7	18kDa
27.	16kDa	16kDa	15kDa	3.7	16kDa
28.	12kDa	11kDa	10kDa	7.5	11kDa
29.	9kDa	9kDa	10kDa	7.0	10kDa
30.	7kDa	8kDa	8kDa	9.4	8kDa
31.	3kDa	2kDa	2kDa	15.0	2kDa

Table 3B. Molecular mass determinations of TIV polypeptides under reducing conditions.

Virion Polypeptide	Molecular mass determination			Standard error of the mean (%)	Average M _m
	(1)	(2)	(3)		
1.	202kDa	211kDa	220kDa	2.8	211kDa
2.	126kDa	131kDa	133kDa	1.8	130kDa
3.	123kDa	132kDa	123kDa	3.1	126kDa
4.	120kDa	116kDa	120kDa	1.7	119kDa
5.	105kDa	109kDa	110kDa	1.9	108kDa
6.	87kDa	90kDa	90kDa	1.5	89kDa
7.	82kDa	85kDa	85kDa	1.6	84kDa
8.	79kDa	78kDa	83kDa	2.4	80kDa
9.	71kDa	73kDa	78kDa	4.6	73kDa ²
10.	70kDa	72kDa	72kDa	1.6	71kDa ¹
11.	66kDa	70kDa	71kDa	2.9	69kDa
12.	64kDa	64kDa	67kDa	2.1	65kDa
13.	61kDa	63kDa	65kDa	2.1	63kDa
14.	56kDa	59kDa	59kDa	2.3	58kDa
15.	53kDa	56kDa	56kDa	2.4	55kDa
16.	52kDa	55kDa	54kDa	1.9	54kDa
17.	51kDa	54kDa	53kDa	1.9	53kDa
18.	50kDa	53kDa	50kDa	2.6	51kDa
19.	49kDa	52kDa	49kDa	2.3	50kDa
20.	48kDa	50kDa	49kDa	1.4	49kDa ¹
21.	47kDa	49kDa	48kDa	1.4	48kDa
22.	45kDa	48kDa	47kDa	2.1	47kDa
23.	43kDa	46kDa	45kDa	2.2	45kDa
24.	42kDa	45kDa	44kDa	2.3	44kDa
25.	41kDa	44kDa	43kDa	2.3	43kDa
26.	40kDa	43kDa	41kDa	2.4	41kDa
27.	39kDa	42kDa	40kDa	2.5	40kDa ^{1,2}
28.	38kDa	41kDa	39kDa	2.6	39kDa ²
29.	37kDa	40kDa	38kDa	2.6	38kDa
30.	36kDa	38kDa	37kDa	1.8	37kDa
31.	32kDa	32kDa	33kDa	1.0	32kDa
32.	31kDa	31kDa	32kDa	1.1	31kDa
33.	30kDa	30kDa	31kDa	1.1	30kDa
34.	29kDa	29kDa	30kDa	1.1	29kDa
35.	28kDa	28kDa	29kDa	1.2	28kDa

(cont.)

Table 3B. continued.

Virion Polypeptide	Molecular mass determination			Standard error of the mean (%)	Average M _m
	(1)	(2)	(3)		
36.	27kDa	27kDa	28kDa	1.2	27kDa
37.	25.5kDa	26.5kDa	27kDa	2.5	26kDa
38.	24.5kDa	25.5kDa	26kDa	2.6	25.5kDa
39.	23.5kDa	25kDa	25.5kDa	2.7	25kDa
40.	23kDa	24.5kDa	25kDa	3.5	24.5kDa
41.	22kDa	24kDa	24kDa	2.8	24kDa
42.	21.5kDa	23.5kDa	22.5kDa	2.9	23kDa
43.	20.5kDa	23kDa	22kDa	3.8	22kDa
44.	20kDa	22kDa	21kDa	3.2	21kDa
45.	19kDa	20.5kDa	20.5kDa	3.3	20kDa
46.	18kDa	19kDa	19.5kDa	5.4	18.5kDa
47.	17.5kDa	17kDa	18.5kDa	2.0	17kDa
48.	16.5kDa	16kDa	17kDa	3.0	16.5kDa
49.	16kDa	15kDa	16kDa	2.1	16kDa
50.	15.5kDa	14.5kDa	15kDa	2.2	15kDa
51.	13.5kDa	13kDa	13.5kDa	2.6	13kDa
52.	11kDa	10kDa	12kDa	5.5	11kDa
53.	10.5kDa	9.5kDa	10.5kDa	5.0	10kDa
54.	5.5kDa	4.5kDa	5kDa	6.7	5kDa
55.	3.5kDa	3kDa	2.5kDa	11.1	3kDa
56.	2.5kDa	1.5kDa	2kDa	16.7	2kDa

protein than a lower one and under reducing conditions there are fewer high molecular mass proteins to measure, therefore, molecular mass estimation under reducing conditions is likely to be more accurate. The relative molecular masses assigned to individual TIV polypeptides were given from an average derived from three separate determinations. An example of one such separation is shown in Figure 5.

3.1.5 Effect of heat on the dissociation of TIV particles.

An interesting observation was made during the preparation of TIV associated polypeptides for SDS-PAGE with respect to the conditions used for the dissociation of TIV particles. It was consistently noted that TIV particles disrupted by incubation at room temperature in sample lysis buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.0625M Tris-HCL pH 6.8) exhibited different electrophoretic profiles than those particles which were disrupted in the same sample lysis buffer but by heating at 95°C for 3 minutes. The most striking difference was noted with regard to the 49kDa capsid protein whose gene sequence is known from cloning (54). When TIV particles were disrupted in lysis buffer by heating at 95°C for 3 minutes, the capsid protein appeared as a very intense staining band and on this basis the most abundant protein of the virus particle. However, when TIV particles were disrupted in sample lysis buffer at room temperature for 30 minutes (or longer) the majority of the capsid protein did not appear as a 49kDa species but rather as a relatively prominent high molecular mass protein of ~240kDa. This observation suggests that the capsid protein may be a pentameric oligomer in its native state. Also the SDS/2-mercaptoethanol treatment further suggests that the capsid oligomer is stabilized by disulphide bonds and thermolabile non-covalent hydrogen bonds and hydrophobic interactions which together play an integral role in the subunit stabilizing force.

The effect of increasing temperature upon the dissociation of TIV polypeptides is shown in Figure 6A. Oligomerization of polypeptides was also found in other examples such as the complex of viral proteins 37kDa, 38kDa and 39kDa. These polypeptides appeared in the profile following disruption of TIV particles at 80°C and stained more intensely following disruption at 95°C. Similarly, a 73kDa polypeptide appeared in the profile after disruption at 70°C. Two other polypeptides which became more evident with increasing temperature were those with relative molecular masses of 84kDa and 119kDa. Consistent with the appearance of these polypeptides, indicated by arrows in Figure 6A, is the disappearance of certain high molecular mass species of approximately 200kDa and 130kDa. Also when TIV particles were disrupted at progressively higher temperatures there was a reduction in the intensity of complexes with molecular masses in excess of 200kDa. In contrast, certain polypeptides appeared to remain constant irrespective of disruption temperature. Seven of these polypeptides are indicated in Figure 6A with dots and have apparent relative molecular masses of 16.5kDa, 18.5kDa, 22kDa, 27kDa, 30kDa, 32kDa, and 53kDa.

The effect of increasing temperature upon the breakdown of the putative capsid oligomeric complex and formation of the 49kDa monomer is plotted in Figure 6B. The relative staining intensities of the capsid oligomeric complex and the 49kDa monomeric species were determined by laser scanning densitometry of each of the lanes shown in Figure 6A. Using the 32kDa and 16.5kDa polypeptides as internal standards, since they remained constant throughout the temperature range used, the relative percentages of the capsid monomer and oligomer were determined. Using the same technique, the effects of increasing temperature upon the appearance of the 73kDa, 84kDa and 119kDa polypeptides were plotted and are shown in Figure 6C.

Figure 6. Effect of disruption temperature of TIV particles on polypeptide composition. Purified TIV particles were disrupted in SDS buffer (2% SDS, 5% 2-mercaptoethanol, 0.0625M Tris-HCL pH 6.8, 10% glycerol) for 5 minutes at temperatures ranging from 22°C-95°C and an aliquot of each fraction electrophoresed through a 12.5% polyacrylamide gel. Following electrophoresis, the gel was fixed in methanol/acetic acid and the polypeptides visualized by silver staining (Figure 6A). Those polypeptides which remained constant throughout the disruption temperature range are indicated by dots. Polypeptides with molecular masses of 37-39kDa, 73kDa, 84kDa and 119kDa, which became more evident with increasing disruption temperatures, are indicated by arrows. Figure 6B plots (i) the dissociation of the putative ~240kDa pentameric capsid oligomer with increasing temperature and (ii) the appearance of the 49kDa capsid monomer. Figure 6C shows the appearance with increasing temperature of the 73kDa, 84kDa and 119kDa polypeptides. The relative percentages of individual polypeptides were determined by laser scanning densitometry of each of the lanes shown in Figure 6A and calculated using the 16.5kDa and 32kDa polypeptides as internal standards.

Figure 6B

Figure 6C

3.1.6 Abundance of TIV associated polypeptides.

At first sight, the SDS-PAGE profile of polypeptides, revealed by staining with either silver or Coomassie brilliant blue, indicates a wide range in variation with respect to amount or relative abundance of individual polypeptides. Determinations of the relative amounts of individual polypeptides were made following disruption of TIV particles under reducing conditions, subsequent SDS-PAGE, staining of the polypeptides with Coomassie brilliant blue and laser scanning densitometry as described in Materials and Methods, section 2.3.2. In an attempt to reduce the statistical error, three determinations were made, each based on a different SDS-PAGE separation.

Although silver staining of TIV associated polypeptides, shown in Figure 2A, visualizes approximately double the number of polypeptides that are seen following Coomassie blue staining (Figure 2B), the basis of silver staining is not as well-defined and extreme caution needs to be exercised for the quantitation of proteins following silver staining. Nielson and Brown (108) have observed silver staining intensity to be non-linear with different proteins. However, Coomassie blue has been observed to be nearly linear in binding to different proteins and so densitometry of Coomassie blue stained proteins in gels is useful for determining the stoichiometry of multisubunit proteins.

Relative amounts were calculated from the copy number of individual polypeptides, copy number being based upon unit staining per kDa of polypeptide, and their abundance relative to a polypeptide which was used as an internal standard. The choice of the latter was based upon its consistency in terms of both size and quantity following the disruption of TIV particles over an increasing temperature range (as determined in Figure 6A). The abundances of individual polypeptides were therefore calculated relative to both the 16.5kDa and 32kDa polypeptide species and are summarized in Table 4.

Table 4. The relative amounts of individual TIV polypeptides. TIV particles were disrupted at 95°C in 2% SDS/5% 2-mercaptoethanol buffer and electrophoresed through a 12.5% polyacrylamide gel. Following electrophoresis the gel was fixed in methanol/acetic acid and the TIV polypeptides visualized by Coomassie brilliant blue staining. The amounts of individual TIV polypeptides were determined by laser scanning densitometry and expressed as individual copy numbers based upon the unit staining per kDa of each polypeptide. The amount of each polypeptide was calculated relative to the 16.5kDa and 32kDa polypeptides, these being used as internal standards owing to their consistency as determined in Figure 6A. Polypeptides are referred to by relative molecular mass and are expressed as a percentage of the total polypeptide population detected by Coomassie blue staining. The relative amounts of individual polypeptides are based upon the average of results obtained from a total of three separate determinations.

Table 4. The relative amounts of TIV polypeptides.

M _m (kDa)	Copy number (unit staining per kDa)	Amount Relative to		Relative amount (%)	Standard error (%)
		16.5kDa	32kDa		
84	0.0039	0.072	0.19	0.52	2.1
73	0.0036	0.061	0.17	0.46	1.8
65	0.0049	0.090	0.23	0.65	2.4
63	0.0038	0.068	0.18	0.50	3.1
58	0.0020	0.036	0.10	0.27	3.0
55	0.0029	0.052	0.14	0.38	2.7
53	0.0096	0.171	0.46	1.26	1.9
49	0.2400	4.278	11.42	31.39	2.3
48	0.0025	0.045	0.12	0.33	2.9
47	0.0054	0.096	0.26	0.71	2.6
43	0.0130	0.232	0.62	1.70	2.9
39	0.0245	0.437	1.17	3.21	2.4
37	0.0170	0.303	0.81	2.22	2.7
32	0.0210	0.374	1.00	2.74	2.1
31	0.0034	0.061	0.71	0.46	1.8
30	0.0065	0.116	0.31	0.85	2.9
27	0.0039	0.070	0.19	0.52	2.8
26	0.0050	0.089	0.24	0.67	4.0
24	0.1215	2.165	5.79	15.89	2.7
22	0.0610	1.087	2.90	7.97	3.6
18.5	0.0083	0.148	0.40	1.09	2.9
16.5	0.0561	1.00	2.67	7.23	3.4
16	0.0656	1.169	3.12	8.57	3.1
11	0.0228	0.406	1.09	2.99	2.0
10	0.0563	1.004	2.68	7.36	2.5

Total 99.97

The putative 49kDa capsid polypeptide dominates the SDS-PAGE profile and in terms of relative amount represents 31.39% of the TIV associated polypeptide population. Polypeptides with relative molecular masses of 16.5kDa, 10kDa, 22kDa, 16kDa and 24kDa also exhibit relatively high levels of relative amount with values of 7.23%, 7.36%, 7.97%, 8.57% and 15.89% respectively. Levels of relative abundance representing 2-3% were seen in polypeptides with molecular masses of 37kDa, 32kDa, 11kDa and 39kDa and between 1-2% in polypeptides of 18.5kDa, 53kDa and 43kDa. The remaining polypeptide species integrated by densitometry exhibit somewhat less than 1% in content.

3.2.1 2-D PAGE of TIV associated polypeptides.

The high resolution two-dimensional gel electrophoresis procedure of O'Farrell (100), which combines isoelectric focussing in the first dimension with sodium dodecylsulphate gel electrophoresis in the second dimension, was used in an attempt to delineate the features, with respect to component polypeptides, in both 'filled' and 'empty' TIV capsids. To ensure homogeneity in the enriched fractions of 'filled' and 'empty' capsids obtained from the initial bandings during the centrifugal preparation of these virus particle fractions, both fractions were banded a second time on sucrose density gradients prior to use.

In Figures 7A and 7B the silver stained 2-D separations of 'filled' and 'empty' TIV particles respectively are shown. Attempts were also made to visualize polypeptides by staining the gel with Coomassie brilliant blue. However, owing to the decrease in sensitivity only a fraction of the polypeptides were detected. Numerically the 'empty' capsid fraction contains a higher number of silver stained polypeptides, a total of 116, in contrast to 103 stained spots seen in the 'filled' capsid fraction. However, these totals include individual spots which may represent charge variants (see section 3.2.2). Figure 7C is a diagrammatic

Figure 7. The separation of TIV associated polypeptides by isoelectric focussing and SDS (2-D) gel electrophoresis. TIV particles purified by sucrose density gradient centrifugation were disrupted by heating at 95°C for 3 minutes in sample application buffer (2% SDS, 5% 2-mercaptoethanol, 0.0625M Tris-HCL pH 6.8, 10% glycerol), and polypeptides were separated in the first dimension by isoelectric focussing (IEF) as described in section 2.3.3. Separation in the second dimension was performed by SDS-PAGE in a 12.5% acrylamide gel. Molecular mass markers were applied in the standard well and run in tandem in the second dimension. Following electrophoresis, the gel was fixed in methanol/acetic acid and polypeptides subsequently visualized by silver staining. Figure 7A shows the 2-D profile of polypeptides obtained from 'filled' TIV capsids. Figure 7B shows the 2-D separation profile of polypeptides obtained from 'empty' TIV capsids. A composite diagrammatic representation of a comparison of the 2-D polypeptide profiles obtained from 'filled' and 'empty' TIV capsid fractions is shown in Figure 7C. Those polypeptides found only in 'filled' TIV particles are represented as black filled spots and those specific to the 'empty' capsid fraction as open spots. Polypeptides common to both 'filled' and 'empty' capsids are represented as hatched spots.

7A

7B

7C

representation of a composite of the 2-D separations obtained from 'filled' and 'empty' capsid fractions. This allows a correlation to be made between polypeptides which are (i) common to both 'filled' and 'empty' capsids, (ii) found only in 'filled' capsids, and (iii) those which are specific to the 'empty' capsids fraction.

A total of forty-one polypeptide spots are common to both capsid fractions; the majority of these spots were found to range in sizes above 25kDa. Some gel to gel variation was observed in migration profiles of polypeptides in the IEF dimension. However, in any one gel the shifts in migration of the polypeptides do not change their relative mobilities.

3.2.2 Variation in component polypeptides of 'filled' and 'empty' TIV particles.

A highly distinctive feature of the 'empty' capsid profile was apparently extensive charge heterogeneity within certain clusters of proteins. This beaded 'necklace' effect was most pronounced amongst polypeptide species with molecular masses of approximately 46kDa or higher. Distinctive of 'filled' capsids were two polypeptides which migrated with apparent molecular masses of approximately 29kDa and 26kDa and which exhibited negative staining properties in that an unstained area considerably lighter than the background was seen in place of a stained spot. Consequently, as seen in Figure 7A, the polypeptides acquired a 'ghost-like' appearance in the stained gel. These polypeptides also displayed what appeared to be charge heterogeneity since a string of up to six of the 'ghost' spots were observed. Furthermore, the spacing between the consecutive spots was relatively consistent. It was also observed that although the two series of spots differ in molecular mass by somewhere in the order of 3kDa, the individual series were virtually superimposable upon each other.

A further series of spots with a molecular mass of approximately 23kDa was also observed as being restricted to 'filled' capsids and which displayed IEF migration profiles suggestive of charge heterogeneity. These spots were somewhat weaker in silver staining intensity which may have some significance with regard to their stoichiometry.

A polypeptide observed to be notably present in 'filled' capsids and equally notably absent in 'empty' capsids was one with a molecular mass of 32kDa. This appeared in the 'filled' capsid profile as a relatively intensely silver staining spot second only to the putative capsid polypeptide. Also observed to be absent in 'empty' capsids were the weaker staining 23kDa series mentioned above.

A constellation of polypeptide spots which migrated with a slightly higher molecular mass and a lower pI value than the major capsid protein was observed to be present in 'empty' and absent from 'filled' capsids. A further three relatively prominent polypeptides which migrated with approximately the same pI as this constellation but with molecular masses of approximately 37kDa, 39kDa and 41kDa, were observed to be present in 'empty' capsids but absent from the 'filled' variety. Another polypeptide which appeared to be exclusive to 'empty' capsids was one of approximately 42kDa and which migrated in the IEF dimension between the two putative capsid isomers. In this general vicinity of the 2-D map a further collection of exclusively 'empty' capsid polypeptides ranged in molecular masses from ~45kDa-49kDa and which appeared to form an arch over the top of the capsid isomer with the higher pI value. In contrast, several clusters of polypeptides, which migrated with molecular masses ranging from ~25kDa up to 32kDa in 'filled' capsids were noticeably absent or at best present in much reduced quantity in 'empty' capsids.

An artifact consistently observed following silver staining of 2-D gels was a series of spots occurring in the track for the capsid polypeptide. Figure 8 shows

Figure 8. Artifacts associated with putative capsid polypeptide following 2-D PAGE. Artifactual spots occurring in the path of migration of the putative capsid polypeptide after the second dimension SDS gel. Four artifactual spots were consistently observed behind the putative capsid polypeptide and are indicated by arrows. The occurrence of these spots is further discussed in Chapter 4. Relative positions of the molecular mass markers were determined by extrapolation of the standard molecular mass markers shown in Figure 7a.

one example of these artifacts in an enlargement of the area of the gel in which they occur. The artifactual spots are indicated by arrows in figure 8 and exhibit apparent molecular masses of approximately 80kDa, 140kDa, 200kDa and 240kDa. The occurrence and a possible explanation of these spots is further discussed in chapter 4.

3.3 Surface labelling of TIV particles.

Polypeptides exposed on the surface of TIV particles can potentially be tagged or labelled with radioactive probes using at least three different methods which are described below. The mechanisms involved in each of the methods are described in chapter 4. The purpose of the surface labelling experiments was to detect which proteins are located on the surface of the TIV particles, this being potentially useful information for classification purposes.

3.3.1 Enzymatic iodination of TIV particles using lactoperoxidase.

The method, described by Philips and Morrison for the labelling of surface proteins of erythrocytes (109), utilizes lactoperoxidase, hydrogen peroxide and radioactive iodine, to detect putative TIV surface proteins. To carry out labelling by this method the experimental conditions were optimized. Table 5 and Figure 9 present the results of an experiment designed to determine the optimal time required for the iodination reaction with proteins of TIV particles. The reaction conditions were as described in section 2.2.1 and aliquots were removed at 10 minute intervals up to an elapsed reaction time of 120 minutes. The incorporation of ^{125}I proceeded at a relatively steady rate up to approximately 30 minutes after which the rate of incorporation slowly declined. The objective of the radioiodination of TIV surface proteins was to obtain as high a specific activity as possible without causing denaturation of proteins by destruction of

Table 5. Time course of lactoperoxidase catalyzed ^{125}I -labelling of TIV particles. The reaction conditions used for the time course labelling experiment are described in the Methods section 2.2.1. 20 μl aliquots of the radioiodination mixture were removed at time intervals and the reaction stopped by pelleting of the immobilized lactoperoxidase at 1,000 x g for 5 minutes. Virus particles were collected at 15,000 x g for 30 minutes, washed three times in 0.01M Na_2HPO_4 pH 7.5 buffer and resuspended in 0.01M phosphate buffer. The resuspended virus particles were transferred to scintillation vials and counted in 2.5mls of Aquasol liquid scintillation cocktail. Data was collected from three consecutive experiments as indicated by (1), (2) and (3). An average value was taken for each time aliquot for construction of the line graph shown in Figure 9 representing the incorporation of ^{125}I into TIV particles as a function of time.

Table 5. Time course of Lactoperoxidase catalyzed ^{125}I -labelling of TIV particles.

Reaction Time (minutes)	Incorporated ^{125}I cpm $\times 10^{-3}$			Mean incorporated ^{125}I cpm $\times 10^{-3}$	Standard Error of the mean (%)
	(1)	(2)	(3)		
5	5.1	5.6	5.7	5.5	2.4
10	15.4	15.9	15.7	15.7	1.2
20	47.9	43.6	46.8	46.1	3.6
30	74.0	75.3	70.0	73.1	2.9
40	88.4	89.2	80.8	86.1	4.1
50	96.0	97.2	89.1	94.1	3.5
60	103.8	104.2	93.3	100.4	3.5
70	106.1	106.8	101.0	104.6	2.3
80	110.5	110.7	102.8	108.0	3.2
90	114.0	113.1	106.5	111.2	2.8
100	116.7	116.2	111.8	114.9	1.8
110	118.9	118.8	112.6	116.8	2.4
120	121.1	120.9	114.6	118.9	2.6

Figure 9. Incorporation of ^{125}I into TIV particles as a function of time. The rate of incorporation of ^{125}I into purified TIV particles was plotted from measurements as described in the legend for Table 5.

labile amino acids. Hydrogen peroxide is an obligate reactant of the radioiodination procedure and the potential exists for destructive oxidation although it is produced only in small quantities from the immobilized glucose oxidase. If oxidative destruction of amino acids occurs, there will be a subsequent progressive breakdown of the virus particle structure which could potentially give erroneous information about the pattern of radiolabelled protein. Therefore, a delicate balance between maximum incorporation of radioactivity and the maintenance of the integrated structure of the virus particle is required. Subsequent radioiodination experiments of TIV surface structures were therefore performed under the conditions stated in section 2.2.1 with reaction times of 30 minutes.

To test if labelling causes denaturation of TIV particles and if differences in specific activity of various polypeptides is due to denatured versus undenatured TIV, purified virus particles were labelled and then fractionated by sucrose density gradient centrifugation. The fractionation of radioiodinated TIV is shown in Figure 10A. The gradient resolved two distinct peaks with a third banding as a trailing shoulder on the major peak. Radiolabelled material was also found near the meniscus of the gradient. Analysis of the protein components of each of the three major radiolabelled peaks by SDS-PAGE is shown in the autoradiograph in Figure 10B. Fractions 9-14 (lane 1) and 16-24 (lane 2) contained essentially the same radioiodinated proteins while fractions 25-30 (lane 3) contained only traces of label, although according to their position in the gradient, they should also contain 'filled' TIV capsids. Since the material which banded near the meniscus of the gradient (lane 4) contained no detectable radiolabelled proteins it is concluded that the radioactivity in these fractions is due to unreacted residual ^{125}I .

As shown in Figure 10B, eleven major radiolabelled polypeptide species are evident and some are more heavily labelled than others. Since the intensity of

Figure 10. Distribution of ^{125}I -labelled polypeptides in lactoperoxidase-catalyzed radioiodinated TIV particles. (A) ^{125}I labelled virus particles were banded on a 10-45% (w/v) sucrose gradient before electrophoresis to separate possibly disrupted and intact virus particles. A 20 μl aliquot was removed from each fraction and counted in 2.5ml of liquid scintillant. (B) Fractions comprising peaks of radioactivity were pooled and dialysed extensively against 0.01M Na_2HPO_4 buffer. The radiolabelled polypeptides from disrupted TIV particles were subjected to SDS-PAGE and radiolabelled polypeptides detected by autoradiography; Lane 1, Fractions 9-14; Lane 2, Fractions 16-24; Lane 3, Fractions 25-30. Lane 4, Fractions 52-60. (C) Sucrose density gradient centrifugation as in (A) except that TIV particles were disrupted with 2% (w/v) SDS prior to the radioiodination procedure. (D) Fractions containing radioactivity were pooled and subjected to SDS-PAGE as in (B); Lane 1, ^{14}C -labelled molecular mass standards; Lane 2, Fractions 32-45; Lane 3, Fractions 46-60.

labelling was not uniform among these polypeptides, this suggests that those which were more heavily labelled are more abundant than others, more accessible on the surface of the TIV particle or contain a higher proportion of surface exposed tyrosyl residues.

In parallel with the surface labelling of TIV by radioiodination, TIV particles previously disrupted with 2% (w/v) SDS were subjected to an identical procedure. Sucrose density gradient centrifugation of this material resulted in all of the radioactive material banding near the meniscus of the gradient (Figure 10C) and none in the position of intact virus particles. The radioactive material was pooled into two fractions (fractions 32-45 and 46-60), dialyzed in 1,000 molecular weight cut-off membrane and analyzed by SDS-PAGE. The polyacrylamide gel profiles and subsequent autoradiography (Figure 10D, lanes 2 and 3) indicated extensive labelling of a heterogeneous mixture of polypeptides which presumably represent integral polypeptide species of the TIV particle.

3.3.2 Iodination of TIV particles using chloramine-T.

Various applications of the chloramine-T oxidation method of Hunter and Greenwood (110) have enabled highly specific labelling of hormones (110, 111) and immunoglobulins (112, 113) by using carrier free [^{125}I] iodide. However, these methods have drawbacks, because the iodination is carried out in the presence of high concentrations of a powerful oxidizing agent. Protein denaturation is a potential problem when chloramine-T is used and results from the oxidation of labile amino acids such as methionine (114). This can manifest itself in several ways such as an altered electrophoretic mobility and the loss of biological activity.

In an attempt to minimize particle damage, intact TIV particles were iodinated using chloramine-T for a short period of 90 seconds. Following iodination the

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particles were banded to equilibrium on a 10-40% sucrose density gradient to detect virus particles that may have been disrupted during the iodination procedure. The pattern obtained following sucrose density gradient centrifugation (Figure 11A) was virtually identical to that obtained following enzymatic iodination using lactoperoxidase. This indicated that no extensive disruption of virus particles occurred during the iodination procedure. However, one distinguishable feature of the chloramine-T based iodination was the low level of incorporation of ^{125}I in comparison to that obtained using lactoperoxidase. This probably indicates that the chloramine-T iodination procedure is not as efficient as the lactoperoxidase reaction, possibly because of the time required for the incorporation of iodine. It has been suggested that iodide concentration is the critical factor in labelling of the viral polypeptides with time (91). TIV particles were also iodinated following disruption with 2% SDS in a similar manner to the lactoperoxidase labelling procedure. The banding profile obtained following sucrose density gradient centrifugation was essentially the same as that obtained from lactoperoxidase-labelled, disrupted virus particles; all of the radioactive material banded near the meniscus of the gradient (Figure 11C). The SDS-PAGE profile of fractions pooled into two lots, consisting of fractions 30-45 and 46-60 shown in the autoradiograph in Figure 11D, displayed an identical pattern with that of the SDS-disrupted TIV particles labelled by lactoperoxidase (Figure 10D). However, the patterns of polypeptides seen in the SDS-PAGE profile of intact virus particles labelled by chloramine-T and by lactoperoxidase differ to some extent. Additional labelled polypeptides were seen following chloramine-T radioiodination. Also a polypeptide of ~200kDa incorporated a relatively higher level of ^{125}I when TIV particles were iodinated using lactoperoxidase than when labelled using chloramine-T. A plausible explanation for this difference is that a rearrangement or repositioning of certain tyrosyl residues resulted from

Figure 11. Fractionation of TIV particles labelled by radioiodination using chloramine-T. (A) ^{125}I labelled virus particles were banded on a 10-45% (w/v) sucrose gradient prior to electrophoresis to remove any virus particles disrupted during the iodination procedure. A 20 μl aliquot was removed from each fraction and counted in 2.5mls of liquid scintillant. (B) Fractions comprising peaks of radioactivity were pooled and dialysed extensively against 0.01M Na_2HPO_4 buffer. The radiolabelled polypeptides from disrupted TIV particles were subjected to SDS-PAGE and radiolabelled polypeptides detected by autoradiography; Lane 1, ^{14}C -labelled molecular weight standards; Lane 2, Fractions 1-4; Lane 3, Fractions 5-10; Lane 4, Fractions 12-20; Lane 5, Fractions 52-60. (C) Sucrose density gradient centrifugation as in (A) except that TIV particles were disrupted with 2% (w/v) SDS prior to the radioiodination procedure. (D) Fractions containing radioactivity were pooled and subjected to SDS-PAGE as in (B); Lane 1, ^{14}C -labelled molecular mass standards; Lane 2, Fractions 30-45; Lane 3, Fractions 46-60.

a conformational change in the polypeptide following chloramine-T treatment which exposed them on the surface of the molecule prior to iodination.

A further noticeable difference between the two types of labelled products is in the cluster of polypeptides of molecular weight ~46kDa-49kDa. Using the lactoperoxidase procedure three polypeptides with apparent molecular masses of 46kDa-49kDa incorporated ^{125}I . However, in the chloramine-T iodination only two out of three of these polypeptide species incorporated label. Polypeptides of ~22kDa and 26kDa were also observed to incorporate ^{125}I following radioiodination utilizing chloramine-T. However, polypeptides with corresponding molecular masses were not seen to incorporate label following lactoperoxidase-catalyzed radioiodination.

3.3.3 Radiolabelling of TIV particles using ^{35}S .

^{35}S radiolabelling of purified, intact TIV particles was performed using a general purpose ^{35}S labelling reagent t-Butoxycarbonyl-L-(^{35}S)methionine N-hydroxysuccinimidyl ester (^{35}S -LR). This reagent permits the incorporation of ^{35}S , at high specific activity, into proteins and other molecules containing $-\text{NH}_2$ groups such as unblocked N-termini, lysine, etc. This reagent reacts with amino groups via an active ester reaction under very mild conditions. The use of this reagent takes advantage of the attractive features of ^{35}S as an isotope for autoradiography, which generally yields low backgrounds, high sensitivity and good discrimination of bands in protein blots and polyacrylamide gels.

Since labelling with ^{35}S -LR takes place under very mild conditions, it would be expected that very little damage and disruption of virus particles would occur. However, as a precautionary measure, ^{35}S -labelled virus particles were subjected to sucrose density gradient centrifugation following the labelling reaction under conditions identical to those used for radioiodinated TIV particles. The ^{35}S -

Figure 12. Sucrose density gradient centrifugation of TIV particles following putative surface labelling with ^{35}SLR . (A) ^{35}S labelled virus particles were banded on a 10-45% (w/v) sucrose gradient (see Materials and Methods) to remove any virus particles disrupted during the radiolabelling procedure. A 20 μl aliquot was removed from each fraction and counted in 2.5 mls of liquid scintillant. Fractions comprising the major peak (fractions 15-19) were pooled and dialysed extensively against 0.01M Na_2HPO_4 buffer pH 7.5. (B) The radiolabelled polypeptides from disrupted TIV particles were subjected to SDS-PAGE through a 12.5% polyacrylamide gel. The gel was dried and radiolabelled polypeptides detected by autoradiography following an overnight (16 hr) exposure. Lane 1, ^{14}C -labelled molecular mass standards; Lane 2, Fractions 15-19.

MW
(kDa)

97.4-

66.3-

43-

29-

18.4-

14.3-

B

1

labelled profile shown in Figure 12A suggested that no disruption of TIV particles occurred during the labelling procedure since radioactivity was associated only with intact virus particles. The autoradiograph of the SDS-PAGE profile of the major labelled fraction (Figure 12B) indicates that a more extensive level of labelling occurs with the ^{35}S -LR than with either of the two radioiodination reagents. The level of ^{35}S incorporated into virus particles is approximately double that of ^{125}I incorporated using lactoperoxidase and approximately four times that when using chloramine-T. Since more polypeptides became labelled when using ^{35}S -LR than are labelled by either radioiodination method, this suggests that the labelling of proteins other than those which were exposed on the surface of the TIV particles also occurred. This pattern of ^{35}S labelled polypeptides was seen consistently in a total of six labelling experiments performed on the same preparation of purified TIV particles.

3.3.4 Surface labelling of 'empty' TIV capsid shells.

Putative TIV 'surface' polypeptides of empty capsids were detected by labelling with ^{125}I using lactoperoxidase under conditions identical to those used for intact virus particles. ^{125}I labelled 'empty' capsids (Figure 13A) banded at an expected lower density than their 'filled' counterparts (Figure 13B). A comparison of the SDS-PAGE profiles of radioiodinated polypeptide components of 'empty' and 'filled' capsids reveals a high degree of similarity although certain differences are apparent (Figure 13C). The polypeptide of 46kDa incorporated relatively more radioactivity in 'empty' capsids than in 'filled' capsids. Conversely, the polypeptide of 30kDa incorporated relatively more radioactivity in 'filled' capsids than the corresponding polypeptide in 'empty' capsids. The autoradiograph of the SDS-PAGE profile of the 'empty' capsid fraction also revealed the presence of a polypeptide with an approximate molecular mass of

Figure 13. Comparison of Lactoperoxidase/ ^{125}I labelled polypeptides of 'empty' and 'filled' TIV capsids. ^{125}I -labelled 'empty' capsids (A) and 'filled' capsids (B) were each banded on a 10-45% (w/v) sucrose density gradient prior to electrophoresis. The gradients were fractionated as described in Materials and Methods and a 20 μl aliquot was removed from each fraction and counted in 2.5ml of liquid scintillant. Fractions comprising the major peaks of radioactivity (fractions 27-42 in A and Fractions 16-22 in B) were pooled, extensively dialysed against 0.01M Na_2HPO_4 buffer pH 7.5 to remove any free ^{125}I ; the capsids were disrupted and subjected to SDS-PAGE (C). The gel was dried and radiolabelled polypeptides were detected by autoradiography for 16 hours using an intensifying screen. Lane 1, ^{14}C labelled molecular mass standards; Lane 2, 'empty' capsid ^{125}I labelled polypeptides; Lane 3, 'filled' capsid ^{125}I labelled polypeptides.

~70kDa which became radioiodinated following surface labelling. However, no incorporation of radioactivity into this polypeptide was apparent in the 'filled' capsid fraction.

3.3.5 Molecular mass determination of surface labelled polypeptides.

Prior to the molecular mass determination of surface labelled polypeptides, a calibration curve was constructed using the relative mobilities of ^{14}C labelled standard molecular mass markers. The curve was constructed from relative mobilities determined in triplicate. Similarly, the molecular masses of radioiodinated surface polypeptides were determined in triplicate by interpolation from the constructed calibration curve shown in Figure 14. Determinations were made under both non-reducing and reducing conditions. Values from the three determinations are summarized in Table 6.

3.3.6 Comparison of surface labelled polypeptides under non-reducing and reducing conditions.

To determine which of the surface located polypeptide species were covalently attached by intermolecular disulphide bonds, ^{125}I labelled TIV particles were disrupted and electrophoresed under both reducing and non-reducing conditions. The profile of ^{125}I labelled polypeptides obtained following electrophoresis of TIV particles disrupted under non-reducing conditions (lane 2) and reducing conditions (lane 3) are shown in the autoradiograph of the gel in Figure 15A. In the polypeptide profile obtained under reducing conditions (lane 3) a discrete band was seen of approximately 39-40kDa which was absent in the pattern from virus particles disrupted under non-reducing conditions (lane 2). In addition a labelled polypeptide of molecular weight ~19kDa was present only in the reduced sample. This band was almost contiguous with other labelled proteins

Figure 14. Relationship of electrophoretic mobility and molecular mass of known ^{14}C -labelled polypeptides through a 12.5% acrylamide, 0.34% bisacrylamide slab gel at 20V/cm for 45 min at 22°C. The molecular masses of proteins (data provided by the manufacturer, see section 2.5.2 of Materials and Methods) were plotted as $\log_{10}$.

Points: 1, lysozyme (14.3kDa); 2, β -lactoglobulin (18.4kDa); 3, carbonic anhydrase (29kDa); 4, ovalbumin (43kDa); 5, bovine serum albumin (66.3kDa); 6, phosphorylase b (97.4kDa); 7, myosin heavy chain (200kDa).

Table 6. Molecular masses of radioiodinated TIV putative surface polypeptides: Following labelling of TIV particles by radioiodination and their banding to equilibrium by sucrose density gradient centrifugation, the particles were disrupted under both reducing and nonreducing conditions. Polypeptides were separated by SDS-PAGE in a 12.5% acrylamide gel, fixed in methanol/acetic acid, dried and autoradiographed at -70°C with an intensifying screen for 24 hours. The molecular masses of ^{125}I -labelled polypeptides were calculated by interpolation from a calibration curve constructed from the relative mobilities of ^{14}C labelled molecular mass markers (Figure 14). The average molecular mass of individual polypeptides was calculated from a total of three determinations.

Table 6. Molecular masses of radioiodinated TIV putative surface polypeptides.

Non-reducing conditions			Reducing conditions		
(1)	(2)	(3)	(1)	(2)	(3)
209kDa	211kDa	213kDa	209kDa	211kDa	211kDa
124kDa	120kDa	121kDa	-	-	-
76kDa	79kDa	78kDa	77kDa	79kDa	81kDa
61kDa	61kDa	63kDa	-	-	-
49kDa	49kDa	49kDa	49kDa	49kDa	50kDa
44kDa	46kDa	45kDa	45kDa	46kDa	46kDa
-	-	-	38kDa	39kDa	40kDa
30kDa	30kDa	29kDa	30kDa	30kDa	31kDa
25kDa	25kDa	24kDa	24kDa	25kDa	25kDa
-	-	-	18kDa	19kDa	19kDa
16kDa	16kDa	16kDa	16kDa	16kDa	15kDa
11kDa	11kDa	11kDa	11kDa	11kDa	10kDa
-	-	-	4kDa	5kDa	5kDa

of lower molecular mass. Conversely, a polypeptide with an apparent molecular mass of 59kDa was detected only in particles disrupted under non-reducing conditions. The 39kDa and 19kDa polypeptides were also evident in the densitometer scan of reduced polypeptides, Figure 15C, as peaks 7' and 4 respectively. They were absent or significantly reduced in quantity in the scan of unreduced material, (Figure 15B) in which the 59kDa polypeptide was detected as peak 8' and which was observed to be absent in the scan of reduced material.

A diffuse band with an approximate molecular mass of 120kDa, detected as peak 9' in Figure 15B, was observed in the unreduced material (lane 2) and appeared to be absent in the profile of reduced polypeptides (Figure 15C). Since no reduced polypeptides with molecular masses totalling 120kDa can be accounted for, this may possibly represent a trimer of the 39kDa species (peak 7' Figure 15C).

Under reducing conditions a polypeptide with an apparent molecular mass of approximately 5kDa was observed which incorporated ^{125}I at an intensity comparable to that displayed by the 49/46kDa complex and 30kDa species. In the autoradiograph this band was seen as an elongation of the heavily labelled 11kDa band but was discernible in the densitometer scan as a distinct shoulder on the lower molecular mass side of the 11kDa polypeptide. Owing to the high intensity of the neighbouring 11kDa polypeptide, it is difficult to discern whether or not a labelled 5kDa species was present in the unreduced sample. Scanning densitometry indicated the presence of a similar shoulder on the 11kDa band but at a considerably lower intensity.

Accurate quantitation of surface labelled polypeptides was a somewhat difficult parameter to calculate. The level of labelling intensity is not necessarily representative of either the polypeptide abundance in the TIV particle or of its accessibility on the surface of the virus particle. More accurately, labelling

Figure 15. SDS-PAGE of ^{125}I -labelled TIV surface polypeptides under reducing and non-reducing conditions. (A) ^{125}I labelled TIV particles were disrupted in SDS-PAGE application buffer in the absence of reducing agent (lane 2) and in the presence of 2% β -mercaptoethanol (lane 3). Following electrophoresis the gel was fixed in methanol/acetic acid, dried and exposed to X-ray film at -70°C for 20 hours using an intensifying screen. The developed autoradiographs of unreduced TIV polypeptides (lane 2) and reduced polypeptides (lane 3) were scanned using a computing laser densitometer to produce the profiles (B) and (C) respectively. Peaks are numbered from the fastest migrating (i.e. smallest bands) and are indicated by arrows in (A).

intensity should be correlated directly with, and is dependent upon, the number of tyrosyl residues which are exposed on the outer surface of the polypeptide molecule.

3.3.7 2-D PAGE of ^{125}I labelled surface polypeptides of TIV particles.

The separation of surface labelled polypeptides by one-dimensional electrophoresis (Figure 15) using a discontinuous system in the presence of SDS, allowed an accurate estimation to be made of apparent molecular mass. To determine whether each of the radiolabelled bands observed in the one-dimensional SDS-PAGE separation was homogeneous, surface-labelled TIV polypeptides were further characterized by 2-D PAGE.

The 2-D separation shown in Figure 16 indicates that several of the radiolabelled bands contain more than one component. However, careful inspection of the autoradiograph revealed that in spots migrating in the second dimension with identical molecular weights, the spacing between the consecutive spots is relatively uniform. This can probably be attributed to artifactual charge heterogeneity introduced during the radioiodination procedure, the potential sources of which are discussed in chapter 4.

3.4.1 N-terminal sequence analysis of selected TIV polypeptides.

TIV associated polypeptides were initially selected for characterization by NH_2 -terminal amino acid sequence analysis on the basis of their abundance as determined by Coomassie brilliant blue staining following transfer to a sequencer-stable support (PVDF). Previous studies on the characterization of the major 49kDa capsid protein have shown its amino terminus to be blocked (54) and unsuitable for NH_2 -terminal sequence analysis. A total of seventeen polypeptides were selected for initial sequencing trials and are indicated with small arrow heads

Figure 16. 2-D PAGE of ^{125}I /lactoperoxidase-labelled, putative surface polypeptides of TIV particles: Following the labelling of TIV particles by radioiodination and their banding to equilibrium by sucrose density gradient centrifugation, the particles were disrupted and the polypeptides analyzed by 2-D gel electrophoresis (A). Polypeptides were separated in the first dimension by isoelectric focussing (IEF) as described in section 2.3.3, and in the second dimension by SDS-PAGE in a 12.5% acrylamide gel. To correlate the separation profile obtained in the second dimension with that obtained by one dimensional SDS-PAGE, a sample of the radioiodinated TIV particles was electrophoresed separately by SDS-PAGE in parallel with the second dimension separation under identical conditions (B). Following separation in the second dimension, the gels were fixed in methanol/acetic acid, dried and autoradiographed at -70°C with an intensifying screen for 48 hours.

in Figure 17A. Corresponding spots from a total of five consecutive 2-D separations were sequenced as described in section 2.6.1 of Materials and Methods. Of the seventeen polypeptides selected for sequence analysis, sixteen failed to yield a readable sequence. However, the 32kDa polypeptide, indicated by the large arrow head in Figure 17A, sequenced successfully for a total of twenty cycles as shown in Figure 17B.

Since the relative abundance of certain polypeptides, which failed to yield a sequence, was comparable to the one that was sequenced successfully, it would appear that insufficient quantity of material was not the cause for sequence failure. Similarly, suggested precautions were taken as preventive measures against artifactual amino terminal blockage. These included pre-ageing of the SDS-polyacrylamide gel and the inclusion of thioglycolic acid in the gel running buffer to act as an anti-oxidant and scavenger for free radicals, which can potentially produce undesirable effects such as NH₂-terminal blockage. In addition, artifactual blockage of NH₂-termini can be discounted because one polypeptide, subjected to identical preparative conditions to those which failed, was sequenced. Therefore, it is concluded that those polypeptides which failed to yield informative sequence data possibly did so owing to post-translationally blocked amino termini.

A subsequent computerized data bank search of protein sequences for similarities with the sequence obtained, produced several positive alignments particularly involving the two cysteinyl residues. Proteins exhibiting sequence similarity with the 32kDa TIV polypeptide are shown in Appendix A at the end of this thesis. In some cases these were known DNA binding proteins (see Discussion, Chapter 4).

Figure 17. Amino acid sequence analysis of TIV associated polypeptides: NH₂-terminal amino acid sequence analysis was performed on TIV associated polypeptides following their separation by 2-D polyacrylamide gel electrophoresis. Separated polypeptides from 'full' TIV particles were electroblotted onto PVDF sequencer stable membrane and visualized by staining with Coomassie brilliant blue. Seventeen polypeptides displaying medium to relatively high levels of staining intensity and indicated by small arrow-heads in 17A, were excised and individually subjected to automated amino acid sequence analysis. The amino acid sequence shown in 17B, was obtained from the 32kDa polypeptide marked with the large arrow in panel 17A. Of the remaining sixteen polypeptides selected for amino terminal sequence analysis, all failed to yield a discernible sequence. 17A is a silver stained gel representative of the 2-D polypeptide separation obtained; it was run in parallel with those gels from which polypeptides for sequence analysis were taken.

17A

1. 5. 10. 15. 20.
NH₂-Phe-Leu-Pro-Ser-Met-Thr-Tyr-Cys-Val-Asp-Cys-Val-Ala-Ala-Pro-Asn-Glu-His-Asp-Ser-

17B

3.4.2 Amino acid composition analysis of the 32kDa TIV associated polypeptide.

In an effort to gain more insight into the structural properties of this polypeptide its total amino acid composition was determined following acid hydrolysis and automated amino acid analysis. The combined results of analyses performed in triplicate are summarized in Table 7. Calculation of the number of amino acids present in this polypeptide reveals certain striking features, perhaps the most noticable of which is the high proportion of glutamic acid. However, this is to some extent misleading since the conditions used for acid hydrolysis of the protein (6N HCL at 155°C for 75 minutes), a prerequisite for composition analysis, ultimately results in the deamidation of glutamine to glutamic acid as well as asparagine to aspartic acid. Subsequently, the content of glutamic and aspartic acid residues is in all probability somewhat lower than the analysis indicated.

The polypeptide also contained relatively high proportions of proline, valine and glycine, but even more striking was its comparatively high content of cysteine and histidine. The high content of these two amino acids is consistent with its potentially containing a 'zinc finger' motif, such as those found in some DNA binding proteins (see Discussion). The tryptophan content of this TIV polypeptide was not determined owing to tryptophan instability under the hydrolysis conditions used. While hydrolysis procedures are available which retain tryptophan intact they are not particularly suitable for 'on-line' automated analysis. This coupled with the fact that losses of tryptophan, due to oxidation, are known to exceed 30% during electroblotting procedures used for the transfer of proteins from SDS gels to sequencer-stable PVDF membrane (103, 104), renders the quantitation of tryptophan far from accurate under these conditions.

Table 7. Amino acid composition of 32kDa TIV associated polypeptide. The 32kDa polypeptide, which was partially sequenced as described in Figure 16, was purified to homogeneity by 2-D polyacrylamide gel electrophoresis. Samples for automated amino acid composition analysis and amino-terminal sequencing were obtained by electroblotting directly from the gel onto ProBlot™ PVDF membrane. Coomassie blue stained spots of the 32kDa polypeptide were excised from the PVDF membrane and subjected to automated hydrolysis/analysis. The determined values of individual amino acids are expressed as moles % and nearest integer values as determined by a best fit analysis. The molecular mass of 32,055 was calculated from the summation of the monoisotopic molecular mass values of the individual amino acids.

Table 7. Amino acid composition of TIV 32kDa polypeptide.

Amino acid	Residues/100 (moles %)	Integral residues
Aspartic acid	6.78	(19)
Threonine	3.04	(9)
Serine	5.43	(15)
Glutamic acid	14.95	(42)
Proline	13.68	(38)
Glycine	11.98	(34)
Alanine	6.28	(17)
*Cysteine	6.80	(19)
Valine	10.14	(28)
Methionine	N.D ^a	N.D ^a
Isoleucine	6.36	(18)
Leucine	6.07	(17)
Tyrosine	1.43	(4)
Phenylalanine	2.35	(7)
Histidine	4.17	(12)
Lysine	4.72	(13)
Tryptophan	N/D ^b	N/D ^b
Arginine	3.98	(11)

Calculated $M_m = 32,055$ DaN.D ^a Not detectedN/D ^b Not determined

* determined as cysteic acid following performic acid oxidation

3.5 Detection of glycoprotein in TIV particles.

Glycoproteins in PVDF transfers of electrophoretically separated mixtures of cellular and viral proteins can be rapidly and sensitively located by sequential incubation with the lectin concanavalin A (Con A) and the enzymatically active glycoprotein horseradish peroxidase. Subsequently the bound enzyme is located by incubation with a substance which is converted to a highly insoluble coloured product. The method has been demonstrated as being capable of detecting as little as 60ng of purified model glycoprotein after electrophoresis and has been applied to the analysis of the glycoproteins of Lassa virus and Japanese encephalitis virus (115).

Utilizing this technique, the polypeptides associated with purified TIV particles were analyzed for glycoproteins as shown in Figure 18B. A positive reaction was obtained with seventeen polypeptide species under reducing conditions (lane 2) and eleven polypeptides under non-reducing conditions (lane 3). The pattern of staining of TIV polypeptides was found to be reproducible and five separate experiments performed on different purified preparations of TIV particles resulted in identical profiles.

The staining intensities of the glycoproteins varied with the highest intensity being observed in the putative capsid polypeptide and a low molecular weight polypeptide, which under reducing conditions had a molecular weight of approximately 5kDa. This low molecular weight polypeptide was absent under non-reducing conditions, implying that it was intermolecularly disulphide bonded to another polypeptide species as a higher molecular weight dimer or complex. Under reducing conditions a further five polypeptides exhibited a slightly less intense staining response and corresponded to polypeptides with molecular masses of 79kDa, 39kDa, 25kDa, 22kDa and 16.5kDa. Polypeptides with molecular masses of 84kDa, 65kDa, 53kDa, 18.8kDa and 11kDa were also

Figure 18. Lectin staining of glycoproteins associated with TIV particles: Sucrose density gradient purified TIV particles were disrupted with 2% SDS both with and without 2-mercaptoethanol and the viral proteins were separated by electrophoresis on a 12.5% polyacrylamide gel. For direct comparison, one gel was fixed in methanol/acetic acid and stained with Coomassie brilliant blue (A) and TIV polypeptides from a second gel (B) were transferred to ProBlot™ PVDF membrane and stained with the concanavalin-A/peroxidase/3,3' diaminobenzidine-H₂O₂ procedure for glycoprotein detection. (see Materials and Methods section 2.8). (A) Lane 1, standard molecular mass markers; Lane 2, TIV particles disrupted under non-reducing conditions; Lane 3, TIV particles disrupted under reducing conditions. (B) Lane 1, Mouse IgG Fab fragment plus β -Lactoglobulin; Lane 2, TIV particles disrupted under reducing conditions; Lane 3, TIV particles disrupted under non-reducing conditions. Arrows directed at lane 1 of (B) indicate the slight non-specific staining observed in mouse IgG Light chain and β -lactoglobulin neither of which contain carbohydrate.

discernible as less intensely stained bands in addition to species with molecular masses corresponding to 211kDa, 119kDa, 108kDa, 46kDa, 44kDa, 42kDa, 36kDa and 23kDa, which stained only very faintly. Under non-reducing conditions the eleven polypeptides which exhibited a positive staining response did so with various levels of intensity.

A sample containing a mixture of 2 μ g each of mouse IgG Fab fragment and β -lactoglobulin was used as a negative control in parallel with the TIV polypeptides. A very slight non-specific staining of the Fab fragment light chain and Fd fragment (heavy chain) and also of the β -lactoglobulin was noticeable and is indicated by arrow-heads in Figure 18. This demonstrates that to a limited degree non-specific staining of non-glycosylated proteins can and does in fact occur. However, in consideration of the exceptionally low levels of non-specific staining occurring with 2 μ g of each of the control polypeptides, this strongly suggests that TIV polypeptides, which exhibited a positive staining reaction, do contain carbohydrate.

Discussion.

Previous studies of TIV can generally be divided into two categories. Early studies were primarily centered around the gross structural properties of the virus (1, 9, 15-18, 20-23, 32), while research within the last few years has largely been concerned with the molecular biology of this type 1 iridovirus (51-54).

This thesis describes a reappraisal of polypeptides associated with TIV particles. Initial experiments were performed with the objective of re-assessing the number and class or type of component polypeptides. The second objective was to characterize individual polypeptides. This is an essential prerequisite before any biological insight can be gained into the possible function(s) of individual polypeptides in the process of replication and virion assembly. Both of these processes depend upon the *de novo* synthesis of viral polypeptides and it is therefore important to establish the identity and characteristic properties of the polypeptides which are associated with the virus particle.

4.1 Qualitative and quantitative analysis of TIV associated polypeptides.

Prior to characterizing any of the individual polypeptides, the total number associated with TIV particles was re-examined. This was previously estimated to be between 25-30 as judged by SDS-PAGE (34, 52). The 1-D SDS-PAGE results reported in this thesis using Coomassie brilliant blue staining shown in Figure 2B, were in good agreement with previously published results (34, 52). A total of 28 polypeptides were discerned following Coomassie blue staining. However, increasing the detection sensitivity by utilizing the silver staining technique, a total of 56 polypeptides were visualized following 1-D SDS-PAGE (Figures 2A

and 5). It is apparent therefore that silver staining is preferable for qualitative analysis of complex polypeptide mixtures owing to its inherent sensitivity.

A strong correlation between the intensity of response to Coomassie stain and the basicity of a protein has been reported (116). Hydrophobic interactions of the dye molecule with the polypeptide backbone adjacent to positively charged amino acids have been implicated, since this enhances the binding of the dye (116). The Coomassie R molecule contains two sulphonic groups and three nitrogen, two of which are likely to be positively charged under the acidic staining conditions. Accordingly, the binding of Coomassie R to proteins is a result of electrostatic forces between the sulphonic groups and the basic positively charged amino groups in the protein.

Silver staining of proteins in polyacrylamide gels is reported as being 100-200 times more sensitive than Coomassie blue staining (83). The basis for silver staining is not fully understood; it has been reported that protein-aldehyde adducts, which result from glutaraldehyde pretreatment, are potentially responsible for metallic silver deposition in a fashion resembling latent image formation in the photographic process (117). Nielson and Brown (108) have suggested that silver ions bind only with the side chains of charged amino acids and that this is not only responsible for the sensitivity of silver stain, but also for color formation. In contrast, Chuba and Palchaudhuri (118) have demonstrated that a strong correlation exists between an increased staining with silver and the cysteine content of the protein and that proteins without cysteine exhibit negative staining. On the basis of this information it is evident that while the higher sensitivity of silver staining is advantageous in the qualitative analysis of proteins, the quantitation of proteins using Coomassie blue staining results in a more reliable indication of the relative amounts. For these reasons both techniques were used for the work presented in this thesis.

4.2 Protein heterogeneity.

In order to resolve the maximum number of TIV polypeptides, high resolution separation methods employing the two-dimensional (2-D) system of O'Farrell (100) were used. This technique consists of separations involving both isoelectric focussing and SDS-PAGE. This technique resolved a total of more than 100 putative polypeptide species. However, this number includes those which potentially represent charged variants of identical species which separated by IEF. When these multiple charged species were evaluated as single species, the total number of individual polypeptides numbered 67-70. The 2-D analysis, therefore, reveals that the component polypeptide profile of TIV particles is more complex than had previously been anticipated.

Polypeptides associated with intact TIV particles can be either structural proteins or enzymes. Therefore a proportion of the population of polypeptides resolved by 2-D may have been cytoplasmic in origin and accidentally packaged during the assembly process of the viral capsid. A precedent exists for this suggestion since it has been shown that vaccinia virus will fortuitously package specific polypeptides into virions (77). Covalently linked oligomers are another factor which potentially contributes to the large number of polypeptides seen under reducing and denaturing conditions. Separations of native oligomers under denaturing and reducing conditions give rise to two or more monomeric subunits and it is apparent from the separations performed under comparative non-reducing and reducing conditions shown in Figures 2A and 5, that such oligomeric complexes exist in TIV particles.

Examination of the polypeptide profiles following disruption of particles over a wide range of temperature (Figure 6) indicated that dissociation of oligomeric complexes occurred with increasing temperature. This was particularly evident with the appearance of the putative 49kDa capsid polypeptide and the

concomitant disappearance of a ~240kDa polypeptide species. Similar observations were made with polypeptides of molecular masses of 37kDa, 39kDa, 73kDa, 84kDa and 119kDa which appeared concomitantly with the disappearance of higher molecular weight species above 70°C.

Also contributing to the number of polypeptides found associated with TIV particles is the presumed multitude of enzymes needed to support the transcriptional system. Owing to TIV's property of cytoplasmic replication, and questionable interaction with the nucleus, the intact TIV particle may contain its own DNA-dependent RNA polymerase as well as other gene processing functions e.g. transcription factors. The poxvirus and African swine fever virus, which also replicate cytoplasmically, are the only eukaryotic DNA viruses known to contain their own DNA-dependent RNA polymerase. The vaccinia virus RNA polymerase has a molecular mass of approximately 500kDa and contains subunits of 147kDa, 132kDa, 34kDa, 22kDa, 21kDa, 20kDa and 17kDa (119). If a similar situation exists in TIV, subunits such as these would help contribute to the complexity of polypeptides observed in the 2-D profiles. In addition, TIV particles may contain other transcriptional enzymes to perform complex processes such as mRNA capping and polyadenylation. Many of these enzymes, both viral and cellular, possess subunit compositions and therefore could potentially contribute to the complexity of the TIV profile.

The protein heterogeneity found in TIV particles may be further complicated by polypeptide processing. The formation of viral polypeptides from higher molecular mass precursors has been found to occur during the replication of picornaviruses, notably polio virus, and other related small RNA viruses (120-123). All of these viral polypeptides may arise by multistage cleavage from a few or perhaps a single very large polypeptide. Processing such as this is a common mechanism occurring during the formation of hydrolytic or proteolytic enzymes,

clotting factors, the hormone insulin, small internal T4 phage peptides and viral "gag" proteins. Homes et al. (124) have identified a TIV "gag-like" gene product containing a putative proteolytic cleavage site and it is not difficult to postulate a role for polypeptide cleavage in TIV replication and assembly. The formation of several structural polypeptides from a single precursor would ensure the synthesis and incorporation of equimolar amounts of several polypeptides into virus particles.

Indirect evidence suggesting that polypeptide processing occurred in TIV particles was obtained following examination of comparative 2-D polypeptide profiles of 'empty' and 'filled' capsids (Figures 7A and 7B). In 'empty' capsids, polypeptides with relative molecular masses of 76kDa, 112kDa and 160kDa stained with a relatively high intensity. In contrast the corresponding polypeptides in 'filled' capsids stained a good deal less intensely. Conversely, 'filled' capsids contained additional polypeptide species which were not apparent in the 'empty' capsid profile and which migrated in the second dimension of the 2-D separations with relative molecular masses ranging from 23kDa to 32kDa. The additional lower molecular mass polypeptides observed in 'filled' capsids may have arisen from processing of the higher molecular mass polypeptides observed in the 'empty' capsid fraction. Furthermore, this could have occurred during the DNA packaging with the procapsid. This phenomenon has been observed with a number of viruses such as bacteriophage λ , picornaviruses and adenoviruses. The final step during polio virus maturation is the cleavage of the precursor polypeptide VP₀ into VP₂ and VP₄ (123). It is assumed that the viral RNA penetrates between the capsomeres from the outside triggering the cleavage reaction. The cleavage product VP₄ may be responsible for locking the RNA into the capsid since its loss at uncoating or during heat inactivation is accompanied by separation of RNA from the capsid (120). In adenovirus morphogenesis, the viral

DNA, linked to the 55kDa terminal protein, enters the procapsid together with the core proteins; subsequently proteins of both the procapsid and core undergo cleavage.

It appears, therefore, that the complex mixture of polypeptides observed following 2-D PAGE profiles of TIV could have originated from one or more of three sources; firstly, polypeptides are viral in origin and interact to form oligomers, secondly that some of the polypeptides resolved are representative of host polypeptides that are accidentally or fortuitously packaged in the virus particles, and thirdly that a certain number of the polypeptides arise as a result of proteolytic cleavage of higher molecular mass precursors.

4.3.1 Polypeptides located on the surface of TIV particles.

The topographical location of individual iridovirus proteins is not known although it is presumed that the major or capsid polypeptide makes up most of the surface of the virus particle. To ascertain which polypeptides are located or exposed on the outer surface of TIV particles, intact virions were labeled enzymatically using lactoperoxidase (LPO) and [^{125}I] sodium iodide.

Radioiodination of intact TIV particles resulted in eleven polypeptides incorporating label (Figure 15 and Table 6). From the comparison of radiolabelled TIV particles disrupted under reducing and non-reducing conditions, it was evident that two of the polypeptides with molecular masses of 39kDa and 19kDa are possibly present as a covalently linked dimer through an intermolecular disulfide bond(s). This suggests that among the proteins situated on the surface of TIV particles, the primary stabilizing force is provided by non-covalent interactions between the polypeptide chains. Since the exterior surface of the virus particle must be hydrophilic in nature, hydrogen bonding may occur between individual polypeptides. Ten of these polypeptides are known to be

glycosylated (see section 4.6). This glycosylation may contribute towards polypeptide orientation, stability and surface exposure since the sugar residues would face the hydrophilic exterior. It is of interest to note that the presence of an amorphous 'fuzz' around the periphery of TIV particles has been observed in sectional profiles obtained from electron micrographs (16). This 'fuzz' may be representative of glycan polymers attached to the surface polypeptides. Alternatively, the surface labelled polypeptides and the 'fuzz' may constitute an outer, highly hydrated protein shell similar to that which has been reported as being present in the FV3 virion (17, 23-26).

A striking feature of TIV particle radioiodination is that the putative 49kDa capsid polypeptide was not radioiodinated. This suggests that either it does not contain any surface exposed tyrosyl residues or that it is protected from the exterior environment. Structurally, the amino acid tyrosine lends itself more to being positioned in the interior of a protein's three dimensional structure whereby the highly hydrophobic aromatic ring can provide stabilizing hydrophobic interactions and the hydrophilic hydroxyl groups participate in hydrogen bonding. By doing so, both of these interactions contribute to providing stabilizing forces within the structure of the molecule.

Host cell recognition is a factor which could explain the presence of additional polypeptides being associated with the capsid polypeptide. To gain entry into a cell a virus must be able to recognize and interact with a specific receptor on the surface of a host cell. Having accomplished this the virion must then penetrate into the cell. The function of receptor recognition is performed by proteins in most viruses and more specifically in most cases by glycoproteins. In some viruses, such as influenza virus, a single glycoprotein, hemagglutinin, acts as a mediator in both receptor binding and membrane fusion. It is difficult to imagine

how TIV infection could be initiated unless it possesses specific surface proteins to perform such tasks as receptor recognition.

Evidence suggestive of adverse oxidative effects occurring during the chloramine-T radioiodination of TIV particles came from the observation of additional labeled polypeptides compared to those labelled by the LPO procedure. This observation may be explained as a result of the outer surface of the virus particles having been disrupted and normally inaccessible polypeptides exposed to the ^{125}I .

The extensive labeling of TIV polypeptides observed using ^{35}S -LR suggests that this reagent may have diffused to the interior of the virus particles through surface 'pores' or 'holes'. ^{35}S -LR is a low molecular mass compound (Mol.mass 349Da) and on this account is probably capable of gaining access to the virus interior. ^{35}S -LR binds directly to proteins via an active ester reaction while both chloramine-T and lactoperoxidase act only as indirect participants in the labelling reaction by catalyzing either directly or indirectly the oxidation of iodide ions to free iodine. Additional labelled polypeptides observed following chloramine-T based radioiodination of TIV particles could have arisen from chloramine-T (Mol.mass 227Da) entry into the interior of virus particles. However, lactoperoxidase is physically too large to penetrate TIV due to its attachment, along with glucose oxidase, to spherical beads several microns in diameter.

It has been shown that peroxidases can catalyze the iodination of free tyrosine (125, 126) and that incorporated ^{125}I is bound to tyrosyl residues when utilizing lactoperoxidase (87). A well established fact is that proteins vary in their susceptibility to iodination (87) and that this property probably reflects the accessibility of tyrosyl groups in the molecule. In some highly polymerized molecules, reactive tyrosines may be obstructed in the polymer, a situation which has been found to exist in polymerized bacterial flagellin, but not in its

monomeric form (87). This type of situation may explain why the capsid polypeptide failed to incorporate ^{125}I .

Krohn et. al. (127) reported that the LPO iodination method does not yield as many iodinated by-products, i.e. oxidized iodotyrosine, di-iodotyrosine, and iodophenylalanine, as does the chloramine-T method. It is also reported to produce the highest proportion of monoiodotyrosine which of all the iodinated products contains the most stable covalently bound iodine. LPO catalyzes the substitution of iodine for hydrogen on the phenolic side chains of tyrosine residues in proteins but the mechanism of the reaction, the intermediates and its order are not completely understood. Different methods of iodination appear to depend on different mechanisms (127).

Thomas and Hager (128) and Hager et al. (129) have shown that both molecular iodine and H_2O_2 are obligate reactants of the iodination reaction and spectrophotometric measurements indicate that no labelling of tyrosine occurs until all of the iodide ions are converted to iodine. The reaction is normally conducted by the addition of aliquots of H_2O_2 but this creates the possibility of alteration of membrane structures and their properties by direct oxidation of its components. The use of glucose oxidase, glucose and O_2 leads to the generation of H_2O_2 at a controllable rate and therefore obviates the possibility of adverse effects due to direct addition of peroxide. The coupled iodination reaction is the sum of the following two reactions;

(i) $\text{Glucose} + \text{O}_2 = \text{H}_2\text{O}_2 + \text{gluconolactone}$ (glucose oxidase catalyzed)

(ii) $\text{H}_2\text{O}_2 + \text{I}^- + \text{tyrosine} = \text{iodinated protein}$ (lactoperoxidase catalyzed)

The glucose oxidase method has been used to iodinate red cell membranes (92), murine myeloma cells (97) and erythropoietin (95). Furthermore, iodinated preparations obtained with the glucose oxidase method were reported to not only contain greater biological activity but also to incorporate a higher proportion of

^{125}I than did material labelled by the direct addition of dilute H_2O_2 (93-95, 97). Given these criteria, Enzymobead reagent, which utilizes solid phase technology, was employed for the enzymatic radioiodination of TIV particles. The Enzymobead reagent consists of immobilized preparations of lactoperoxidase and glucose oxidase which are carefully blended to provide optimal enzymatic activity.

4.3.2 Artifacts resulting from lactoperoxidase catalyzed radioiodination of TIV particles.

Artifactual charge heterogeneity introduced during the radioiodination procedure can potentially arise from several sources. The iodination of tyrosyl residues can give rise to two derivatives namely 3-iodotyrosine and 3,5, di-iodotyrosine. In the presence of excess iodine, formation of the 3,5, di-iodo derivative is favored while the formation of 3-iodotyrosine primarily occurs in the presence of excess substrate with limited formation of the di-iodo derivative.

The production of free iodine is dependent upon the peroxide oxidation of [^{125}I]-iodide to iodine. Subsequently, the quantity of free iodine available is dictated by the efficiency of not only the peroxide oxidation of iodide, but also upon the lactoperoxidase which catalyzes the peroxide oxidation. Given these limiting factors as well as the small amount of $\text{Na}[^{125}\text{I}]$ -iodide used in the reaction ($\sim 6\text{ng}/0.1\text{mCi}$), it is highly unlikely that iodine was ever present in an excess. Hence the monoiodo derivative is likely the major product of the reaction along with limited formation of the di-iodo derivative. Dual derivatization results in charge heterogeneity with the result that the differently charged species separate during the IEF dimension of 2-D PAGE. This was observed to occur in certain polypeptides during TIV radioiodination. Furthermore, for the reasons already mentioned, the di-iodo derivatives of the TIV polypeptides, identified by their

being the more negatively charged of the species, were observed to have incorporated less radiolabel than the monoiodo derivative (Figure 16).

Charge heterogeneity may also arise following the iodination of secondary tyrosyl residues which are not exposed on the surface of the polypeptide in its native state. These may become accessible for modification following destabilization of the tertiary structure caused by the iodination of surface exposed residues.

A further cause of heterogeneity may reside in the partial iodination of histidine residues. It has been shown that histidine, in addition to tyrosine, was labeled when an encephalitogenic peptide from ox spinal cord was iodinated by the chloramine-T oxidation method (130). However, under conditions utilizing lactoperoxidase it has been demonstrated that no iodination of histidyl residues is apparent but rather that the incorporation of iodine is restricted to tyrosyl residues (87).

4.4 Sequence analysis of selected TIV polypeptides.

N-terminal sequence analysis was attempted on a total of seventeen TIV polypeptides of which only one, the 32kDa species, gave results. Failure of polypeptides to produce an amino terminal sequence can be attributed to several reasons. Successful sequencing by Edman degradation primarily depends upon the existence of a free α -amino group to which phenylisothiocyanate can bind in the derivatization step of the reaction. Post-translational amino terminal modification such as acetylation results in a blockage of this step. Also, failure to produce a readable amino acid sequence may simply reflect the fact that polypeptide quantities are too low for the sequencing detection system.

Considering that the relative amounts of some of the polypeptides which failed to yield a sequence were comparable to the 32kDa one which was

sequenced, it would appear that insufficient quantity of material was not the cause for sequence failure. To prevent *in situ* amino terminal blockage all recommended preventative measures were taken. These included pre-aging of the SDS-polyacrylamide gels and inclusion of thioglycolic acid in the gel system running buffer to act as an anti-oxidant and scavenger. *In situ* blockage of amino termini can be discounted also on the premise that one polypeptide was sequenced successfully and that this had been subjected to identical preparative conditions as were those which failed. Therefore, the cause of sequence failure was possibly owing to the polypeptides having post-translationally blocked N-termini.

A computer search comparing the N-terminal amino acid sequence of the TIV 32kDa polypeptide to sequences available in Swiss-Prot, Genbank and modified EMBL data bases were analyzed by FASTA search. Varying degrees of identity were found with forty known protein sequences of which fourteen were DNA-binding proteins. Ten of the fourteen proteins contained a Cys-(Xaa)₂-Cys sequence and are shown in Appendix A. The highest level of sequence identity was with the *E.coli* transposition protein, transposon TN7. In a seven amino acid overlap involving the Cys-(Xaa)₂-Cys sequence, an identity of 85.7% was found between the two polypeptides. Sequence identities involving the two Cys residues were also found with polyomavirus T antigens and the E1 protein of papillomavirus. Interestingly, sequence identity was also found with a 5' exodeoxyribonuclease which only involved one of the two Cys residues. However, an identity of 77.8% in a nine amino acid overlap was found which could be further extended to an 84.6% similarity if single base changes in codons were taken into consideration.

4.5 Polypeptides tentatively associated with the nucleoprotein core of TIV particles.

Viruses are made up of two essential components; a genome, which can be either DNA or RNA associated with proteins or polyamines, and a proteinaceous capsid shell. In addition, some virions have enzymes that are needed in the initial steps of genome expression and replication. A proportion of those polypeptides observed in the 2-D profile of 'filled' capsids (Figure 7A), but which were absent in the 'empty' capsid profile (Figure 7B), may be associated with viral DNA. However, their mere presence in 'filled' virus particles does not constitute irrefutable evidence for their being DNA associated proteins.

DNA binding proteins can basically be divided into two categories; those that function in assisting to package the DNA and secondly those which regulate transcription. In order for the large TIV DNA molecule to be packaged within the capsid shell, the DNA has to be folded in an orderly fashion. In this regard, Home et al. (124) have proposed that the L96 gene product may be involved in TIV genomic DNA packaging in a manner related to the "gag" polyproteins of mammalian viruses. In eukaryotes, DNA folding is the task of specific nuclear proteins and the tight complexes formed between DNA and these nuclear proteins are referred to as chromatin. The key proteins in chromatin that serve to fold eukaryotic DNA are the histones. These are relatively small proteins that contain high proportions of positively charged amino acids which help them to bind tightly to the negatively charged DNA double helix.

The 29kDa and 26kDa TIV polypeptides which exhibited 'ghost-like' qualities upon silver staining are worthy of mention as potential DNA associated polypeptides. Initially it was suspected that these proteins possibly represented host *Galleria mellonella* histones which the TIV had fortuitously used to compact the viral DNA into a packageable size. This suspicion was based upon several factors observed following the 2-D PAGE of TIV polypeptides shown in Figure 7A. Firstly, both polypeptides migrated in the first dimension as basic species

with approximate pI's of 9-9.5. The five histone classes are all basic in nature with about one in four residues being either lysine or arginine. Secondly, both polypeptides exhibited extensive charge heterogeneity. Each of the types of histones can exist in a variety of forms owing to post-translational modifications of certain side chains with varying types and extent of modifications involving phosphorylation, acetylation, methylation and ADP-ribosylation. Thirdly, the spots representative of the 29kDa and 26kDa polypeptides exhibited so-called negative staining, a property, according to Chuba and Palchaudhuri (118), which is associated with proteins devoid of any cysteine. With the exception of histone H3, which contains a single residue of cysteine, the remaining four types of histones, namely H1, H2A, H2B and H4, rarely contain any cysteine in their amino acid sequences. Finally, as observed for the 32kDa polypeptide, both the 29kDa and 26kDa polypeptides were only observed to be present in 'filled' capsids.

The evidence suggestive of these polypeptides being *G.mellonella* histones is all circumstantial and is not supported when their relative molecular mass values are taken into consideration. The histones are highly conserved proteins and range in molecular mass from approximately 21kDa to 11kDa. Even taking into consideration the fact that owing to their high content of basic amino acids they tend to migrate anomalously in SDS-polyacrylamide gels, the observed molecular masses of 29kDa and 26kDa are well outside of the range observed for histones. However, this does not exclude the possibility of these polypeptides being histone-like and performing a similar role to histones in the condensation of viral DNA.

Proteins which regulate transcription of DNA recognize specific DNA sequences through discrete DNA-binding domains within their polypeptide chains. Many prokaryotic DNA-binding domains contain a helix-turn-helix motif

that recognizes and binds specific regulatory regions of DNA. The regulation of transcription in eukaryotes is much more complex than the mechanisms for the regulation of prokaryotic genes and often complex sets of regulatory elements control the initiation of transcription. The regulatory elements consist of combinations of specific DNA sequences each of which is recognized by a corresponding site-specific DNA binding protein. These proteins are called transcription factors and each combination of DNA sequence and transcription factor constitutes a control module.

A novel protein motif used for binding to DNA and RNA is the 'Zn finger'. This was originally discovered repeated in tandem in the *Xenopus* transcription factor IIIA (131), but it is now clear that it is ubiquitous. The Zn finger is a motif that is repeated in tandem to recognize DNA sequences of different lengths. Each finger is based on a similar framework, and each interacts with a small number of base pairs. The strength of the interaction can be varied by changes in the sequence of both the protein and the DNA and by varying the length of the spacing between the fingers. These changes allow a high level of specificity in recognition and offer a large number of combination possibilities for specific recognition of DNA. Putative Zn fingers are present in different numbers within the structure of many regulatory proteins and serve as modules for the building up of a specific nucleic acid binding domain. The consensus amino acid sequence of the Zn finger motif is (Phe, Tyr) - Xaa - Cys- (Xaa)₂ or 4 - Cys - (Xaa)₃ - Phe - (Xaa)₅ - Leu - (Xaa)₂ - His - (Xaa)₃ - His - (Xaa)₅, where Xaa is any amino acid.

Similarities exist in amino acid composition and N-terminal sequence between the TIV 32kDa polypeptide and certain characteristics of known DNA-binding proteins. The composition analysis of the 32kDa polypeptide (Table 7) confirms the presence of 19 residues of cysteine and 12 residues of histidine. Therefore, this potentially allows for up to six of the Zn finger motif repeats, given that each

repeat requires 2 cysteines and 2 histidines. Based upon amino acid sequence, a possible Zn finger motif was identified in the N-terminal sequence of the TIV 32kDa polypeptide. Although two cysteine residues conform to the consensus sequence, separated by two amino acids (valine and aspartic acid), the 32kDa TIV polypeptide sequence deviates from the consensus amino acid sequence of the Zn finger motif.

Based upon calculations of data derived from the amino acid composition analysis, the molecular mass value of the protein is 32,055. This is in exceptionally good agreement with the apparent molecular mass value of 32kDa determined by SDS-PAGE. The calculation based upon the composition analysis does not include either methionine or tryptophan even though the partial amino acid sequence indicates at least one methionine. Therefore, depending upon the methionine and tryptophan contents, the calculated molecular mass of the protein will be in excess of 32kDa. However, since these are among the less commonly occurring amino acids in proteins and they would only slightly increase the calculated M_m value of the polypeptide.

A striking feature of the 32kDa TIV polypeptide, illuminated by amino acid composition analysis, was the exceptionally high content of glutamic acid and proline. As explained in section 3.4.2, the calculated 42 residues of glutamic acid represents the total content of both glutamic acid and glutamine. The polypeptide chains of activating transcription factors are usually divided into two functionally different regions, one that binds to specific DNA sequences and another that activates transcription. Three different types of activating regions have been found; those that have a net negative charge, those that contain a large number of proline residues and those that contain a preponderance of glutamine residues (132). From composition analysis, the 32kDa polypeptide possesses at least two of these characteristics, i.e. a high proline content and either a preponderance of

glutamine residues or a high overall net negative charge, if glutamic acid residues predominate.

Strong similarities exist between the observed characteristics of the 32kDa TIV polypeptide and those of known transcription factors. This evidence coupled with its presence in 'filled' capsids and absence in 'empty' capsids make it a strong candidate as a TIV DNA associated protein. More conclusive evidence would be the identification of the histidine residues characteristic of the Zn finger motif consensus sequence, which would confirm the presence of at least one DNA-binding motif within the polypeptide structure.

4.6 TIV associated glycoproteins.

Glycoproteins are a most diverse group of biological polymers that are ubiquitous constituents of all living organisms, with the possible exception of bacteria. The covalent bonds which link certain sugars to amino acids are of two types: N-glycosidic linkages are formed between the anomeric carbon of N-acetylglucosamine and the amide nitrogen of asparagine. Glycosylated asparagine residues are always part of the sequence or "sequon" Asn-X-Ser/Thr, where X can be any of the commonly occurring amino acids with the possible exception of aspartic acid and proline. O-glycosidic bonds occur between N-acetylgalactosamine, galactose, xylose, mannose or arabinose, on the one hand, and the hydroxyl groups of serine, threonine, hydroxylysine or hydroxyproline on the other.

To date, the presence of glycoproteins has not been reported in any members of the iridoviridae family. However, with respect to TIV, it has been demonstrated that lepidoptera insect cells, e.g. *G. mellonella*, contain the necessary glycosyltransferase genes required for the assembly of N-linked oligosaccharides (133).

Glycoproteins were detected in TIV particles following their separation by SDS-PAGE, transfer to PVDF membrane and incubation with concanavalin A (Con A). The plant lectin Con A possesses a remarkably specific capacity to bind primarily to the commonly occurring α -linked mannose and α -linked glucose, and also to their derivatives. The multivalent Con A will bind to carbohydrates, and free binding sites on the attached Con A can bind to horseradish peroxidase, which is a glycoprotein. Since peroxidases may be visualized by reaction with diaminobenzidine, it has been possible using this method to specifically stain carbohydrate residues in TIV polypeptides.

A very prominent feature of the glycan-specific, lectin-stained polypeptide profile is the relatively high staining intensity produced by the putative capsid polypeptide. This signifies one or both of two possibilities: firstly, that the extent of glycosylation is considerably higher in the capsid polypeptide; secondly that this reflects the relatively higher content of α -D-mannose, α -D-glucose sugar residues or their derivatives within the glycan moiety. The deduced amino acid sequence of the TIV capsid polypeptide (54) indicates the potential for up to six N-linked glycosylation sites within the consensus Asn-X-Ser/Thr sequence and this may account for the positive staining reaction.

Glycosylation of capsid polypeptide is an unusual feature which may have several implications. The glycan moiety of the capsid protein would be expected to lie on the exterior of the capsid shell owing to the hydrophilic nature of the sugar residues. On the innermost surface it would in all likelihood encounter a relatively hydrophobic environment if a lipid bilayer or membrane is present. The cost in free energy needed to insert an oligosaccharide chain into a hydrocarbon core is in all probability too high to rotate a glycoprotein from a hydrophilic environment, such as the exterior of the virus particle, to a hydrophobic environment that probably exists inside the capsid shell. Perhaps the

glycosylation of capsid protein serves to orient it in the assembled capsid shell or during assembly. Furthermore, the amorphous 'fuzz' around the periphery of TIV particles, alluded to in section 4.3.1, may be representative of carbohydrate polymers covalently attached to the capsid protein.

The relatively extensive array of polypeptides which produced positive lectin staining for glycoproteins did so with variable levels of intensity. This may reflect the size and composition of the carbohydrate polymer since monosaccharides are polyfunctional molecules that are able to combine with each other glycosidically at different linkage positions to form branched structures.

Table 8 compares those TIV polypeptides detected as surface polypeptides by radioiodination with those which exhibited a positive staining reaction for glycoprotein. A total of ten polypeptides with molecular masses of 211kDa, 79kDa, 46kDa, 44kDa, 39kDa, 25kDa, 19kDa, 16kDa, 11kDa and 5kDa exhibit both characteristics, indicating that these polypeptides are surface located glycoproteins.

4.7 The putative 49kDa capsid polypeptide.

The study of the molecular organization of the capsid shell is important because it uncovers the principles by which biological macromolecules assemble into complex structures. The capsid shell accounts for a large part of the virus mass and is made up of protein molecules which are specified by virus genes. Since viruses have relatively small genomes, they cannot afford too many genes for specifying capsid proteins. Therefore, the capsid must be formed by the economical association of many protein units of a single kind or of relatively few kinds. The repeating protein subunits forming the capsid must be arranged in a regular architecture that utilizes bonds between the same pairs of chemical groups.

Table 8. Molecular mass comparison of TIV surface labelled polypeptides and polypeptides exhibiting a positive glycan staining reaction.

¹²⁵ I surface labelled polypeptides kDa	Glycan (+) polypeptides kDa
211	211
ND	119
ND	108
79	79
ND	73
ND	65
ND	53
ND	49
46	46
44	44
ND	42
39	39
ND	36
30	ND
25	25
ND	23
ND	22
19	19
16	16
11	11
5	5

ND Not Detected

SDS-PAGE comparisons of TIV particles disrupted under reducing and non-reducing conditions reveal that the putative 49kDa capsid polypeptide may be present in its native state as a pentameric oligomer with the individual polypeptide chains linked through intermolecular disulfide bonds. The putative amino acid sequence of the capsid protein (54) indicates that it contains a total of two cysteine residues. This implies that each polypeptide chain of the pentameric oligomer is linked to neighboring chains through two intermolecular disulfide bonds. However, it is also apparent that non-covalent interactions play a role in stabilizing the pentameric complex since complete dissociation of the capsid polypeptide chains only followed heating in SDS buffer at 50°C or higher. Additional evidence for the existence of capsid pentamers comes from observations made following 2-D separations of TIV polypeptides and discussed under artifacts in section 4.8.

Non-covalent interactions must be the major force which stabilizes the overall capsid structure. This assumption is based upon the premise that the putative pentameric capsid oligomer was observed following SDS-PAGE of TIV particles disrupted under non-reducing but denaturing conditions. It is probable that capsid stabilizing interactions consist primarily of hydrophobic interactions between the pentameric capsid oligomers since these form the most stable non-covalent types of interactions. This is not to say that weaker hydrogen bonding, electrostatic bonds and van der Waal interactions do not contribute to the overall stability of the capsid structure, but rather that all four types of interactions act in unison to preserve the structure of the TIV capsid.

4.8 Artifacts associated with the putative 49kDa capsid polypeptide.

Following 2-D separations of disrupted TIV particles, certain polypeptides deviated from their apparent relative molecular mass determined by 1-D SDS-

PAGE (Figure 5 and Table 3). This was particularly noticeable for the putative monomeric capsid protein which migrated in 1-D SDS-PAGE as a 49kDa species while in the second dimension of the 2-D separation, it migrated with an apparent molecular mass of ~40kDa. This irregularity may result from a change in molecular conformation of the polypeptides. The amino acid sequence of the 49kDa capsid protein, predicted from the sequence of the cloned capsid gene (54), contains two cysteinyl residues. Under reducing conditions, such as those used in 1-D SDS-PAGE for molecular mass determination, any existing disulfide bonds would have been broken. Preparation of the sample prior to 2-D PAGE consisted of heat treatment at 95°C for 3 minutes in sample application buffer which contained 0.2% SDS and 5% 2-mercaptoethanol (see Materials and Methods). This effectively ensured the denaturation of the polypeptides for the first dimension IEF separation. Denaturation was also facilitated and maintained by the presence of 8M urea in the polyacrylamide gel. However, upon completion of the first dimension IEF and equilibration of the tube gel in Laemmli buffer, the urea was subsequently removed. Removal of the denaturant in the absence of any reducing agent can effectively result in structural rearrangements leading to more compact conformations due to the formation of intramolecular disulfide bonds. Presumably, in this more compact conformation the mobility of the polypeptide in the second dimension SDS-PAGE was increased giving the impression of a lower apparent relative molecular mass.

The putative capsid polypeptide also exhibited a charge heterogeneity since IEF resolved at least two major components, each of which demonstrated extensive streaking in the SDS-PAGE second dimension. The streaking is attributed to incomplete denaturation prior to separation in the second dimension as described in the previous paragraph. Careful scrutiny of the track in 2-D gels for the putative capsid polypeptide revealed a series of spots of progressively

increasing molecular mass. An example of these spots is given in Figure 8. They were seen consistently in a total of 14 independent 2-D gel profiles obtained from either 'filled' or 'empty' capsid fractions. The spots may represent intermediates of capsid oligomerization resulting from incomplete disruption of intermolecular S-S bonds. Molecular mass values of the putative intermediates, based upon the migrations of the molecular weight standards in the second dimension SDS, indicate that the intermediates are within the range expected for di, tri, tetra and pentameric capsid oligomers.

Conclusions.

In recent years significant progress in TIV research has been made and the biochemical properties of the viral genome and some structural polypeptides have been determined. The initial objectives of this thesis were (i) to examine the complexity of polypeptides associated with TIV and (ii) to partially characterize individual polypeptides as an aid to gaining an insight into their function(s).

The protein component of the TIV particle was found to be considerably more complex than had been previously shown. 2-D separation methods and subsequent silver staining of the gels reveal a total of 67-70 polypeptides associated with TIV particles, nearly three times as many as previously seen by 1-D separations and less sensitive staining methods. These polypeptides represented monomeric species since they were separated under reducing and denaturing conditions, but oligomeric complexes in TIV were demonstrated by comparative disruption of virus particles under reducing and non-reducing conditions. This has not been previously shown.

Observations made on the putative capsid polypeptide led to the conclusion that the capsid polypeptide is complexed as covalently bound pentameric units in the intact TIV particle. When intact TIV particles were disrupted under non-reducing/denaturing conditions, no intensely staining 49kDa polypeptide was apparent, but rather a polypeptide of approximately 240kDa, whose disappearance was concurrent with the appearance of the 49kDa species under reducing/denaturing conditions. Further circumstantial evidence suggestive of a pentameric capsid protein complex was provided by 2-D PAGE.

The pentameric capsid complex is relatively stable, the TIV particles only dissociating at temperatures of 50°C and higher. This property was also observed

with other oligomeric complexes, the monomers of which were only seen with increasing temperature. The inherent stability of TIV oligomeric complexes such as capsid may, therefore, confer intrinsic stability upon the TIV particle.

Eleven polypeptides have been identified as being located on the surface of the TIV particle and ten of these polypeptides appear to be glycosylated. To date no other iridoviruses have been mapped this way. Because of the capsid protein's failure to incorporate ^{125}I surface label, it is concluded that reactive tyrosyl residues are not exposed on the surface of the TIV particle. However, it remains to be conclusively demonstrated that the putative capsid polypeptide is a capsid shell component.

Judging from γ -aman-specific staining, the putative capsid polypeptide is heavily glycosylated, an unusual feature of viruses. ASFV does have glycoproteins, but it is no longer classified as an iridovirus. Owing to the controversial nature of glycoproteins in iridoviruses, confirmatory studies on TIV need to be undertaken involving the use of incorporation of radiolabeled sugars into viral proteins.

This study constitutes the first attempts to sequence polypeptides of iridoviruses on PVDF membrane following their separation by 2-D electrophoresis. As applied to the 32kDa polypeptide, this technique was a success. Similarly, results with amino acid composition analysis from PVDF membrane, a technique still in its infancy, also proved to be highly successful.

These techniques provide a powerful tool in the microanalysis of proteins and peptides. In conjunction with the already proven and impressive resolution capabilities of 2-D electrophoresis, they provide a means by which microanalysis can be performed on individual components of complex protein mixtures. Furthermore, techniques have been perfected for both enzymatic and chemical cleavage of polypeptides directly on the PVDF membrane. These might be

valuable in future research dealing with TIV polypeptides with blocked N-termini or for generating internal sequence data. One approach to cloning genes is through the construction of oligonucleotide probes based upon information obtained from partial amino acid sequence data. This approach, applied to TIV, should allow individual structural genes to be cloned and sequenced.

Potentially, the combined utilization of these techniques can provide the means for a detailed molecular analysis of TIV structural polypeptides, and those of other iridoviruses, which may help in the classification of members of the iridoviridae family. The information currently available on TIV provides only a starting point for our understanding of this genetically and biologically complex virus. Many details of the viral replication process and complexities of virion assembly need to be established. Knowledge of these mechanisms would be useful in order to define the conditions under which TIV could be utilized in nature as a biological insecticide, alone or in synergy with other insect viruses.

Appendix A

TIV 32kDa
 Tnsd_Ecoli
 ID TNSD_ECOLI STANDARD; PRT; 508 AA.
 AC P13991;
 DE TRANSPOSON TN7 TRANSPOSITION PROTEIN TNSD. . . .
 SCORES Init1: 43 Initn: 43 Opt: 43
 85.7% identity in 7 aa overlap

			10		20
TIV 32kDa			FLPSMTYCVDCVAAPNEHDS		
Tnsd_E	IRLMEYQAQGAVHMLGVAASRVKSDNRFYRCPDCVALQLNRYGEAFWQRDWYLPALPYC				
	100 110 120 130 140 150				
Tnsd_E	PKHGALVFFDRAVDDHRHQFWALGHTELLSDYPKDSLSQLTALAAYIAPLLDAPRAQELS				
	160 170 180 190 200 210				

TIV 32kDa
 Swiss-Prot:Vel_Papvc
 ID VE1_PAPVC STANDARD; PRT; 602 AA.
 AC P03112;
 DE PROBABLE E1 PROTEIN. . . .
 SCORES Init1: 43 Initn: 43 Opt: 43
 45.5% identity in 11 aa overlap

		10		20
TIV 32kDa		FLPSMTYCVDCVAAPNEHDS		
		: : :		
Vel_Pa	MAEGTDPLDDCGGFLDTEADCLDCDNLEEDLTELFDA DTVSSLLDDTDQVQGNLSI ?FQH			
	10 20 30 40 50 60			

TIV 32kDa
 Swiss-Prot:Tasm_Povbk
 ID TASM_POVBK STANDARD; PRT; 172 AA.
 DE SMALL T ANTIGEN. . . .
 SCORES Init1: 42 Initn: 42 Opt: 42
 80.0% identity in 5 aa overlap

		10		20
TIV 32kDa		FLPSMTYCVDCVAAPNEHDS		
		:		
Tasm_P	VHCPMLCQLRLRLNRKFLRKEPLVWIDCYCIDCFTQWFGLDLTEETLQWWVQIIGETP			
	110 120 130 140 150 160			
Tasm_P	FRDLKL			
	170			

TIV 32kDa

Swiss-Prot:Tasm_Povha

ID TASM_POVHA STANDARD; PRT; 194 AA.

AC P03080;

DE SMALL T ANTIGEN. . . .

SCORES Init1: 42 Initn: 42 Opt: 44

38.5% identity in 13 aa overlap

```

                                10      20
TIV 32kDa                      FLPSMTYCVDCVAAPNEHDS
                                :: : ||:|| |
Tasm_P RNKGISTCNCILCLRKHLLKKS WRVPCLVLGECYCIDCFALWFGLPVTNMLVPLYAQ
      120      130      140      150      160      170

Tasm_P FLAPIPVDWLDLNVHEVYNPASGP
      180      190

```

TIV 32kD

Swiss-Prot:Tami_Povha

ID TAMI_POVHA STANDARD; PRT; 401 AA.

AC F03079;

DE MIDDLE T ANTIGEN. . . .

SCORES Init1: 42 Initn: 42 Opt: 44

38.5% identity in 13 aa overlap

```

                                10      20
TIV 32kDa                      FLPSMTYCVDCVAAPNEHDS
                                :: : ||:|| |
Tami_P RNKGISTCNCILCLRKHLLKKS WRVPCLVLGECYCIDCFALWFGLPVTNMLVPLYAQ
      120      130      140      150      160      170

Tami_P FLAPIPVDWLDLNVHEVYNPASGMTLMLPPPPADPESSTILTQEDTGPTLMGQQDTLTSR
      180      190      200      210      220      230

```

TIV 32kDa

Swiss-Prot:Tasm_Povba

ID TASM_POVBA STANDARD; PRT; 172 AA.

AC P15000;

DE SMALL T ANTIGEN. . . .

SCORES Init1: 42 Initn: 42 Opt: 42

80.0% identity in 5 aa overlap

```

                                10      20
TIV 32kDa                      FLPSMTYCVDCVAAPNEHDS
                                ||:|| |
Tasm_P VHCPCMLCQLRLRLNRKFLRKEPLVWIDCYCIDCFQWFGLDLTEETLQWWVQIIGETP
      110      120      130      140      150      160

Tasm_P FRDLKL
      170

```

TIV 32kDa
 Swiss-Prot:Exo5_Bpt5
 ID EXO5_BPT5 STANDARD; PRT; 291 AA.
 AC P06229;
 DE EXODEOXYRIBONUCLEASE (EC 3.1.11.3) (5' EXONUCLEASE). . . .
 SCORES Init1: 39 Initn: 39 Opt: 41
 53.8% identity in 13 aa overlap

			10	20	
TIV 32kDa			FLPSMTYCVDCVAAPNEHDS		
			: :::		
Exo5_B	LPLPGKQKYIQNLNASEELLFRNLILVDLPTYCVD	IAA	AVGQDVLDKFTKDILEIAEQ		
	240	250	260	270	280 290

TIV 32kDa
 Swiss-Prot:Hypa_Rnoca
 ID HYPA_RHOC A STANDARD; PRT; 113 AA.
 AC P26409;
 DE HYPA PROTEIN. . . .
 SCORES Init1: 39 Initn: 39 Opt: 39
 50.0% identity in 10 aa overlap

			10	20	
TIV 32kDa			FLPSMTYCVDCVAAPNEHDS		
			: :		
Hypa_R	AGVERAALDFAFDVVLRGSLAEGAAVQIIDLP	GQAACFDCGKTVEIEHRLDICPECGGTR			
	40	50	60	70	80 90

Hypa_R LLVQGGDEMRIKDLEVL
 100 110

TIV 32kDa
 Swiss-Prot:Cnjc_Tetth
 ID CNJC_TETTH STANDARD; PRT; 310 AA.
 AC P16925;
 DE CONJUGATION STAGE-SPECIFIC PROTEIN. . . .
 SCORES Init1: 39 Initn: 39 Opt: 39
 66.7% identity in 6 aa overlap

			10	20	
TIV 32kDa			FLPSMTYCVDCVAAPNEHDS		
			:		
Cnjc_T	KALVNACPTKVFGINPHTNTLEVQDHMKMYCEE	CVYKCAEDFQKPKLIKIDHKKDKFFF			
	210	220	230	240	250 260

Cnjc_T KVE TTGVMKPVEVLRRGFSTLRQKLDFMREQIQENSNNQFEYGN
 270 280 290 300 310

TIV 32kDa
Swiss-Prot:Mcel_Vaccc

ID MCEL VACCC STANDARD; PRT; 844 AA.
AC P20979;
DE MRNA CAPPING ENZYME, LARGE SUBUNIT (CONTAINS: POLYNUCLEOTIDE 5' . .
SCORES Init1: 38 Initn: 38 Opt: 42
35.3% identity in 17 aa overlap

				10	20
TIV 32kDa				FLPSMTYCVDCVAAPNEHDS	
				: : : :~::~:~::	
Mcel_V	IFVEYKKFSNDKGFPKEYGSGKIVLYNGVNYL-NNIYCLEYINTHNEVGIKSVVVPKIFI				
	430 440 450 460 470 480				
Mcel_V	AEFLVNGEILKPRIDKTMKYINSEDDYGNQHNIIVEHLRDQSIKIGDIFNEDKLSDVGHQ				
	490 500 510 520 530 540				

TIV 32kDa
Swiss-Prot:Pols_Rrvt
ID POLS_RRVT STANDARD; PRT; 1254 AA.
AC P08491;
DE STRUCTURAL POLYPROTEIN (CONTAINS: COAT PROTEIN C; SPIKE
GLYCOPROTEINS . . .
SCORES Init1: 38 Initn: 38 Opt: 39
66.7% identity in 6 aa overlap

				10	20
TIV 32kDa				FLPSMTYCVDCVAAPNEHDS	
				: :~::	
Pols_R	EASMTCRNRSRHRRSVTEHFNVYKATRPYLAYCADCGDGYFCYSPVAIEKIRDEAPDGML				
	330 340 350 360 370 380				
Pols_R	KIQVSAQIGLDKAGTHAHTKIRYMAGHDVQESKRDSLRYVYTSAAACSIHGTMGHFIVAHC				
	390 400 410 420 430 440				

TIV 32kDa
Swiss-Prot:Bime_Aspni
ID BIME_ASPNI STANDARD; PRT; 2073 AA.
AC P24686;
DE NEGATIVE REGULATOR OF MITOSIS. . . .
SCORES Init1: 38 Initn: 38 Opt: 44
31.3% identity in 16 aa overlap

				10	20
TIV 32kDa				FLPSMTYCVDCVAAPNEHDS	
				:~::~:~:: ~::	
Bime_A	VQLVTLRTLSTPTGRAMLAFSGRLPLLTEKLPISFSLQCVMKPSNVTISADRASFHEEK				
	1270 1280 1290 1300 1310 1320				
Bime_A	ICWAFFHNGVSTGLAISKNSKGIDTSWILFNKEQLTNRHAGFLLALGLNGHLRSLAKWV				
	1330 1340 1350 1360 1370 1380				

TIV 32kDa
Swiss-Prot:Pols_Sfv

ID POLS_SFV STANDARD; PRT; 1253 AA.
AC P03315;
DE STRUCTURAL POLYPROTEIN (CONTAINS: COAT PROTEIN C; SPIKE
GLYCOPROTEINS . . .
SCORES Init1: 38 Initn: 38 Opt: 45
38.9% identity in 18 aa overlap

```

                                10      20
TIV 32kDa                      FLPSMTYCVDCVAAPNEHDS
                                | :||:| | |::| :|
Pols_S DLLQAALTCRNGTRHRRSVSQHFNVYKATRPYIAYCADCGAGHSCHSPVAIEAVRSEATD
      320      330      340      350      360      370

Pols_S GMLKIQFSAQIGIDKSDNHDTKIRYADGHAIENAVRSSLKVATSGDCFVHGTMGHFILA
      380      390      400      410      420      430

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TIV 32kDa
Swiss-Prot:Mcel_Vaccv
ID MCEL_VACCV STANDARD; PRT; 844 AA.
AC P04298;
DE MRNA CAPPING ENZYME, LARGE SUBUNIT . . .

SCORES Init1: 38 Initn: 38 Opt: 42
35.3% identity in 17 aa overlap

```

                                10      20
TIV 32kDa                      FLPSMTYCVDCVAAPNEHDS
                                :| : :||:|::|::| |
Mcel_V IFVEYKKFSNDKGFPKEYGSGKIVLYNGVNYL-NNIYCLEYINTHNEVGIKSVVVPKFI
      430      440      450      460      470      480

Mcel_V AEFLVNGEILKPRIDKTMKYINSEDDYGNQHNIIVEHLRDQSIKIGDIFNEDKLSDVGHQ
      490      500      510      520      530      540

```

TIV 32kDa
Swiss-Prot:Tasm_Povjc
ID TASM_POVJC STANDARD; PRT; 172 AA.
AC P03083;
DE SMALL T ANTIGEN. . . .
SCORES Init1: 37 Initn: 37 Opt: 37
80.0% identity in 5 aa overlap

```

                                10      20
TIV 32kDa                      FLPSMTYCVDCVAAPNEHDS
                                || || |
Tasm_P VHCPCLMCMLKLRHRNRKFLRSSPLVWIDCYCFDCFRQWFGCDLTQEALHCWEKVLGDTF
      110      120      130      140      150      160

Tasm_P YRDLKL
      170

```


TIV32kDa

Swiss-Prot:Ercl_Mouse

ID ERCL_MOUSE STANDARD; PRT; 298 AA.

AC P07903;

DE DNA EXCISION REPAIR PROTEIN ERCC-1. . . .

SCORES Init1: 37 Initn: 37 Opt: 37

40.0% identity in 20 aa overlap

```

                                10      20
TIV32kDa                      FLPSMTYCVDCVAAPNEHDS
                                ||::| |::| : |: ||
Ercl_M SAEEAGRYLETYRAYEQKPADLLMEKLEQNFLSRATECLTTVKSVNKTDQSQTLLATFGSL
      210      220      230      240      250      260

Ercl_M EQLFTASREDLALCPGLGPQKARRLFEVLHEPFLKVPR
      270      280      290

```

TIV32kDa

Swiss-Prot:Tasm_Sv40

ID TASM_SV40 STANDARD; PRT; 174 AA.

AC P03081;

DE SMALL T ANTIGEN. . . .

SCORES Init1: 37 Initn: 37 Opt: 37

80.0% identity in 5 aa overlap

```

                                10      20
TIV32kDa                      FLPSMTYCVDCVAAPNEHDS
                                || || |
Tasm_S ANCICLLCLLRMKHENRKLYRKDPLVWVDCYCFDCFRMWFGDLCEGTL LLWC DIIGQTT
      110      120      130      140      150      160

Tasm_S YRDLKL
      170

```

TIV 32kDa

Swiss-Prot:Nof1_Drome

ID NOF1_DROME STANDARD; PRT; 633 AA.

AC P15296;

DE 71 KD PROTEIN IN NOF-FB TRANSPOSABLE ELEMENT. . . .

SCORES Init1: 36 Initn: 36 Opt: 45

35.3% identity in 17 aa overlap

```

                                10      20
TIV32kDa                      FLPSMTYCVDCVAAPNEHDS
                                |: :|| || : :|
Nof1_D MAKLIYKKVPLPCAFNFRKAKLSKDVDNIWLRIEGYCNDSSILKGHCLVKPDEQCGIMI
      150      160      170      180      190      200

Nof1_D SVSVPDTRGIPHNNKRRCTGSRRL EIGNELILKKAALWRKEATDNMND DDPEPSYIPNLP
      210      220      230      240      250      260

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