CHARACTERISATION OF TWO APHID PICORNA-LIKE VIRUSES

by

Carolyn Williamson

This thesis was submitted to the Faculty of Science, University of Cape Town in fulfillment of the requirements for the Degree of Doctor of Philosophy.

September 1988

CERTIFICATION BY SUPERVISORS

In terms of Paragraph 9 of the Regulations for the Degree of Doctor of Philosophy at the University of Cape Town, we certify that we approve of the inclusion in this thesis material already published, or submitted for publication, by the candidate Carolyn Williamson.

Signed by candidate

Dr. E.P. Rybicki
Department of Microbiology
University of Cape Town

Ass. Prof. M.B. von Wechmar
Department of Microbiology
University of Cape Town

ACKNOWLEDGEMENTS

A special thanks is due to both of my supervisors; Dr Ed Rybicki for his guidance and encouragement, and to Associate Professor M.B. von Wechmar for her advice, enthusiasm and assistance with maintaining aphids.

I acknowledge and thank Professor Henk Huismans for his guidance with the DNA cloning work. I would also like to thank the Veterinary Research Institute, Onderstepoort for allowing the use of their facilities and space for this work. I also thank Mr G.G.F. Kasdorf for his electron microscopy work.

I thank the staff of the animal and plant rooms for their excellent services, especially Mr Derek Roussouw for his endless hours of maintaining aphids and Mr John Hamman for his much appreciated help with purifying. Also to Mr Peter Buckton for his general assistance with purification and printing.

Thanks also to Dr Anna-Lise Williamson, and friends and colleagues in the Microbiology Department for their help and advice over the years. Lastly I'd like to thank Jorge Cardoso for his encouragement and support throughout this project.

I acknowledge the financial assistance of the Council for Scientific and Industrial Research and the Wheat Board.

ABSTRACT

A new aphid virus, aphid lethal paralysis virus (ALPV), was isolated from laboratory-propagated *Rhopalosiphum padi* aphids co-infected with *R. padi* virus (RhPV). ALPV and RhPV were separated and ALPV was characterised in detail. Virions are isometric with a diameter of 26 nm, a sedimentation coefficient of 164 S and a density in CsCl of 1.34 g/ml. Virions contain a 9.7 kb polyadenylated, single-stranded RNA and three major proteins with molecular weights of approximately 30 kilodaltons.

By characterising RhPV further, two additional putative capsid proteins were found, an RNA poly(A) tract was detected and an RNA size of 10 kb was determined. A South African isolate of RhPV ($RhPV_{OFS}$) was found to be serologically identical but physically distinct from a USA isolate. Complementary DNA was synthesized from $RhPV_{OFS}$ RNA and cloned into the plasmid vector, pBR322. This clone was used for the detection of virus in aphids.

ALPV and RhPV are serologically unrelated. ALPV is serologically distantly related to two insect picornaviruses, cricket paralysis virus (CrPV) and *Drosophila* C virus. No nucleic acid homology was detected between ALPV cDNA and CrPV by dot-blot hybridization. ALPV is serologically unrelated to seven other insect picorna-like viruses. RhPV is serologically unrelated to any of the above mentioned viruses.

ALPV and RhPV RNAs were efficiently translated in rabbit reticulocyte lysate into high molecular weight

polypeptides, the sum of which exceeded the coding capacity of the genomes. Putative capsid precursor proteins of ALPV and RhPV were identified by immunoprecipitation. ALPV translation products were post-translationally cleaved as demonstrated in pulse-chase experiments and in experiments using a translation inhibitor. The efficiency of cleavage was concentration-dependent indicating the action of a protease. In parallel experiments with RhPV RNA, no evidence of post-translational cleavage was observed.

In a survey of aphids collected in South Africa, ALPV and RhPV were detected in aphids from two major small-grain producing areas. Both viruses were found to naturally infect most of the cereal aphid species found in this country. ALPV and RhPV infections of *R. padi* resulted in a marked reduction in longevity and fecundity relative to uninfected aphids. Both viruses were found to be horizontally and vertically transmitted through aphid populations, and aphid host plants and aphid predators could be implicated in virus dissemination.

ALPV and RhPV have many properties in common with each other as well as with insect and mammalian picornaviruses. Based on this data, it is proposed that ALPV and RhPV be classified into the picornavirus group (family *Picornaviridae*).

TABLE OF CONTENTS

ABBREVIATIONS

A_{260}	Absorbance at 260 nm
ALPV	Aphid lethal paralysis virus
BSA	bovine serum albumin (Fraction V)
cDNA	complementary DNA
Ci	Curie
CMV	cucumber mosaic virus
cpm	counts per minute
CsCl	caesium chloride
cv	cultivar
d	dalton(s)
D. noxia	*Diuraphis noxia*
DAS-ELISA	double-antibody sandwich ELISA
DCV	*Drosophila* C virus
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
g	standard gravitational acceleration
h	hours
IFV	infectious flacherie virus
IgG	Gamma-globulin fraction of serum
K	kilodalton
kb	kilobase
LB	Luria-Bertani
M	molar
M. dirhodum	*Metapolophium dirhodum*
M. persicae	*Myzus persicae*
min	minute(s)
mM	millimolar
M_r	relative molecular mass
nm	nanometer
OMV	Ornithogalum mosaic virus
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
R. padi	*Rhopalosiphum padi*
RF	replicative form
RhPV	*Rhopalosiphum padi* virus
$RhPV_{ILL}$	RhPV originating from Illinois (USA)
$RhPV_{OFS}$	RhPV originating from the Orange Free State
RNA	ribonucleic acid
rpm	revolutions per minute

S	Svedbergs
S. avenae	*Sitobion avenae*
S. graminum	*Schizaphis graminum*
SDS	sodium dodecyl sulphate
ss	single-stranded
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TMV	tobacco mosaic virus
Tris	Tris (Hydroxymethyl)-aminomethane
μ	micron
UV	ultraviolet
v/v	volume per volume ratio
v/w	volume per weight ratio

TABLES

Tables appear on the following pages:

FIGURES

Figures appear on the following pages:

CHAPTER 1

INTRODUCTION

1.1 HISTORICAL BACKGROUND

Rhopalosiphum padi virus (RhPV) was discovered in 1980 in South Africa during an investigation of diseases of small grains in the Orange Free State province (OFS), the major wheat growing region of South Africa (von Wechmar and Rybicki, 1981). This wheat disease was aphid transmissible and appeared to be strongly correlated with the presence of a new "invader" aphid, *Diuraphis noxia* (Mordvilko). A virus complex consisting of brome mosaic virus (BMV), barley yellow dwarf virus (BYDV) and RhPV, was isolated from aphid-infested diseased plants (Rybicki, 1984). A virus similar to RhPV was described simultaneously from Illinois, USA, also in association with BYDV (D'Arcy *et al.*, 1981a).

D. noxia aphids were extremely successful in the Eastern OFS and the high number found on plants presented a major problem to farmers (von Wechmar and Rybicki, 1981). A programme was initiated to investigate natural and introduced aphid predators and parasites (Y.K. Aalbersberg, at the Small Grain Center, Bethlehem, OFS). A separate group (M.B. von Wechmar and E.P. Rybicki, Department of Microbiology, University of Cape Town, South Africa) investigated plant pathogenic viruses transmitted by aphids. A preliminary characterisation of the South African isolate of RhPV was published by this group (von Wechmar and

Rybicki, 1981; Rybicki and von Wechmar, 1982a; Rybicki, 1984). This isolate was physically similar to the isolate reported from Illinois, USA (D'Arcy *et al.*,1981a).

1.2 PROJECT AIMS

The initial aims of this project were to characterise RhPV in detail and to investigate its replication strategy. During these investigations, a second virus was inadvertently introduced into the aphid stock colonies. This virus was named aphid lethal paralysis virus (ALPV), after the symptoms expressed by infected aphids, and occurred as a mixed infection with RhPV. The project was modified to include the characterisation of this new virus and the elucidation of its *in vitro* translation. Overall objectives were to place these viruses in the classification scheme of RNA viruses of insects and to conduct preliminary investigations on their epidemiology and pathogenicity.

1.3 LITERATURE REVIEW

1.3.1 INTRODUCTION

Over a hundred virus pathogens have been isolated from at least nine different taxonomic groups of insects. Many of these viruses have been subjected to preliminary investigations but have not been sufficiently well characterised to warrant their classification into existing groups, or into new virus groups. To illustrate the diversity of insect viruses, a brief overview of the different groups is presented. This review and thesis are primarily concerned with small, single-stranded, monopartite RNA viruses of insects, particularly the picorna- and picorna-like viruses. As aphids have played an integral part in this project, aphid biology and the use of viruses in the control of insect pests are also briefly reviewed.

1.3.2 CLASSIFICATION OF INSECT VIRUSES

Generally, viruses are classified according to their common physicochemical and biological properties, and replication strategies (Matthews, 1982). Viruses can be broadly divided into vertebrate, invertebrate, plant, fungal and bacterial virus groups; however, there are a number of viruses that cross these barriers. The family *Rhabdoviridae*, for example, includes members that infect

mammals (rabies virus), and plants (lettuce necrotic yellows virus), and probably insects (Sigma virus) (Matthews, 1982).

Most insect viruses have been classified into pre-existing vertebrate and plant virus groups (Table 1.1). Only three groups have been created whose members are exclusively invertebrate viruses: these are the *Baculoviridae*, the *Polydnaviridae* and the *Nudaurelia β* virus group (Matthews, 1982; Brown, 1986).

The unclassified DNA insect viruses include viruses such as a rod shaped, non-enveloped virus isolated from tsetse flies (*Glossina pallidipes*) (Odindo *et al.*, 1986); an ovoid virus (CmV2) isolated from parasitic wasps (*Cotesia melanoscela*) (Stolz *et al.*, 1988), and an enveloped filamentous virus isolated from honey bees (*Apis mellifera*) (Bailey and Milne, 1978, Bailey *et al.*, 1983).

1.3.3 SMALL RNA VIRUSES OF INSECTS

Most of the small RNA insect viruses described, belong to, or are potential members of, the picornavirus, Nodavirus and *Nudaurelia β* virus groups. Picorna- and picorna-like viruses are reviewed in detail in Sections 1.3.4 and 1.3.5.

The Nodavirus genome is divided into two segments, both of which are packaged into one particle. The type member of this group, Nodamura virus, is pathogenic to various insects, replicates in baby hamster kidney (BHK) cells and is lethal to suckling mice (Bailey *et al.*, 1975; Moore *et al.*, 1985). The virions are non-enveloped and isometric, with an approximate diameter of 29 nm, sedimentation

coefficient of 135 S and buoyant density in CsCl of 1.34 g/ml. The virions contain a single major structural protein with relative molecular mass (M_r) of approximately 40 000 and two segments of single-stranded (ss) RNA with M_rs of 1.15×10^6 and 0.46×10^6 (Longworth, 1978). The second member of the *Nodaviridae*, black beetle virus (*Heteronychus arator*) has physical properties very similar to Nodamura virus. Although it infects a range of insects, this virus is not infective for mice (Longworth and Carey, 1976).

The type member of the *Nudaurelia* β virus group is *Nudaurelia capensis* β virus (NβV) isolated from the pine emperor moth, *Nudaurelia cytherea capensis*. NβV is an isometric virus with an approximate diameter of 35 nm, a sedimentation coefficient of 210 S and a buoyant density in CsCl of 1.30 g/ml. The virions contain a single protein with an approximate M_r of 61 000 and an undivided ssRNA genome with an approximate M_r of 1.8×10^6 (Struthers and Hendry, 1974; Moore *et al.*, 1985). Additional members of the *Nudaurelia* β family include *Nudaurelia* viruses (γ, δ, β, α, ε and ω), *Antheraea eucalypti* virus, *Darna trima* virus, *Thosea asigna* virus, *Philosamia cynthia* x *ricini* virus, *Trichoplusia ni* virus, *Dasychira pudibunda* virus and *Psudoplusia includens* virus (Reinganum *et al.*, 1978; Juckes, 1979; Morris *et al.*, 1979; Moore *et al.*, 1985 and Hendry *et al.*, 1985).

There are also numerous unclassified small RNA viruses that are physically distinct from the picornaviruses, Nodaviruses and the *Nudaurelia* β viruses. Arkansas bee virus (ABV), for example, is an isometric virus with a

diameter of 30 nm and a sedimentation coefficient of 135 S. The virions contain one major polypeptide (M_r 43 x 10^3) and one ssRNA species (M_r 1.8 x 10^6). Based on these characteristics, ABV was tentatively classified as a tombusvirus of which tomato bushy stunt virus is the type member. The previous identification of two genome segments was thought to be due to contamination of virus preparations with a picorna-like virus, Berkley bee picornavirus (BBPV), frequently found in association with ABV (Lommel *et al.*, 1985).

Kelp fly virus is a unique virus in that it is 29 nm in diameter with surface projections of 8 nm. Virions have a sedimentation coefficient of 158 S and a density of 1.425 g/ml at pH 7.0 and 1.467 g/ml in CsCl at pH 9.0. Virions contain two major proteins (M_r 73 x 10^3 and 29.4 x 10^3) and single-stranded RNA of approximate M_r 3.5 x 10^6 (Longworth, 1978). Kelp fly virus is unlike any previously described virus.

Chronic bee paralysis virus particles are ellipsoid, of varying lengths and constant modal width of 20 nm. The modal lengths are 65 nm, 55 nm, 40 nm and 30 nm with corresponding sedimentation coefficients of 125-136 S, 110-124 S, 97-106 S and 82 S and corresponding RNA M_r of 1.35 x 10^6, 0.9 x 10^6, 0.35 x 10^6 and 0.35 x 10^6. The virions have a density in CsCl of 1.33 g/ml and contain one major structural protein of M_r 23 500 (Ball *et al.*, 1985; Bailey and Woods, 1977). A similar ellipsoid virus, Drosophila RS virus, was isolated from *Drosophila* flies. However, these particles were a regular size, contained two

polypeptides (*M* 19.5 x 10^3 and 45 x 10^3) and had a density of 1.26 g/ml (Longworth, 1978).

Several very small RNA viruses ("miniviruses") with a diameter of less than 20 nm have been identified in insects (Longworth, 1978). One such virus was isolated in association with chronic bee paralysis virus and appears to be dependent on the latter for replication (Ball *et al.*, 1985). This agent was named chronic bee paralysis virus associate and is isometric, contains three species of RNA all approximately 0.35 x 10^6 in M_r, has a sedimentation coefficient of 41 S and is serologically unrelated to chronic bee paralysis virus. A "minivirus" was also isolated from *Antherea eucalypti* (*Antherea* satellite virus) which is 14 nm in size and has a sedimentation coefficient of 45 S (Reinganum, 1975). A third isometric virus, crystalline array virus, was isolated from the grasshopper, *Melanoplus bivattatus*. It is 13 nm in diameter, has a sedimentation coefficient of 42 S. Unlike the previous viruses, no other virus was detected in association with it (Longworth, 1978).

1.3.4 CHARACTERISATION, STRUCTURE AND EXPRESSION OF THE MAMMALIAN PICORNAVIRUSES

The vast majority of characterised and assigned picornaviruses are human and animal viruses. They cause a diversity of diseases in their hosts, ranging from the severe paralytic disease caused by polioviruses to the common cold caused by rhinoviruses. They are one of the

most extensively studied virus groups: many of the viruses have been cloned and sequenced and their translation strategies elucidated; several virion structures have been determined to atomic resolution, and extensive data are available on their antigenic properties and the molecular basis of virulence. The definition of picornaviruses in general and the genera in particular, is based on the properties of these animal and human viruses. Only aspects pertinent to a comparison of mammalian viruses with insect picorna- and picorna-like viruses, are reviewed here.

Picornaviruses are small, non-enveloped, icosahedral viruses (22 - 30 nm) containing a single-stranded, positive sense RNA genome (7 to 8.5 kb). The RNA has a 3′ poly(A) tail which is heterogeneous in length between viruses. Direct sequencing of the 3′ end indicated that the approximate tail length of encephalomyocarditis virus (EMCV) RNA was 35 bases, of poliovirus RNA was 62 bases and of rhinoviruses was 74 bases (Ahlquist and Kaesberg, 1979). Genomic, replicative form, negative sense and nascent plus sense RNAs all have a small protein attached to the 5′ termini (VPg) (reviewed by Wimmer, 1982). This VPg is covalently linked via a tyrosine residue to 5′ end of poliovirus RNA by a phosphodiester bond (Ambros and Baltimore, 1978). VPg may serve as a primer for RNA synthesis by a virus encoded, primer-dependent, RNA-dependent RNA polymerase. RNA synthesis *in vitro* requires the addition of either HeLa cell host factor or an oligo(U) primer (Hey *et al.*, 1986; Morrow *et al.*, 1985; reviewed by Flanegan *et al.*, 1987).

The genomic RNA has a single open reading frame and is translated into a polypeptide which approximates the coding capacity of the genome. This "polyprotein" is subsequently cleaved into structural and non-structural proteins (detailed below). Virions are composed of four major capsid proteins present in equimolar amounts; three with an approximate M_r of 24 000 to 41 000, and a fourth low M_r protein with an approximate M_r 5 000 to 13 000. These proteins are referred to as VP1, VP2, VP3 and VP4, with VP1 having the highest M_r and VP4 the lowest (Rueckert and Wimmer, 1984). Small amounts of the VP2/VP4 precursor (VP0) are occasionally detected in intact virions.

Classification

The picornaviruses are currently divided on the basis of differing physicochemical properties into four genera: enteroviruses, cardioviruses, rhinoviruses and apthoviruses (main sources of reference are Spier, 1962; Mak *et al.*, 1970; Newman *et al.*, 1973; Rueckert, 1976; Matthews, 1982; Palmenberg, 1987).

Enteroviruses

The type member of this genus is poliovirus (three serotypes). Other members of this group include human coxsackie virus A and B, human echovirus, hepatitis A virus, murine poliovirus (Theiler's encephalomyelitis virus), swine vesicular virus, and simian, porcine and bovine enteroviruses. The main feature that differentiates enteroviruses from other picornaviruses is their stability

at pH 3. The average buoyant density in CsCl is 1.34 g/ml and virions have a sedimentation coefficient of 156-160 S.

Cardioviruses

The type member is encephalomyocarditis virus (EMCV). Other members include mengovirus, encephalomyelitis virus, Maus Elberfeld virus and Colombia SK virus. These viruses are unstable at pH 6 in the presence of 0.1 M Cl^- or Br^-. The RNA contains an internal poly(C) tract which varies in length from 80 to 250 bases and is located approximately 150 bases from the 5′ terminus. The virion buoyant density ranges from 1.33-1.34 g/ml and virions have a sedimentation coefficient of 156-160 S.

Rhinovirus

There are more than a hundred different human rhinovirus serotypes and at least 2 bovine rhinovirus serotypes. These viruses are unstable below pH 5-6 and are denatured by heat in the presence of $MgCl_2$. The virion buoyant density ranges from 1.38-1.45 g/ml and virions have a sedimentation coefficient of 150-156 S.

Aphthoviruses

There are seven different aphthovirus (or foot-and-mouth disease virus, FMDV) serotypes. These viruses are unstable below pH 5-6. As with cardioviruses, the RNA contains a poly(C) tract which varies in length from 100-170 bases and is located about 400 bases from the 5′ terminus.

The virion buoyant density ranges from 1.42-1.44 g/ml and virions have a sedimentation coefficient of 141-145 S.

The Relationship Between Picorna- and Picorna-like Viruses

The present criteria used for virus classification do not necessarily reflect the phylogenetic or evolutionary relationships between members of a particular group or between different groups of viruses (Palmenberg, 1987; Goldbach, 1987).

Antigenic relationships have been detected between structural and non-structural proteins of viruses belonging to the same genera and between viruses of different genera. Antisera to poliovirus VP3 (structural) and 2C protein (non-structural) precipitated the corresponding coxsackie virus, echovirus (enteroviruses) and rhinovirus proteins. No EMCV proteins were precipitated with antisera to either poliovirus structural or non-structural proteins (Emini *et al.*, 1985). A degree of antigenic relationship was detected between FMDV $3D^{pol}$ (polymerase), VP1, VP2, VP3 proteins and the corresponding EMCV proteins; but no antigenic relationship was detected between FMDV proteins and the corresponding proteins of bovine enterovirus and swine vesicular disease virus (Grubman *et al.*, 1987).

Nucleic acid sequence homology studies and comparisons of their predicted amino acid sequences show a high degree of homology between the 2C, VPg, $3C^{pro}$ (protease) and $3D^{pol}$ proteins of poliovirus, EMCV and FMDV (Argos *et al.*, 1984). On the basis of genome organization, capsid structure, virion antigenicity and sequence homology, Palmenberg (1987)

proposed an alternative classification system. Group 1 would include FMDV and related serotypes. Group 2 would include EMCV, mengovirus and Theiler's murine encephalomyocarditis virus. Mengo virus, for example, shares more than 93% peptide sequence homology with EMCV whereas EMCV and poliovirus share less than 40% sequence identity (Palmenberg, 1987). Group 3 would contain hepatitis A virus (HAV) which appears to be different from all other picornaviruses. Group 4 would contain poliovirus, coxsackie virus, rhinovirus-14 and rhinovirus-2. No mention was made of the classification of insect picornaviruses.

On the basis of similarities in viral genome structure, translation strategies and genome sequence homologies, Goldbach (1987) placed poliovirus (a picornavirus), cowpea mosaic virus (CPMV, a comovirus), tomato black ring virus (a nepovirus) and tobacco vein mottling virus (a potyvirus) into a "picorna-like super-group". Picornaviruses and comoviruses share several structural and functional properties. The genomic RNAs of both groups of viruses have a 5' VPg, a 3' poly(A) tract, are positive sense RNA and translate into a "polyprotein" which is subsequently cleaved into functional proteins. The gene order is also similar and in addition the capsid proteins fold to form β-barrel domains (Hogle *et al.*, 1985; Goldbach, 1987). Significant nucleic acid sequence, and predicted amino acid sequence homology was detected between CPMV RNA-dependent RNA polymerase, 24 K protease, 58 K membrane-bound protein and VPg and the corresponding picornavirus proteins $3D^{pol}$,

$3C^{pro}$, 2C and VPg (Franssen *et al.*, 1984; Argos *et al.*, 1984). The major differences between the groups lies in the genome structure, whereas comoviruses have a divided genome, the picornaviruses do not.

Two plant viruses were recently described which have several properties in common with the *Picornaviridae* (Murant *et al.*, 1987). Parsnip yellow fleck virus (PYFV) and dandelion yellow mosaic virus (DYMV) are small, monopartite, RNA viruses with a similar protein profile to picornaviruses. Their viral RNA is polyadenylated and PYFV probably has a small protein covalently linked to the genome. It would be of interest to determine where these viruses are situated in a phylogenetic tree determined on nucleic acid homology.

Virion Structure

The structures of poliovirus, mengovirus and rhinovirus have been studied at atomic resolution by X-ray crystallography (Hogle *et al.*, 1985; Rossmann *et al.*, 1985 and Lou *et al.*, 1987). Information from these studies indicates that picornaviruses are structurally very similar. The virions are composed of 60 repeating units, each unit containing a copy of VP1, VP2, VP3 and VP4. The major capsid proteins, VP1, VP2 and VP3, are folded into an eight-stranded anti-parallel β-barrel. VP1 is situated around the five-fold rotational axes of the icosahedral particles, and is particularly well exposed. VP2 and VP3 are less exposed and VP4 is internally situated. The interior of the protein

shell contains the RNA which is spatially disorganized relative to the icosahedral capsid.

The spatial relationship of the proteins provides useful information on virus evolution, protein processing, receptor recognition and virus neutralization by antibodies and antiviral agents (for a review, see Rossman and Rueckert, 1987).

Translation and Protein Processing

All functional picornaviral proteins are produced by the same basic translation strategy. Translation is initiated near the 5′ terminus of the genomic RNA, and proceeds along the entire length of the genome. The resulting polypeptide is cleaved in a series of controlled events to produce functional proteins and mature virions (Shih *et al.*, 1978 and 1979; Gupta *et al.*, 1985; Jackson, 1986; Korant, 1972). In this review, poliovirus is used to illustrate the translation of picornaviruses. For convenience, a standard nomenclature, termed the L434 convention, was adopted for all picornaviruses (Rueckert and Wimmer, 1984). The symbol L refers to the leader region. The polyprotein is subsequently divided into three regions - the P1, P2 and P3 - which generate proteins 1A to 1D, 2A to 2C and 3A to 3D respectively (Fig. 1.1).

Poliovirus (type 1) translation starts 743 bases from the 5′ end and proceeds for approximately 7.5 kb. The first cleavage event occurs between the P1 and P2 regions while the polyprotein is still nascent on the ribosome. This cleavage is catalysed by the intramolecular action of the 2A

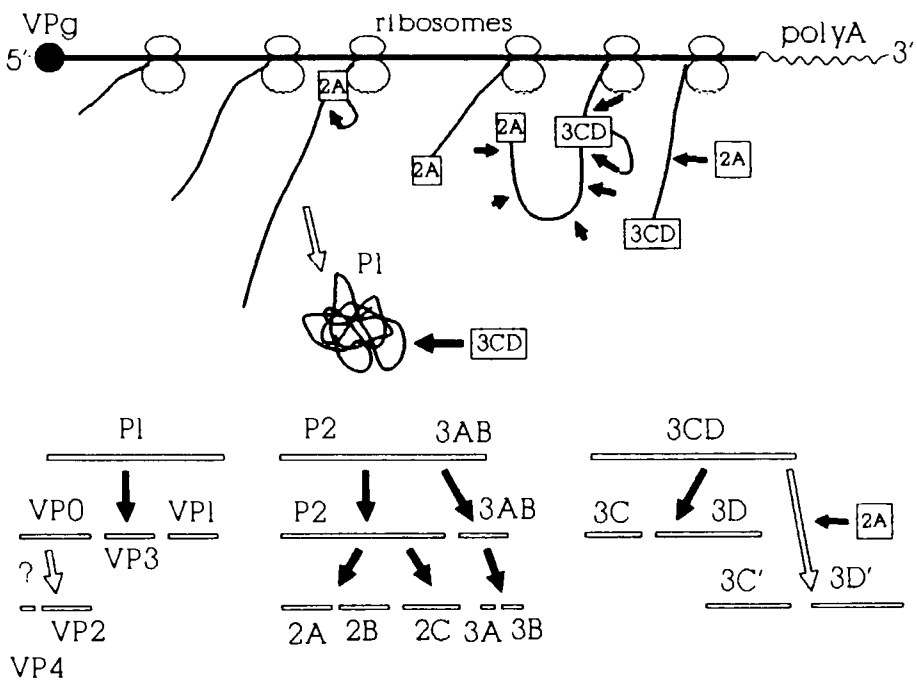


Figure 1.1
Model of proteolytic processing of the initial translation products of poliovirus by two virus-encoded proteases (adapted from Toyoda *et al.*, 1986).

protease ($2A^{pro}$) which cleaves between tyrosine and glycine amino acid residues (Toyoda *et al.*, 1986). Based on homology studies with other proteases, this protease is thought to belong to the thiol class of cysteine-type enzymes.

Most of the remaining cleavage events are catalysed by the 3C and/or 3CD protease which cleaves between the amino acid residue pair glutamine and glycine. This enzyme belongs to the thiol class of enzymes and has an affinity for its own polyprotein substrate, cleaving intramolecularly. This enzyme also functions intermolecularly (Arnold *et al.*, 1987).

The 3CD protein, *in vitro*, appears to be the active protease responsible for efficient cleavage of the P1 region into VP0, VP3 and VP1 (Jore *et al.*, 1988). The $3C^{pro}$ alone is sufficient for efficient cleavage of the P2 region into $2A^{pro}$, 2B and 2C (which have unassigned functions). The 3CD protein can be cleaved by both proteases; by $3C^{pro}$ into $3C^{pro}$ and 3D polymerase ($3D^{pol}$), or by $2A^{pro}$ to generate 3C′ and 3D′ proteins (Toyoda *et al.*, 1986). The 3B protein (VPg) is linked to the 5′ terminus during RNA synthesis ($3B^{VPg}$).

The final cleavage event occurs between the asparagine and serine residues in VP0, the VP2/VP4 precursor protein (Larsen *et al.*, 1982). Cleavage of VP0 is essential for the final maturation of the capsids and only occurs in the final stages of morphogenesis. In the mature capsids the VP2/VP4 cleavage sites are internal and are shielded from exogenous protease activity. Cleavage is thought to be an

autocatalytic event in a serine-protease type cleavage event. The cleavage reaction is unusual in that catalysis is thought to result from RNA-protein interaction where the RNA basic groups serve as proton abstractors (Arnold *et al.*, 1987).

Although the translation strategies of picornaviruses are basically the same, it should be noted that differences do exist. Only apthoviruses and cardioviruses translate a leader (L) peptide preceding the capsid precursor proteins. This L protein has no known function. The 3C proteases of different viruses have specificity for different amino acid pairs (Arnold *et al.*, 1987). The gene order location on the genome and size of the proteins translated is not identical between virus genomes; for example, FMDV 2A protein is either very small or absent, and FMDV RNA appears to code for three VPg's in tandem (Forss *et al.*, 1984).

1.3.5 INSECT PICORNAVIRUSES

Introduction

Most investigations of insect viruses have been performed on viruses pathogenic to insects that are of some importance to man. *Gonometa* virus and *Lymantria ninayi* virus were isolated from pests of the pine tree, *Pinus patula* (Harrap *et al.*, 1966; Pullin *et al.*, 1984). Kawino virus was isolated from mosquitos (Pudney *et al.*, 1978) which are vectors of serious tropical diseases such as malaria and yellow fever. Extensive investigations of

beneficial insects, like bees and silk worms, have revealed numerous viral pathogens.

Of the more than 20 RNA insect viruses that have been proposed as members of the *Picornaviridae*, only three have been accepted by the International Committee on Taxonomy of Viruses (ICTV) as picornaviruses of unassigned genera. These are *Gonometa* virus, cricket paralysis virus (CrPV) and *Drosophila* C virus (DCV) (Matthews, 1982). Subsequent studies indicate that other viruses, such as infectious flacherie virus (IFV), should be included in the *Picornaviridae* on the basis of similar physicochemical characteristics, RNA translation strategy and the presence of a VPg (Hashimoto and Kawase, 1983; Hashimoto *et al.*, 1984; Hashimoto *et al.*, 1986).

Virus Discovery and Incidence

CrPV was first isolated from laboratory populations of the field crickets *Teleogryllus oceanicus* and *T. commodus* (Reinganum *et al.*, 1970). Virus-induced symptoms included uncoordinated movements caused by an apparent paralysis of the hind legs. This paralysis was shortly followed by death. Symptoms were first observed in early instar nymphs, and CrPV was subsequently found to infect early-to-mid instar nymphs, with the crickets developing an apparent resistance as they approached adulthood. The virus has been detected in the following insects: in the Orthopterans, *Gryllus bimaculatus*, *Chortoicetes teminifera*, *Melanoplus bivittatus*, *Schistocera americana*; in the Lepidopterans, *Choristoneua fumiferana*, *Anteraea eucalypti*, *Galleria*

melonella, *Phalaeonoides glycine*, *Orgyia anartoides*, *Entometa apicalis*, *Clania ignobilis*, *Oenochroma rinana*, *Pieris brassicae*; in the Hymenoptera, *Apis cerana*; and in the Dipterans, *Drosophila melanogaster* and *Ceratitis capitata* (Reinganum, 1975; Plus and Scotti, 1984 and Roberts *et al.*, 1987; Anderson and Gibbs, 1988). CrPV, or serologically related viruses, are endemic in insect populations (Wigley and Scotti, 1983) and has a wide geographical distribution. It has been detected throughout Australia (Reinganum *et al.*, 1981), in New Zealand (Wigley and Scotti, 1983), Canada (Roberts *et al.*, 1987), the Canary Islands and the United States (Harrap and Berkeley respectively, pers. comm. in Scotti *et al.*, 1981). A virus physically similar and serologically related to CrPV, $CrPV_{ark}$, was isolated from *Pseudoplusia includens* (Chao and Young, 1986).

Neutralising antibodies to CrPV have been detected in numerous mammalian species (Scotti and Longworth, 1980; Moore *et al.*, 1981a, Moore *et al.*, 1987). These antibodies were of the IgM type and were thought to be due to passive exposure to low levels of CrPV or a related virus. There is no evidence that CrPV replicates in mammalian hosts.

Drosophila C virus (DCV) was first isolated and characterised by Jousset *et al.* (1977). Flies naturally infected with DCV had no apparent symptoms, injected virus however, was lethal to flies. DCV has been isolated from wild *Drosophila* populations from France (Vigier, Charolles and Gif; DCV_V, DCV_C and DCV_G respectively); Morocco (Ouarzazate, Taroudant, Marrakech and Zagora; DCV_O, DCV_T, DCV_M and DCV_Z respectively); the French Antilles (DCV_{AN});

Yugoslavia (DCV_Y); and Germany (DCV type B) (Plus *et al.*, 1978; Scotti *et al.*, 1981; Moore *et al.*, 1982; Pullin *et al.*,1982).

Gonometa virus was isolated from *Gonometa podocarpi*, a serious pest of *Pinus patula*. Infected larvae become limp and flaccid and hang from the food plant by one or more pairs of their prolegs. Reports of *Gonometa* virus are restricted to isolations made from *G. podocarpi* collected in Uganda (Harrap *et al.*, 1966; Longworth *et al.*, 1973).

Many of the insect viruses identified have not been characterised in any detail and cannot be placed with certainty in the picornavirus group. These viruses have been listed under the *Picornaviridae* as "unclassified small RNA viruses of invertebrates" (Matthews, 1982). They include acute bee paralysis virus (APV), bee slow paralysis, bee virus X, sacbrood virus (for review, see Bailey, 1976) and *Drosophila* viruses P and A (Teninges and Plus, 1972; David and Plus 1971; Plus *et al.*, 1976).

Other potential members of this picornavirus group are infectious flacherie virus (IFV) isolated from the silk worm, *Bombyx mori* (Hashimoto and Kawase, 1983); *R. padi* virus (RhPV) isolated from aphids (D'Arcy *et al.*, 1981a; von Wechmar and Rybicki, 1981; Rybicki and von Wechmar, 1982a); Kawino virus isolated from the mosquito *Mansonia uniformis* (Pudney *et al.*, 1978); and *Lymantria ninayi* virus isolated from the Tussock moth larvae (Pullin *et al.*, 1984).

Viruses of bees are numerous; including black queen cell virus (BQCV), Berkeley bee virus (BBV), Egypt bee virus, acute paralysis virus, at least two isolates of

sacbrood virus (SBV), and at least five isolates of Kashmir bee virus (KBV) (reviewed by Bailey, 1976; Lommel *et al.*, 1985). Most of the bee viruses have a restricted host range and have not been detected in insect species belonging to any other order. Inapparent infections of bee communities are common (Dall, 1985; Anderson and Gibbs, 1988), and have been known on occasion to be stimulated to virulence resulting in epizootics and collapse of the population (Dall, 1985).

General Characterisation

Most of the insect viruses tentatively classified as picornaviruses have been assigned to this group on the basis of the physicochemical properties of the virions. (Table 1.2). Although similarities between these viruses and the enteroviruses have been drawn, classification on the genus level is premature. All these viruses are approximately 30 nm in size and most have three proteins, approximately 30 K in size. The density in CsCl of these insect picorna-like viruses ranges from 1.32 to 1.38 g/ml and sedimentation coefficients range from 153 to 183 S. CrPV has an RNA size of 8.5 kb (King *et al.*, 1987) and the reported value of $CrPV_{ark}$ RNA is 9.7 kb, which is significantly larger than the average picornavirus genome (Chao and Young, 1986). However, most of the RNA sizes of these insect viruses have not been accurately assessed by denaturing gel electrophoresis. An RNA poly(A) tract has been detected on DCV, CrPV, $CrPV_{ark}$, *Lymantria ninayi*, and Kawino virus genomes (see Table 1.2). CrPV, DCV and IFV RNAs all have

Table 1.2. A comparison of the properties of insect picorna- and picorna-like viruses*.

Virus#	Diameter (nm)	RNA M_r x 10^{-6}	Capsid Protein M_r x 10^{-3}	Buoyant Density (g/ml)	$S_{20.w}$
DCV	27	3	(37)x,31,30,9	1.34	153
Gonometa	32	ND	(48),37,32,29,12	1.35	180
CrPV	27	2.9	(43),33,30,31	1.3	167
$CrPV_{ARK}$	25	3.3	(38),34,31,30	1.37	178
IFV	26	2.4	(44),35,33,31,12	1.38	183
DPV	ND	ND	48,29,26	1.36	ND
DAV	ND	ND	73,41,32	1.37	ND
RhPV	28	2.6	32,30,28	1.37	165
Lymantria ninyi	29	2.8	(43),38,33,32	1.32	ND
Kawino	28	2.6	33,30,27,7	1.33	165
BBPV	ND	2.8	37,35,33	ND	165
APV	28	ND	32,24	1.34	160
SBV	28	2.6	32,28,25	1.33	159
Thai SBV	30	2.8	39,34,30	1.35	160
Egypt Bee	30	ND	41,30,35	1.37	165
Kashmir Bee	30	ND	41,37,25	1.37	167-170
BQCV	30	ND	30	1.34	153
SPV	30	ND	46,29,27	1.37	173-178

*Jousset *et al.*, 1977; Chao and Young, (1986); Hashimoto and Kawase, (1983), Rybicki and von Wechmar, (1982a); Pullin *et al.*, (1984); Bailey and Woods, (1977); Lommel *et al.*, (1985); Bailey *et al.*, 1979 Moore *et al.*, (1985); King *et al.*, (1987); Longworth *et al.*, (1973); Plus *et al.*, (1976); Pudney *et al.*, (1985); Chao and Young, (1986).
#*Drosophila* C virus (DCV); cricket paralysis virus (CrPV); infectiou flacherie virus (IFV); Drosophila P and A viruses (DPV and DAV respectively); *R. padi* virus (RhPV); Berkley bee picornavirus (BBPV) bee acute paralysis virus (APV); sacbrood virus (SBV); black queen-cell virus (BQCV); bee slow paralysis virus (SPV).
xMinor amounts present presumed to be equivalent to VP0 of mammalian picornaviruses.

genome linked proteins (VPgs) as determined by ^{125}I labelling of the genomes (King *et al.*, 1986; Hashimoto *et al.*, 1986). For all these viruses, a protein co-purified with the RNAs, which was degraded by proteinase K and was resistant to RNAse. The M_r values of these proteins are 3.9×10^3 for CrPV and DCV and 11.5×10^3 for IFV. As the size of IFV VPg was estimated on 8.75% and 10% polyacrylamide gels using M_r markers greater than 14×10^3, this size is probably an overestimation. IFV putative VPg was serologically unrelated to the capsid proteins and was not required for efficient RNA translation *in vitro*.

Relationship Between Viruses

The majority of insect viruses are apparently serologically unrelated. Tests done by agar gel double diffusion and by immuno-osmophoresis showed that CrPV and DCV were serologically related (Plus *et al.*, 1978; Reinganum and Scotti, 1976), but were not related to termite paralysis virus, acute bee paralysis virus, *Drosophila* P virus, *Gonometa* virus or sacbrood virus (Reinganum and Scotti, 1976). No serological relationship was detected between termite paralysis virus, acute bee paralysis virus, *Drosophila* P virus, *Gonometa* virus or sacbrood virus (Reinganum and Scotti, 1976).

Comparison of CrPV and DCV at the nucleic acid level showed no similarities in oligonucleotide maps of their respective genomes (Pullin *et al.*, 1982) and no significant nucleic acid homology was detected by *in vitro* hybridization using cDNA (King *et al.*, 1984).

CrPV was shown to be distantly serologically related to EMCV, a mammalian cardiovirus, and was found to enhance the immune response in guinea pigs to EMCV when injected prior to, or after inoculation of EMCV (Tinsley *et al.*, 1984). This serological relationship could explaing the 'CrPV' antibodies in mammals (Scotti and Longworth, 1980).

Most of the CrPV genome has been cloned and a 1 600 bp 3' terminus nucleotide sequence has been determined (King *et al.*, 1987). The restriction enzyme map of overlapping clones comprising 7.5 kb of the 8.5 kb genome, shows no conservation of restriction enzyme sites with poliovirus and EMCV virus. No sequence homology or predicted amino acid sequence homology was detected between the 3'end of CrPV and the corresponding regions of poliovirus, FMDV or EMCV. In mammalian picornaviruses this region codes for the viral RNA polymerase, and would be expected to be conserved (King *et al.*, 1987). One conserved region, postulated to be the RNA polymerase binding site (Argos *et al.*, 1984) was, however, detected. This consisted of a Gly-Asp-Asp triplet (GDD) surrounded by hydrophobic amino acids and contained several conserved amino acids or amino acid types 20-30 bases upstream (King *et al.*, 1987).

Site of Virus Multiplication and Associated Pathology

The pathology associated with picorna- and picorna-like viruses of insects has not been extensively studied. The most detailed work has been done on sacbrood virus (SBV) and Kashmir bee virus (KBV) infections of the honey bee, *Apis mellifera* (Mussen and Furgala, 1977; Dall, 1987).

As with mammalian picornaviruses, virus multiplication of insect picorna- and picorna-like viruses appears to occur exclusively in the cytoplasm. Infections of honey bee larvae with KBV resulted in the induction of membrane-bound structures in which virions accumulated (Dall, 1987). These vesicular structures appeared to be intimately involved with virus replication and have been observed in other insect virus-infected cells, including *Gonometa* virus infections of *Gonometa podocarpi* (Longworth *et al.*, 1973), and $CrPV_{ark}$ infections of *Pseudoplusia includens* (Chao and Young, 1986). A common pathological response observed in these virus infected cells was the accumulation of granular, electron-dense inclusions in the cytoplasm.

Vesicles containing KBV were observed in the epithelial tissue of the foregut and hindgut, in the alimentary canal musculature, epidermis, tracheal epithelium, haemocytes, oenocytes and tracheal end cells. No evidence of multiplication in the tissue of the nervous system was obtained although degeneration of glial cells was apparent. Cytoplasmic organelles in virus-infected cells lost their integrity, and condensation of the chromatin and sloughing of tubules of the cytoplasm was observed. Although infections were accompanied by a significant rise in haemolymph osmolarity, this alone was not the cause of degeneration of the glial cells (Dall, 1987).

Larvae of the honey bee are more susceptible to SBV infection than adult bees. This was reflected in the pathology of virus-infected cells. While adult cells had minimal damage, severe cytopathic effects were observed in

larval cells where total disruption of the cytoplasm was frequently observed (Mussen and Furgala, 1977). In larvae, SBV appeared to have accumulated in the gut lumen adjacent to the peritropic membrane. Particles were also observed in the perineurium, glial cells, neurons and axons of larval nervous tissue and in the haemocytes. In adults, SBV was observed in the tracheal epithelial cells of the air sacs around the brain, and in the hypopharyngeal glands, as well as in the abdominal ganglia.

CrPV particles were observed in crystalline arrays and also as scattered particles in large numbers in the epidermal cells. Particles were also observed in the alimentary canal and in nerve ganglia cells (Reinganum *et al.*, 1970).

Virus-like particles (VLPs) have been observed in the cytoplasm and nucleus of *Drosophila* tissue and *Drosophila* cell lines. Particles were observed in the cells from the midgut, trachea, connective tissue, paragonia and nerve cells (Rae and Green, 1967), in brain tumors (Akai *et al.*, 1967), fat body cells, oenocytes, melenotic masses, central nervous tissue (Perotti and Bairati, 1968; Philpott *et al.*, 1969), gut cells and larval imaginal discs (Wehman and Brager, 1971). The identity of these particles was not established. DCV has been detected in tracheal cells and cells surrounding the ganglia in *Drosophila melanogaster*, and in the cytoplasm of cells surrounding the intestine and in the Malpighian tubules of *Certitis capitata* (Scotti *et al.*, 1981). *Drosophila* P virus appears to accumulate in the ovaries and Malpighian tubules in *Drosophila melanogaster*

and particles were also observed in male accessory glands (Teninges and Plus, 1972).

Gonometa virus accumulated in columnar cells, and infections were associated with degeneration of the mitochondria and cell membrane. Particles were also observed in goblet cells of the midgut (Longworth *et al.*, 1973).

$CrPV_{ark}$ was found in most tissues studied including the epidermal cells, haemocytes, fat body and midgut epithelial cells. The extent of associated cell damage was dependent on the cell type; epithelium cells showed no signs of degeneration, whereas fat body cells contained disrupted organelles and/or disorganized cytoplasm. Infected cells had an increased number of microtubules and fibril-containing vesicles (Chao and Young, 1986).

Although VLPs have been identified in numerous aphid species, the only ones so far identified are plant rhabdoviruses, luteoviruses (Gildow and Rochow, 1980) and a putative plant picornavirus (Murant *et al.*, 1976). VLPs were observed in crystalline array in the cytoplasm of infected cells of *Rhopalosiphum maidis* (Parrish and Briggs, 1966), and were also observed in the nerve ganglia, salivary gland cells and in midgut cells of *Myzus persicae* (Kitajima, 1976).

Virus Replication

CrPV and DCV have been successfully introduced into tissue culture and have adapted sufficiently to replicate to high titres in *Drosophila* cells (Scotti, 1976; Moore *et al.*, 1981b,c; Moore and Pullin, 1982). CrPV-infected cells show distinct cytopathic effects (CPE), and infections result in

the efficient shut down of host protein synthesis (Scotti, 1977; Moore *et al.*, 1980). In contrast, DCV infections do not completely shut down the synthesis of cellular proteins and infections do not cause distinct CPE (Moore *et al.*, 1981b; Moore *et al.*, 1981d). In addition to virion RNA, a high M_r double-stranded (ds) RNA, presumably the replicative form, was detected in CrPV-infected cells (Eaton and Steacie, 1980). There is no evidence of intracellular sub-genomic RNA in DCV or CrPV infected cells (Reavy *et al.*, 1983a; for a review on DCV and CrPV replication see Moore *et al.*, 1985 and Moore *et al.*, 1987).

CrPV infection of cells in culture results in the production of approximately 20 virus-induced polypeptides (Moore *et al.*, 1980). The major proteins detected in cells are the structural proteins VP1 and VP3 (picornavirus equivalents) and a putative VP0. By four hours after infection, minor amounts of high molecular weight proteins (M_r 144 000, 125 000, 115 000; proteins A, B and C) and proteins ranging in M_r between 63 000 and 50 000 were found. These proteins were chased into lower M_r proteins and, in addition to VP0, were immunoprecipitated with antiserum to mature capsids, indicating that they were capsid protein precursors (Moore *et al.*, 1980; Moore *et al.*, 1981c). VP2 was only apparent after extended incubation times suggesting that it is produced by a different processing event to VP1 and VP3 which are processed extremely rapidly. All three proteins are present in roughly equimolar amounts in mature virions (Moore *et al.*, 1980, Moore *et al.*, 1981c). There is no evidence of a VP4 in purified or partially purified

virions. Although a 12 K protein was detected in infected cells using ^{35}S-methionine; no low molecular weight protein was identified using ^{14}C-labelled amino acids (Moore *et al.*, 1980; Moore *et al.*, 1981c).

Pre-treatment of cells with iodoacetamide before virus infections resulted in the appearance of two additional proteins with M_r values of 205 000 and 190 000. These proteins could be equivalent to the mammalian picornavirus polyprotein. A group of polypeptides with an M_r range between 63 000 and 50 000 were also detected. The 124 K and 115 K proteins were not detected in cells pre-treated with iodoacetamide (Moore *et al.*, 1981c).

Elevation of the temperature of *Drosophila* cells from 28^0C to 37^0C resulted in a reduction in the number of cellular proteins synthesized. In CrPV-infected heat shocked cells, there were no significant levels of cellular proteins synthesized (Moore *et al.*, 1981d). This suppression of protein synthesis occurs, at least in part, at the translational level, as poly(A) RNA extracted from heat-shocked infected cells was efficiently translated *in vitro* (Reavy *et al.*, 1983b). Heat-shocked infected cells also resulted in the production of proteins of approximate M_r of 200 000, indicating that processing is, to a certain extent, heat sensitive. Cleavage of precursor proteins was inhibited by N-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which are inhibitors of trypsin and chymotrypsin proteases respectively (Moore *et al.*, 1981e). Canavanine, a arginine

analogue, also reduced the efficiency of proteolytic cleavage.

The major DCV-induced polypeptides in *Drosophila* cells, as with CrPV, were the capsid proteins which appeared to be produced in supramolar amounts (Moore *et al.*, 1981b). The structural proteins, VP1 and VP3, were produced rapidly, while VP2 and the lowest M_r protein, VP4, only becoming evident after prolonged incubation in the presence of an excess unlabelled amino acids. Eight proteins were detected with M_r values higher than the structural proteins, the largest protein being 146 K. Treatment of infected cells with iodoacetamide prior to pulsing, resulted in the appearance of a protein approximately 200 K in size (Moore *et al.*, 1981b). A comparison of eight different DCV isolates from France, Morocco and the French Antilles showed that although the isolates produced different virus-induced polypeptides, their peptide maps were similar (Moore *et al.*, 1982).

As DCV infections, even in combination with actinomycin D, did not efficiently shut off host protein synthesis, the reduction of cellular protein synthesis after heat shock was particularly useful in the analysis of DCV-induced polypeptides (Moore *et al.*, 1981d; Moore and Pullin, 1983). The high molecular weight DCV proteins produced by elevated temperature could be chased into lower molecular weight proteins by the addition of an excess unlabelled amino acids and a temperature shift to 28^0C. As with CrPV, the addition of canavanine and/or TLCK reduced the efficiency of processing. A combination of TLCK, canavanine and elevated

temperature stopped the appearance of virus structural proteins and VP0, indicating that, at least at elevated temperatures, the structural proteins are a product of proteolytic processing and not of premature termination of translation, nor of illegitimate internal initiation (Moore and Pullin, 1983).

Both CrPV and DCV RNAs were efficiently translated *in vitro* in rabbit reticulocyte lysate into high molecular weight products (Reavy and Moore, 1981b; Reavy and Moore, 1983b). The translation products were different to those produced *in vivo*; the high molecular weight polypeptides predominated, with relatively lower amounts of capsid proteins being produced and there was no evidence of the production of a VP0. Analysis of translation products in a time-course experiment showed that the high molecular weight proteins were processed, and that this processing could be diluted out with untreated lysate. This indicated that at least some of the proteolysis was due to a virally-encoded protease (Reavy and Moore, 1983b). Processing of CrPV capsid protein precursors was sensitive to leupeptin, indicating that the CrPV protease was possibly a serine protease. Supplementation of the CrPV translation mix with *Drosophila* cell extract resulted in more efficient production of the capsid proteins indicating that, unlike picornaviruses, a cellular protease may play a role in post-translational cleavage (Reavy and Moore, 1983b). In a series of dilution experiments the CrPV protease was shown to process some of the DCV precursor proteins and *vice versa* (Reavy and Moore, 1983b).

Both CrPV and DCV virus genomes function as monocistronic messengers *in vitro* and *in vivo*. The excess of structural proteins relative to non-structural proteins could be due to rapid processing of precursor proteins. It has been suggested that the accumulation of capsid proteins, in supramolar excess over other viral proteins, is a result of a "pause" in protein synthesis after the ribosomes have traversed the coat protein coding region. Translation may then terminate at this point with occasional read through occurring (Moore *et al.*, 1985). Shih *et al.* (1979) suggested a similar mechanism in the translation of EMCV proteins.

The only other insect virus to be studied, in any detail, at the level of translation is IFV. IFV RNA was efficiently translated in both rabbit reticulocyte lysate and wheat germ extract into high molecular weight proteins, the sum of which exceeded the coding capacity of the genome. Two of these proteins, a 200 K and 130 K protein, were immunoprecipitated with antiserum to mature virions indicating that they are capsid protein precursors (Hashimoto *et al.*, 1984).

Genome Organization

The genomic organization of CrPV was mapped using pactamycin (Reavy and Moore, 1983c). Pactamycin is an initiation inhibitor, therefore the amount of protein produced after addition of pactamycin will be proportional to the distance of the coding region from the initiation site. Using this method, CrPV VP0-VP1-VP3 were shown to be

encoded by the 5′ end of the genome and proteins A, B and C were coded by the 3′ end. The 1.6 kb sequence of the 3′ end of CrPV contained only one open reading frame and a 242 base pair non-coding region preceding the 3′ poly(A) tract (King *et al.*, 1987). Based on its position on the genome as compared to other picornaviruses, as well as the presence of a putative polymerase binding site, this region is postulated to code for CrPV polymerase (King *et al.*, 1987).

1.3.6 APHID BIOLOGY

Aphids are small (1-10mm), soft-bodied insects that feed on phloem sap. There are more than 4 000 described aphid species, most of these occurring in the temperate regions of the world. Approximately 59% of these species belong to the subfamily Aphidinae, of which *R. padi* (Linnaeus) is a member (Dixon, 1985). *R. padi* (Fig. 1.2) belongs to the tribe Aphidini, subfamily Aphidinae, family Aphididae, superfamily Aphidoidea and order Homoptera (Eastop, 1977). *R. padi* aphids are usually dark green in colour tinged with orange between the siphunculi (Miyazaki, 1987).

In nature most aphids reproduce sexually and asexually. The sexual cycle is important, not only to ensure genetic diversity, but also to increase the chances of survival into the next season by the production of cold-resistant eggs. The sexual cycle is usually interrupted by several generations of parthenogenetic reproduction in which

Figure 1.2
Rhopalosiphum padi aphid

fertilization is not required, and aphids produce live nymphs. Approximately 3% of all aphid species are totally parthenogenetic (Dixon, 1987b). Sexual reproduction is triggered off by internal and/or external cues. Under constant laboratory conditions, aphids usually only reproduce by parthenogenesis.

During the asexual cycle, the embryo developing within the parthenogenetic female may also contain an embryo developing within itself (Dixon, 1985). The ability to reproduce in this manner enables rapid colonization of an area and is the major reason why aphids are such serious pests. Although aphids become numerous extremely quickly, they are also very vulnerable to environmental stresses such as temperature, rainfall and wind, and to predators, parasites and pathogens.

Most aphids live on one or a few species of a particular plant genus (autoecious). Approximately 10% of aphid species alternate between primary host plants on which the overwintering egg is laid, and secondary hosts on which the parthenogenetic stages occur (heteroecious) (Dixon, 1987a). In South Africa the following aphids are commonly found on graminaceous host plants: *Diuraphis noxia* (Mordvilko), *Metapolophium dirhodum* (Walker), *R. padi* (L.), *Rhopalosiphum rufiabdominalis* (Sasaki), *Schizaphis graminum* (Rondani) and *Sitobion avenae* (Fitch) (Durr, 1983). In South Africa, *R. padi* is a common pest of oats, barley, wheat and maize and may be anholocyclic (i.e. does not go through a sexual cycle but overwinters viviparously) on

these hosts (von Wechmar, Department of Microbiology, University of Cape Town, South Africa, pers. comm.).

Plants and aphids may be of mutual benefit. Aphids, in order to reduce the osmotic stress due to the difference between the osmotic concentration of aphid haemolymph and the phloem sap they ingest, convert mono- and disaccharides into trisaccharides, the major trisaccharide being melezitose (Klinghauf, 1987; Dixon, 1985). This is excreted as honeydew which drops onto the ground and stimulates the growth of nitrogen-fixing bacteria, which in turn supply nitrogen to the plants (Dixon, 1985).

Nevertheless, aphids are far more a liability to plants than they are an asset. Serious crop damage occurs due to the spread of viral diseases by aphids, which are the most common vectors of plant viral disease (Dixon, 1985). Heavy aphid infestations also cause a reduction in plant growth due to nutrient drain. The saliva of some aphids may also be toxic to the host plant, resulting in galls, crumpled leaves and discoloration. The compounded effect of aphid feeding and plant virus infections causes a reduction in plant growth and reproductive potential of crop plants, ultimately resulting in reduced yields and poor seed quality.

The aphid problem has been tackled at several levels. Chemical insecticides used are mainly synthetic organic substances. The use of these insecticides has several drawbacks. Their broad range of activity results in elimination of beneficial insects such as predators, which play an important role in controlling insect populations

(Chambers *et al.*, 1986; Griffiths *et al.*, 1985). The chemicals often persist in nature for long periods of time, preventing recolonization of the affected area by predators. In addition they have deleterious effects on organisms higher up the food-chain. There is also the problem of the increasing number of insects developing insecticide resistance. Physical deterrents used to control aphids include reflective surfaces which repel aphids, and yellow sticky traps that attract aphids (Harpaz, 1982). Biological control of aphids by predators, parasites or pathogens is inherent in nature. Aphids have a large number of natural enemies, and are preyed on by birds, bats, reptiles, spiders, and many insects. They are also vulnerable to protozoal, bacterial, fungal and viral infections (Harpaz, 1982; Hall, 1976; Griffiths *et al.*, 1985 and Chambers *et al.*, 1986).

1.3.7 **VIRUSES FOR BIOLOGICAL CONTROL OF INSECTS**

There are over a million different insect species, only a limited number of which have been investigated for viruses. The potential pool of naturally-occurring pathogenic viruses is, therefore, enormous. Many of these viruses are endemic to insect populations, often causing chronic infections which are occasionally stimulated to virulence (Podgwaite and Mazzone, 1986).

Of the ten virus families known to infect invertebrates (Table 1.1), the baculoviruses have been the most exploited as microbial pesticides. However, any of these viral pathogens are potential candidates as viral pesticides. Of

the picorna-like viruses, many exist in nature as inapparent infections and do not have a noticeable effect on insect populations. *Drosophila* P virus was isolated after extracts of apparently healthy aphids were injected into "healthy" flies (Teninges and Plus, 1972). There are several reports on inapparent infections of the bee viruses including sacbrood virus, Kashmir bee virus (Dall, 1985), acute bee paralysis virus (Bailey *et al.* 1963) and Black queen cell virus (Anderson and Gibbs, 1988).

Some of the small RNA viruses have severe effects on insect populations. CrPV was isolated from the Australian field cricket (*Teleogryllus oceanicus*) after 95% of laboratory-propagated crickets died (Reinganum *et al.*, 1970) and frequent epizootics of bee virus have been reported which have resulted in marked declines of bee populations (Dall, 1985). Little detailed information is available on the effect of picorna-like viruses on insect populations. Often biological observations are consequential to other investigations and are not done in a detailed or orderly manner. However, preliminary investigations on CrPV have shown that it may have potential as a field control agent of the olive fruit fly *Dacus-oleae Gmel*; CrPV was shown to kill 80% of the flies within 12 days of exposure to the virus (Manousis and Moore, 1987).

Reservations have been expressed about using insect picornavirus pesticides because of their similarities to viruses that infect vertebrates (Flexner *et al.*, 1986), especially with the discovery that CrPV is antigenically related to EMCV (mammalian picornavirus) (Tinsley *et al.*,

1984) and IgM antibodies to CrPV and *Gonometa* virus were detected in mammalian sera (Longworth *et al.*, 1973; and Scotti and Longworth, 1980). A similar immunological response to granulosis virus (GV) of the codling moth (*Cydia pomonella*) was detected in wood mice (*Apodemus sylvaticus*) (Bailey and Fujita, 1987) - GV has been extensively investigated and found not to infect non-target organisms (Summers *et al.*, 1975). The presence of virus-specific antibodies in both cases was thought to be due to passive exposure and not to replication. The fears of utilizing insect picornaviruses for insect control are probably unfounded as there is no evidence to indicate that CrPV replicates in vertebrate hosts or in mammalian cell lines (Scotti and Longworth, 1980). Also, no conservation of 3' terminal sequences or of genome restriction enzyme endonuclease map were observed between CrPV and other picornaviruses (King *et al.*,1987), indicating that CrPV is only very distantly related to these viruses.

Several viruses, all of them baculoviruses, have been registered by the United States Environmental Protection Agency (EPA). Prior to commercial application of viral pesticides, the safety of these viruses has to be thoroughly checked to ensure that they are harmless to non-target organisms, harmful to target organisms, do not damage the environment and can be economically viable (Flexner *et al.*, 1986).

The numerous problems encountered using these viral insecticides have resulted in their limited application. These viruses often infect only a certain stage of insect

development, therefore the timing of application has to be precise for successful control. They are also very host-specific and therefore do not kill more than one insect pest. The virus dose is often very important for effective control thus large amounts need to be applied. Stability is often a major problem with environmental factors such as UV irradiation, temperature and pH of the plant surface being detrimental to survival. In comparison to chemical pesticides which result in an almost instantaneous elimination of insect pests, virus infected insects die more slowly and may only result in a reduction in insect numbers (Falcon, 1985).

Many of these problems could be overcome. Using recombinant DNA technology it may be possible to extend the host range of insect viruses, increase virulence and increase virion stability (Faulkner and Boucias, 1985). Much research is necessary on the physical, genetic and biological properties of insect viruses in general - and picornaviruses in particular - before their potential as pest control agents can be realised.

CHAPTER 2

CHARACTERISATION OF *RHOPALOSIPHUM PADI* VIRUS

SUMMARY

A South African isolate of *Rhopalosiphum padi* virus (RhPV) was directly compared with an isolate from the United States of America. A difference in molecular weights was found between their respective VP3 capsid proteins, and particles had different buoyant densities indicating that the two isolates are distinct viruses. The South African isolate was further characterised; two additional putative capsid proteins were found and a 10 kb RNA molecule with an RNA poly(A) tract was detected. Virions were found to be acid-stable and heat-labile. Double-stranded RhPV-specific RNA was isolated from infected aphids and characterised. RhPV cDNA was synthesized from this dsRNA and cloned into the plasmid vector pBR322. Plasmids containing RhPV sequences were identified by dot-blot hybridization and a clone containing a 1.5 kb insert was characterised by restriction enzyme mapping.

CHARACTERISATION OF *RHOPALOSIPHUM PADI* VIRUS

2.1 INTRODUCTION

Although isometric, virus-like particles have been observed in several aphid species (Parrish and Briggs, 1966; Peters, 1967; Kitajima, 1976), only a few aphid viruses have been isolated and even partially characterised. Fraval and Lapierre (1970) described an isometric virus from *R. padi* (L.), and Allen and Ball (1986) have reported the partial characterisation of a small isometric virus from the aphid *Sitobion avenae*. *R. padi* virus (RhPV) is the only aphid virus to be characterised in any detail (D'Arcy *et al.*, 1981a; Rybicki and von Wechmar, 1982a; Rybicki, 1984). RhPV has been isolated from two geographically separate areas. D'Arcy *et al.* (1981a) described a virus of *R. padi* aphids collected in Illinois, USA; and in an independent study in South Africa, von Wechmar and Rybicki (1981) isolated a similar virus from *R. padi* and *Diuraphis noxia* aphids. Recently, a similar virus was described by Eweida and Oxelfelt (1985) in Sweden. In all the reported incidences, RhPV was found during investigations of barley yellow dwarf virus (BYDV). RhPV has been detected in the aphids *R. padi*, *Rhopalosiphum rufiabdominalis*, *Schizaphis graminum* (D'Arcy *et al.* 1981b), *D. noxia* (von Wechmar and Rybicki, 1981), *R. maidis* and *Acyrthosiphum dirrhodum* (Rybicki, 1984). It has been proposed that RhPV may influence the efficiency of BYDV transmission (D'Arcy *et al.*, 1981a; Rybicki and von Wechmar, 1982a), however, subsequent investigations showed this not to be the case (Gildow and D'Arcy, 1988).

This chapter reports a direct comparison of the physical properties of the Illinois isolate of RHPV ($RhPV_{ILL}$) (D'Arcy *et al.*, 1981a) and the Orange Free State (OFS, South Africa) isolate ($RhPV_{OFS}$) (von Wechmar and Rybicki, 1981). The $RhPV_{OFS}$ isolate was investigated in more detail and additional properties are reported. RhPV double-stranded (ds) RNA was isolated, characterised and DNA sequences complementary to both strands of this dsRNA were synthesized and cloned into the plasmid vector pBR322.

2.2 MATERIALS AND METHODS

2.2.1 Viruses

$RhPV_{OFS}$ originated from aphids collected from the Bethlehem region of the OFS (von Wechmar and Rybicki, 1981; Appendix C.2). Purified $RhPV_{ILL}$ was obtained from C.J. D'Arcy (Dept. of Plant Pathology, University of Illinois, USA). Cricket paralysis virus (CrPV) originated from the NERC Institute of Virology, Oxford, and was propagated and purified in this laboratory by K. Struthers (Department of Community Health, University of the Witswatersrand, S. Africa). Ornithogalum mosaic virus (OMV), a potyvirus, was obtained from J.T. Burger (Department of Microbiology, University of Cape Town, South Africa). Tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV) were propagated in tobacco plants (*Nicotiana tabacum* cv. Soulouk).

2.2.2 Virus Propagation and Purification

$RhPV_{OFS}$ was propagated in the aphid, *Rhopalosiphum padi* (L.), reared on barley (*Hordeum vulgare* (L.) cv. Clipper). Virus was purified from plants bearing virus-infected aphids or from aphids alone (Appendices B.1 and B.3). Approximate virus yields were 0.4 mg/kg of plant material or 0.10 mg/g of aphids. Greater yields were obtained when virus was purified from plants with aphids compared with purification from aphids separated from the same number of plants, hence the former method was employed routinely.

2.2.3 Isopycnic Density Gradient Centrifugation

Buoyant densities of $RhPV_{OFS}$ and $RhPV_{ILL}$ were determined on caesium chloride gradients (Appendix B.4) and fractions containing low virus concentrations were screened by DAS-ELISA (Appendix B.14).

2.2.4 Single-Stranded RNA Isolation

RNA was extracted from virions of $RhPV_{OFS}$ by SDS disruption and phenol/chloroform extraction (Appendix B.8).

2.2.5 Double-Stranded RNA isolation

Double-stranded (ds) RNA was extracted as described by Valverde *et al.* (1986). One to 100 g of fresh or frozen aphids or plant material was ground to a fine powder in liquid N_2. To 4 g of material extracted, 14 ml of double strength STE (50 mM Tris, 0.1 mM EDTA, 100 mM NaCl, pH 8.0), 2 ml 10% (w/v) SDS, 18 ml STE-saturated phenol and 16 mg bentonite were added and the mixture was shaken vigorously

for 30 min. The phases were separated by centrifugation (16 000 x *g*). The upper aqueous phase was removed and adjusted to 16.5% (v/v) ethanol and the dsRNA purified by cellulose (Whatman CF-11) affinity chromatography. A 50 ml syringe column was prepared containing 2.5 g cellulose suspended in STE containing 16.5% (v/v) ethanol (16.5% EtOH-STE). The sample was applied to the column which was then washed with 3 X 30 ml of 16.5% EtOH-STE. The dsRNA was eluted from the column with STE, the sample was adjusted to 16.5% (v/v) ethanol and the column cycle repeated. The dsRNA was concentrated by ethanol precipitation (Appendix B.8) and analysed by non-denaturing gel electrophoresis in 6% polyacrylamide gels or 0.8% agarose gels (Appendix B.10)

Where necessary the dsRNA was further purified by precipitation with LiCl. The RNA was adjusted to a final concentration of 2 M LiCl and incubated for 16 h at 0^0C. The contaminating single-stranded RNA was removed by centrifugation (15 min in an Eppendorf centrifuge) and the LiCl was removed by dialysis against water (0.025 μm pore size dialysis filters, Millipore).

2.2.6 **RNA Molecular Weight Estimations**

The molecular weights of $RhPV_{OFS}$ single-stranded RNA were estimated by electrophoresis in formaldehyde or glyoxal denaturing agarose gels (0.8 - 1.0%) (Appendix B.10). Double-stranded RNA molecular weights were estimated by electrophoresis in non-denaturing agarose gels (0.8 -1.0%) (Appendix B.10).

2.2.7 Oligo(dT)-Cellulose Chromatography

Oligo(dT)-Cellulose chromatography was performed as recommended by the manufacturers (Collaborative Research, Waltham, Mass., USA.).

The oligo(dT) cellulose was suspended in elution buffer (0.01 M Tris.HCl pH 7.5; 0.05% w/v SDS; 1 mM EDTA) and poured into a 1 ml syringe column. The column was washed with 10 x bed volume of high salt concentration binding buffer (0.5 M NaCl; 0.01 M Tris.HCl pH 7.5; 0.5% w/v SDS; 1 mM EDTA). The RNA, in binding buffer, was applied to the column and 300 μl fractions were collected. The column was thoroughly rinsed with binding buffer (approximately 12 fractions) before the addition of low salt concentration elution buffer. The A_{260} of the fractions was determined by UV spectrophotometry.

2.2.8 Protein Iodination

Samples were labelled immediately after purification and electrophoresed the same day to prevent decomposition of the proteins by radiation (Lonberg-Holm and Butterworth, 1976).

Iodination using lactoperoxidase

Lactoperoxidase was immobilised on CNBr-activated Sepharose 4B as specified by the manufacturers (Pharmacia Fine Chemicals). To 100 μl of virus suspension (0.1 to 0.5 mg/ml), 5 μl H_2O_2, 10 μl immobilised lactoperoxidase (approx. 100 μg/ml) and 5 μl ^{125}I (4 mCi/ml) (Amersham) were added. After 1 h agitation at 4^0C the Sepharose-

lactoperoxidase was removed by centrifugation (1 min in a Eppendorf centrifuge) and the free ^{125}I was removed by dialysis against 0.1 M phosphate buffer, pH 7.0 or by Sephadex G25 column chromatography.

Iodination with Chloramine-T

To 50 μl of virus suspension (0.1 to 0.5 mg/ml) were added 3 μl of ^{125}I (4 μCi/μl) and one Iodobead (chloramine-T derivatised polystyrene bead; Pierce Chemical Co., Rockford, IL, USA). The mixture was incubated for 5 min at 22^0C. The Iodobead was removed and the free ^{125}I was removed as described above. Where necessary, virions were disrupted prior to labelling by heating for 5 min at 95^0C in 0.1% (w/v) SDS.

2.2.9 **SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Polypeptides of both RhPV isolates ($RhPV_{ILL}$ and $RhPV_{OFS}$) were fractionated by SDS-PAGE on 12.5% or 15% gels (Appendix B.6). Radioactive gels were dried on a vacuum gel-drier (Hoefer, SE540) and autoradiographed (Appendix B.7). Page blue stained gels and autoradiographs of labelled $RhPV_{OFS}$ were scanned at 550 nm and 700 nm respectively on a DU-8 spectrophotometer equipped with a scanning densitometer (Beckman Instruments). The relative amount of each protein or the relative extent of labelling was estimated from the peak areas of densitometer tracings.

2.2.10 **Serology**

Antisera to $RhPV_{OFS}$ were raised in rabbits (Appendix B.11) and antiserum to $RhPV_{ILL}$ was obtained from C.J. D'Arcy

(Department Plant Pathology, University of Illinois, USA). Double-antibody sandwich (DAS-) ELISA, indirect ELISA and immunoelectroblotting (Western blotting) were performed as described (Appendices B.14 and B.15).

2.2.11 **The Effect of pH on Virion Stability**

Equal amounts of purified $RhPV_{OFS}$ virions were resuspended, after centrifugal pelleting (Appendix B.1), in each of the following 0.2 ionic strength buffers: pH 2, 3, 4, 5, 7 and 8.4 (Appendix A.2.2). The absorbance at 260 nm of each preparation was checked to ensure the concentrations were equivalent. The virus suspensions were incubated at 22°C and monitored daily by DAS-ELISA. After 3 days samples were examined by electron microscopy and their sedimentation velocities were measured in a Beckman Model E analytical ultracentrifuge (Appendix B.5). The sedimentation coefficients ($S_{20.w}$) were determined by the method of Chervenka (1969).

2.2.12 **The Effect of Heat on Virions**

$RhPV_{OFS}$ suspensions in 0.1 M phosphate buffer (pH 7) were incubated at 56°C for 10 min. RNase A (700 U/ml) was included in the incubation mix to minimise possible interference by RNA with antibody reaction and sedimentation rate (Icenogle *et al.* 1981). The effect of heat was monitored by changes in antigenicity, sedimentation coefficient and morphology.

2.2.13 Synthesis and Cloning of cDNA

Complementary DNA synthesis and cloning from dsRNA of $RhPV_{OFS}$ was performed essentially as described by Huismans and Cloete (1987).

Polyadenylation of dsRNA

Approximately 10-15 μg dsRNA was denatured in 10 mM methyl mercury hydroxide for 30 min at room temperature. Denatured RNA (25 μl) was added to the following sterile mixture and incubated at 37^0C for 15min:

1 M Tris.HCl pH 8.0	7.5 μl
1 M $MgCl_2$	1.5 μl
0.25 M $MnCl_2$	1.5 μl
4 M NaCl	9.4 μl
5 mM ATP	7.5 μl
poly(A) polymerase (BRL)	1.5 μl (6 units)
water	99 μl
[2-^{3}H]-ATP	20 μCi

The polyadenylated RNA was purified and un-incorporated nucleotides were removed by chromatography on a Sephadex G-75 column. The purified RNA was lyophilized and resuspended in 20 μl of water.

Synthesis of cDNA

The standard reaction conditions for the synthesis of cDNA were as recommended by the suppliers of the avian myeloblastosis virus reverse transcriptase (Seikagaku America, USA). Double-stranded RNA was denatured in 10 mM methyl mercury hydroxide for 30 min at room temperature before use.

The reaction mix for cDNA synthesis contained:

10 mM dATP	10 µl
10 mM dGTP	10 µl
10 mM dTTP	10 µl
10 mM dCTP	1 µl
20-30 µCi (α-^{32}P)dCTP (3 000 Ci/mmol)	2 µl
5 x RT buffer	20 µl
RNasin (1250 U/ml, Amersham)	2 µl
200 mM dithiothreitol	5 µl
1 mg/ml oligo(dT) (12-18mer, Pharmacia)	10 µl
RNA (1-5 µg poly(A) RNA)	10 µl
Reverse transcriptase (400 U/ml)	2 µl
water	20 µl

5 x reverse transcriptase (RT) buffer.

KCl	250 mM
$MgCl_2$	30 mM
Tris.HCl, pH 8.3	250 mM

After 1h incubation at 42^{0}C, the cDNA/RNA was purified and un-incorporated radioactive nucleotides removed by Sephadex G75 column chromatography.

Size fractionation of cDNA

Lyophilised cDNA was resuspended in 100 μl of 0.3 M NaOH, 20 mM EDTA and incubated at 22^{0}C for 20 min. The denatured cDNA was layered onto a 5 ml 10-40% (w/v) sucrose gradient which had been poured with 1 ml steps of 10, 17.5, 25, 32.5 and 40% (w/v) sucrose in 30 mM NaOH and 2 mM EDTA. Centrifugation was for 15 h at 50 000 rpm in a Beckman SW 50.1 rotor. Gradients were drop fractionated (approx. 180 μl/fraction) and fractions assayed for ^{32}P by scintillation counting. The fractions containing the largest DNA fragments were pooled and purified by chromatography on NENSorb columns (New England Nuclear, Berkley, California). The DNA was eluted from the columns with 50% (v/v) methanol, and the cDNA fractions pooled, lyophilised and resuspended in 22 μl water.

dC-Tailing of cDNA

Poly-dC tails were added to the 3' termini of cDNA molecules by incubation for 30 min at 37^{0}C in the following mixture:

^{32}P dCTP (Amersham)	1.5 μl (30 μCi)
1 mM dCTP	2 μl
5 X tailing buffer (BRL)	4 μl
deoxynucleotidyl transferase (BRL)	1 μl (15 units)
cDNA	11 μl

C-tailed cDNA was purified on a Sephadex G-75 column and lyophilised.

Cloning of the cDNA

Lyophilised cDNA was resuspended in 30 μl of ligation buffer (0.15 M NaCl, 10 mM EDTA and 10mM Tris.HCl, pH 8.0). G-tailed *Pst*1-digested pBR322 (approx. 0.1 μg, BRL) was added and the mixture heated in a capillary glass tube for 5 min at 95^0C. This was followed by an annealing step for 2 h at 65^0C and 1 h at 56^0C. The hybridized DNA was then used to transform competent cells of *Escherichia coli* HB101.

2.2.14 **Preparation and Transformation of Competent cells**

E. coli strain HB101 has the following genotype: F^-, *dSd20* (R_Bm_B), *recA13*, *ara-14*, *proA2*, *proA2*, *lacY1*, *galK2*, *rspL20* (Sm^r), *xyl-5*, *mtl-1*, *supe44*, $^{\lambda-}$ (Maniatis *et al.*, 1982).

Competent cells of *E. coli* HB101 were prepared essentially by the method of Mandel and Higa (1970). A 10 ml overnight Luria-Bertani (LB) liquid culture was diluted 1/100, into 50 ml LB broth (Appendix A.3.1) containing 0.1% glucose. The preculture was vigorously shaken at 37^0C, until the culture had reached early exponential phase (A_{600} = 0.2). The preculture was diluted 1/50 into 400 ml prewarmed LB broth containing 0.1% glucose and grown to early exponential phase as previously described. The culture was cooled on ice for 5 min and the cells harvested by centrifugation at 4 000 x *g* for 5 min at 4^0C. Cells were resuspended in 200 ml ice-cold 0.1 M $CaCl_2$ and left on ice

for 20 min. The cells were harvested by centrifugation at 3 000 x *g* for 5 min and resuspended by pipetting in 4 ml of ice cold 0.1 M $CaCl_2$. Cells were placed on ice and used either immediately or aged overnight at 0^0C. Competent cells were stored in aliquots in 15% glycerol at -70^0C.

DNA (approx. 2 μl) in TE buffer was added to 100 μl competent cells and the sample left on ice for 10 min. Cells were heat-shocked for 3 to 5 min at 42^0C and returned to ice for 10 min. One ml of LB broth was added and the cells were incubated for 60 min at 37^0C to allow expression of transferred DNA. Transformants were selected on LB agar plates containing 15 μg/ml tetracycline. After 16 to 48 h incubation, colonies were checked for sensitivity on LB agar plates containing 50 μg/ml ampicillin. A transformation efficiency of the control sample of DNA was typically 10^6 -10^8 transformants per μg DNA.

2.2.15 **Plasmid DNA Isolation**

Plasmid DNA was isolated by the method of Ish-Horowicz and Burke (1981) (Appendix B.9).

2.2.16 **Restriction Endonuclease Digestion**

DNA was typically digested in a reaction volume of 20 μl using 2 units of restriction enzyme (Boehringer Mannheim) per μg of DNA. Digestions were performed in high, medium or low salt as recommended by Boehringer Mannheim, using the buffers supplied. Double digestions were carried out simultaneously in the same buffer, or if dissimilar conditions were required, the DNA was first restricted with

the enzyme requiring the buffer of lower ionic strength. The second enzyme and the appropriate amount of salt were added and the incubation continued for the second reaction. If necessary the first enzyme was inactivated by heating to 65^0C or as recommended by Maniatis *et al.* (1982).

The sizes of the DNA fragments were estimated by electrophoresis in agarose gels (Appendix B.10) using lambda DNA digested with *Pst*1 endonuclease and phage ϕ-X174 DNA digested with *Hae*III endonuclease (BRL) as standards.

2.2.17 **Nick Translation**

Nick translations were performed essentially as described by Rigby *et al.* (1977), using an Amersham Nick Translation Kit (PB5025). Plasmid DNA (0.5 to 1 μg) was labelled in Amersham nick translation buffer combined with polymerase I - pancreatic DNAse solution and 20-30 μCi of (α-^{32}P)dCTP (Amersham, 3000 Ci/mM). After 2 h incubation at 15^0C, the reaction was terminated by addition of 0.5 M EDTA to a final concentration of 20 mM, and the labelled DNA was purified by Sephadex G50 column chromatography and stored at -20^0C. Probes with specific activities of approximately 10^7 cpm/μg were usually obtained and were used within two weeks of labelling.

2.2.18 **5' RNA End-Labelling**

Single-stranded or dsRNA was resuspended in a solution of 25 mM glycine and 2 mM $MgCl_2$ (pH 9.0) and hydrolysed by incubation at 60^0C for 30 to 60 min. The RNA was purified by ethanol precipitation (Appendix B.8). To 5 μl hydrolysed

RNA was added 5 μl of 4 X end-labelling buffer (45 mM $MgCl_2$, 50 mM Tris.HCl, pH 9.5, 20 mM dithiothreitol), 25 μCi γ-^{32}P-ATP (synthesized by the Department of Biochemistry, University of Cape Town) and 10 units of phage T_4 polynucleotide kinase (BRL). The volume was adjusted to 20 μl and the solution incubated at 37^{0}C for approximately 3 h. The RNA was purified on Sephadex G50 columns and stored at -20^{0}C. A specific activity between 10^6 to 10^7 cpm/μg was usually obtained.

2.2.19 **DNA Dot-Blotting**

DNA samples were heated to 95^{0}C for 5 min, chilled on ice and approximately 2 μl samples were spotted onto nylon membrane (Hybond-N, Amersham). The membrane was wetted in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 min and transferred to neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M EDTA) for 1 min. The blot was dried before exposure to UV (254 nm) on a transilluminator for 2-5 min.

2.2.20 **DNA Hybridization**

Blots were prehybridized at 65^{0}C for 4-16 h in pre-hybridization solution [5 x SSPE (Appendix A.2.11), 5 x Denhardt's solution (Appendix A.2.10), 0.5 mg/ml denatured herring sperm DNA (Apendix A.2.12) and 0.5% (w/v) SDS]. DNA probes were denatured with the herring sperm DNA by boiling for 5 min followed by chilling on ice, prior to addition to the hybridization solution. RNA probes were not denatured. Probes were incubated with blots for 16 h at

65^{0}C in hybridization solution (5 x SSPE, 5 x Denhardt's, 0.5% w/v SDS and 0.1 mg/ml herring sperm DNA). Blots were washed four to six times for 15 min at 65^{0}C in 1 x SSPE containing 0.1% (w/v) SDS.

2.2.21 **Tranfer of RNA from Gels to Membrane Filters**

Gel-fractionated dsRNAs, for hybridization, were denatured in an excess volume of 50 mM NaOH for 40 min with constant agitation. The gel was neutralised by soaking for 60 min in several changes of transfer buffer (0.025 M sodium phosphate buffer, pH 6.5). RNA was transferred by electroblotting onto nylon membrane (Hybond-N, Amersham) for 4h at 0.8 A in transfer buffer. RNA was cross-linked to the membrane by exposure to UV as described in Section 2.2.19.

2.2.22 **RNA Hybridization**

RNA blots for hybridization were pre-hybridized for 4 to 16 h at 42^{0}C in pre-hybridization solution (50% v/v formamide, 5 x Denhardt's solution [Appendix A.2.10], 5 x SSPE [Appendix A.2.11], 0.5 mg/ml denatured herring sperm DNA [Appendix A.2.12] and 0.5% w/v SDS). Labelled DNA or RNA probes were incubated with blots for 16 h at 42^{0}C in hybridization solution [50% v/v formamide, 5 x Denhardt's solution, 5 x SSPE, 0.5% w/v SDS and 0.1 mg/ml herring sperm DNA]. Probes were heat denatured with heterologous DNA as described (Section 2.2.20)

RNA blots were washed four to six times for 15 min at 65^{0}C in 1 x SSPE containing 0.1% (w/v) SDS.

2.3 RESULTS

2.3.1 Direct Comparison of Two RhPV Isolates

2.3.1.1 Virus Proteins

$RhPV_{OFS}$ and $RhPV_{ILL}$ virion proteins were fractionated by SDS-PAGE and visualised by PAGE blue staining. $RhPV_{OFS}$ proteins had M_rs of 31.5 $\pm$ 0.8 K, 30.0 $\pm$ 0.6 K and 28.4 $\pm$ 0.8 K (six determinations). In accordance with picornavirus nomenclature (Rueckert and Wimmer, 1984), these proteins will be referred to as VP1, VP2 and VP3 respectively. When $RhPV_{ILL}$ and $RhPV_{OFS}$ were co-electrophoresed, the respective VP1s co-migrated, as did the VP2s. However, $RhPV_{ILL}$ VP3 consistently migrated slightly faster than the $RhPV_{OFS}$ VP3 (Fig. 2.1a).

2.3.1.2 Buoyant Density

$RhPV_{OFS}$ virions had a buoyant density of 1.373 $\pm$ 0.005 g/ml (six determinations), which was not significantly different to the approximate density of 1.370 g/ml which was calculated for $RhPV_{ILL}$ run in parallel. However, when different concentrations of the two viruses were loaded onto one gradient and centrifuged, and 150 μl fractions screened by DAS-ELISA, the viruses separated into two peaks with $RhPV_{OFS}$ being slightly less dense than $RhPV_{ILL}$. CrPV, run as a standard, had an approximate density of 1.360 g/ml.

2.3.2 **Additional properties of RhPV**

2.3.2.1 Virus Proteins

Virus preparations, purified by rate zonal centrifugation and two cycles of isopycnic centrifugation, were radioiodinated and analysed by SDS-PAGE. In addition to the capsid proteins VP1, VP2 and VP3 detected by PAGE blue staining, the most prominent proteins detected by iodination and autoradiography were proteins of M_r 11 $\pm$ 0.5 K (VP4), 40 $\pm$ 1 K and occasionally one of 59 $\pm$ 4 K (four determinations) (Fig. 2.1c). The amounts of the 40 K and 59 K proteins varied between different virus preparations. Parallel extractions were done from plants bearing uninfected aphids and labelled similarly to virus preparations (negative control). None of the above proteins were present in the negative control. Occasionally a small amount of an 18 K protein was identified in both the virion samples and the negative control; this was assumed to be of host origin.

2.3.2.2 Virion Structure

The relative amounts of VP1, VP2 and VP3 present in virions were approximately 38%, 31% and 31% respectively, as estimated from densitometer scans of Page blue-stained gels (Fig. 2.2a). When intact virions were surface-labelled by iodination using lactoperoxidase, the percentage of radioactivity associated with VP1 and VP3 were 79% and 21% respectively. VP2 did not label (Fig. 2.2b). However, when virions were disrupted prior to iodination, all three

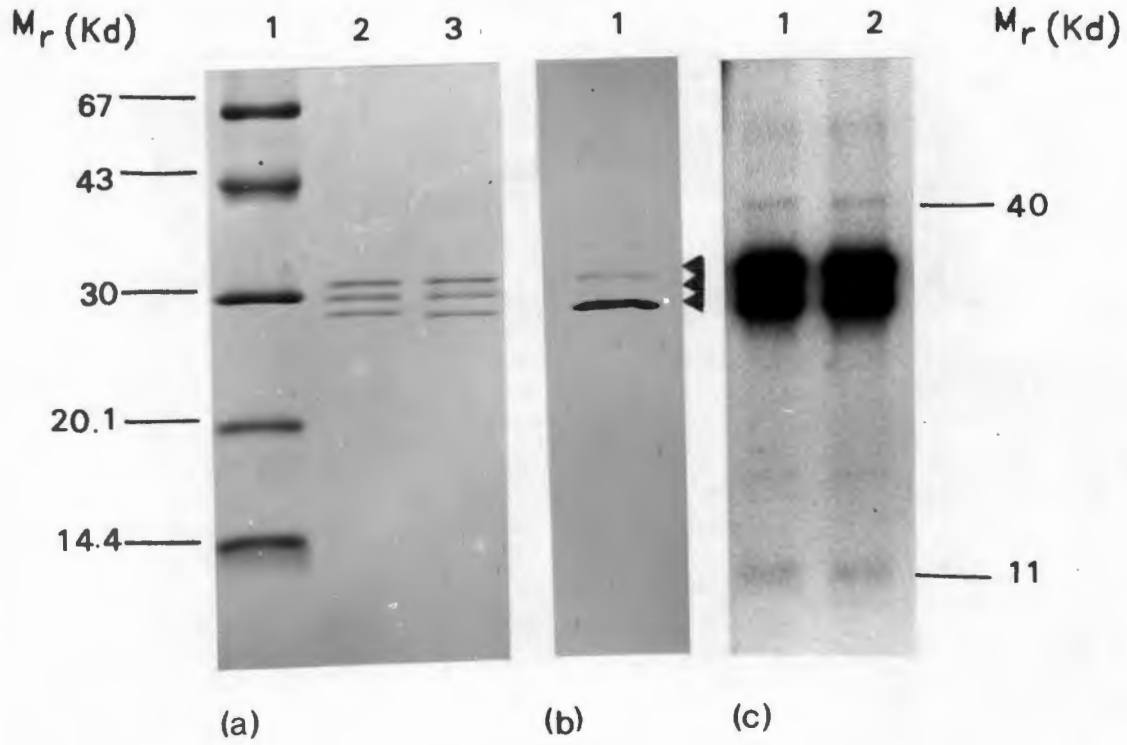


Figure 2.1

a) Comparison of polypeptides of $RhPV_{OFS}$ with $RhPV_{ILL}$ by SDS-PAGE in a 15% gel.
Lane 1, molecular weight markers (Pharmacia);
Lane 2, $RhPV_{OFS}$;
Lane 3, RhPV $RhPV_{ILL}$.

b) Immunoelectroblot of $RhPV_{OFS}$ fractionated by SDS-PAGE in a 15% gel, probed with $RhPV_{OFS}$ antiserum (1/100 dilution).

c) Autoradiograph of SDS-disrupted $RhPV_{OFS}$ radioiodinated using chloramine-T and fractionated by SDS-PAGE in 15% gels (lanes 1 and 2).

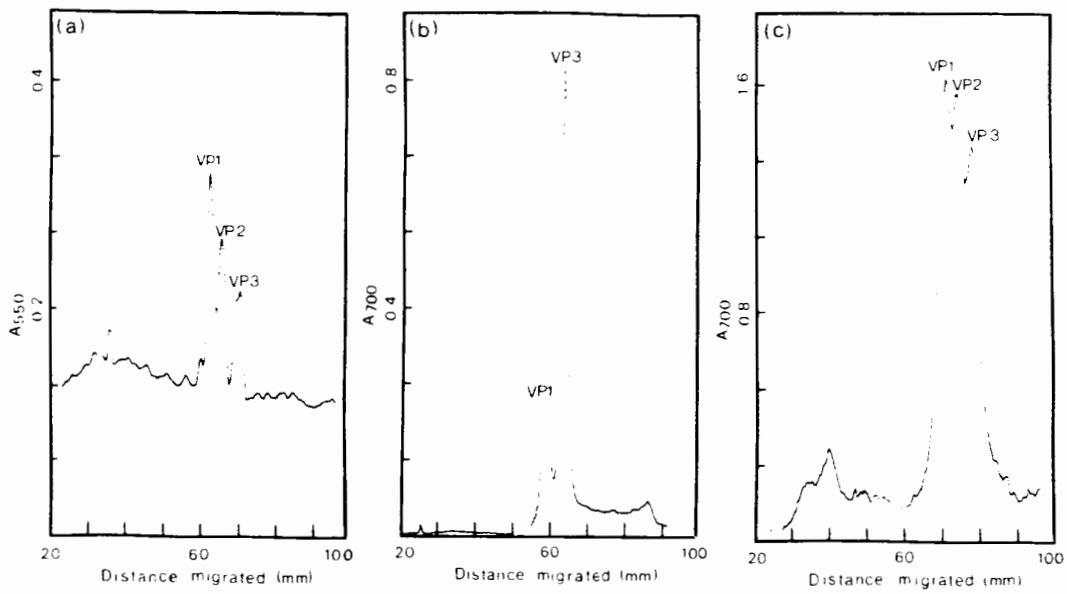


Figure 2.2

Densitometer scans of $RhPV_{OFS}$ polypeptides fractionated by SDS-PAGE in 12.5% gels.

a) Scan of a PAGE blue stained gel.

b) Scan of an autoradiograph. Intact virions were radioiodinated prior to electrophoresis using lactoperoxidase. Proteins were identified by superimposition of a co-electrophoresed PAGE blue stained gel.

c) Scan of an autoradiograph. Disrupted virions were radioiodinated prior to electrophoresis using chloramine-T.

proteins labelled to an approximately equal extent (Fig. 2.2c).

Antisera from four different rabbits, made to intact virions, reacted predominantly in Western blots with VP3, to a lesser extent with VP1, and very weakly with VP2 (Fig. 2.1b). However, antiserum to SDS-PAGE fractionated capsid proteins reacted equally well with all three proteins (data not shown).

2.3.2.3 Virus RNA

The size of the $RhPV_{OFS}$ genomic RNA was 9.98 ± 0.13 kb (M_r 3.39 x 10^6; six determinations). RhPV RNA migrated slightly slower than ALPV RNA run in parallel (Fig. 2.3) (see Chapter 3, Section 3.3.7). Glyoxal gels gave very similar results to formaldehyde gels.

Forty-four ± 7% (four determinations) of the input RhPV RNA bound to oligo(dT) cellulose as opposed to 65 ± 5% (four determinations) of the poly(A)$^+$ RNA positive control (OMV RNA), and 4 ± 1% of the poly(A)$^-$ RNA negative control (TMV RNA). The integrity of the RNA was checked by agarose gel electrophoresis and the RNA was found to be intact, prior to chromatography. There was efficient reverse transcription of the RNA when oligo(dT) was used as a primer. DNA synthesis occurred only in the presence of the oligo(dT) primer. These results indicate the presence of a poly(A) tract in viral RNA .

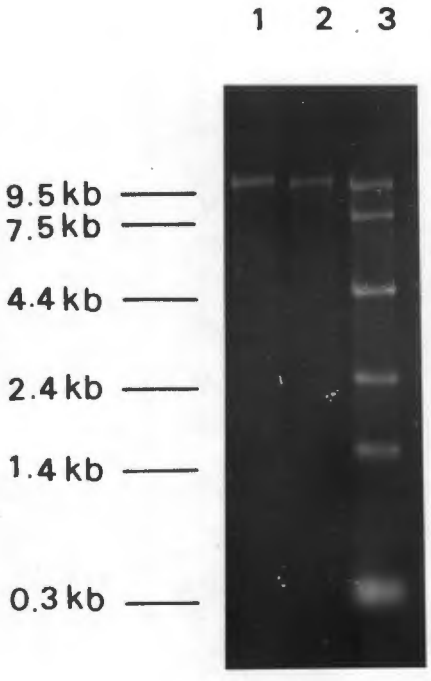


Figure 2.3
1% agarose formaldehyde denaturing gel stained with ethidium bromide. Lane 1, $RhPV_{OFS}$ virion RNA; Lane 2, ALPV virion RNA; Lane 3, Single-stranded RNA molecular weight markers (RNA ladder, BRL).

2.3.2.4 The Effects of pH on Virions

Antigenicity: Virions incubated for seven days in buffers of pH 2, 3, 5 and 8.4 were antigenically less reactive than virions incubated at pH 7. Virions incubated in buffers at pH 5 and 8.4 maintained a constant antigenic reactivity relative to virions incubated at pH 7. Below pH 5, virions became markedly less reactive with time. Virions incubated at pH 2 did not react at all in this system (Fig. 2.4). All three antisera tested gave qualitatively similar results. All antigens were tested at pH 7.0.

Morphology: The effect of pH on the morphology of RhPV after 3 days exposure to buffers of various pHs was studied by electron microscopy. At pH 2, no intact particles were seen although approximately equal numbers of particles could be seen in preparations in buffers of pH between 3 and 8.4. There was an apparent increase in the number of particles penetrated by stain from 3-7% at pHs 5, 7 and 8.4 to approximately 17% at pH 3 (results not shown).

Sedimentation Rate: The sedimentation coefficients after 3 days incubation in buffers of pH 3, 5, 7 and 8.4 were: 180 $\pm$ 2 S, 181 $\pm$ 1 S, 172 $\pm$ 4 S and 177 $\pm$ 3 S respectively (results are the average of four determinations at pH 3, 5 and 8.4; and 7 determinations at pH 7).

2.3.2.5 Effect of Heat on Virions

After heat treatment, RhPV virions were found to increase or decrease in antigenicity by DAS-ELISA and to decrease in sedimentation rate from 172 $\pm$ 4 S to 70 $\pm$ 3 S

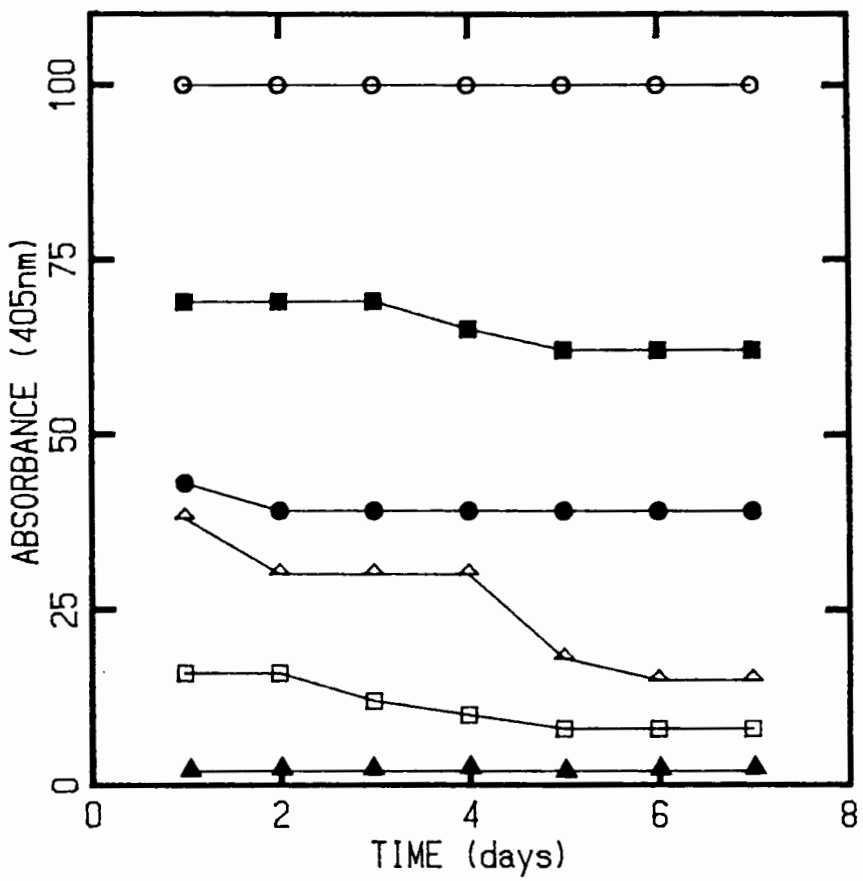
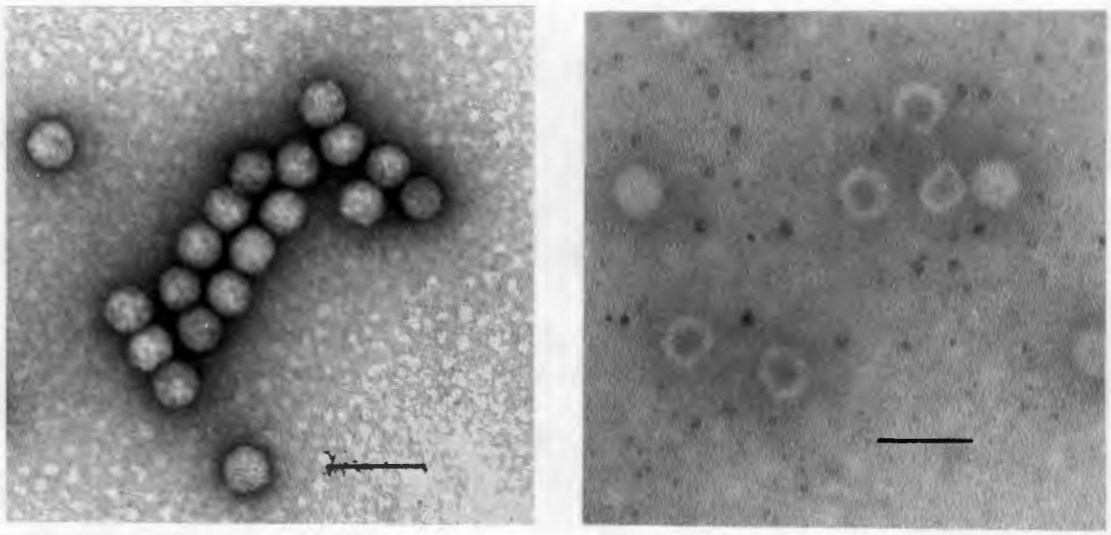


Figure 2.4
pH-induced changes in antigenicity of $RhPV_{OFS}$ monitored by DAS-ELISA. Relative absorbance was calculated as the percentage of the A_{405} of 10 μg/ml of virus incubated at pHs 2, 3, 4, 5 and 8.4 relative to the A_{405} of 10μg/ml of virus incubated at pH7. (▲) pH 2, (□) pH 3, (△) pH 4, (●) pH 5, (○) pH 7 and (■) pH 8.4.



(a) (b)

Figure 2. 5

Electron micrograph of virions negatively stained with 2% (w/v) uranyl acetate pH 4.2. Size bars indicates 50 nm.

a) Intact virions in 0.1 M phosphate buffer, pH 7.
b) Heat-treated virions in 0.1 M phosphate buffer, pH 7.

(four determinations). A high percentage of the particles were penetrated by stain when visualised by electron microscopy (Fig. 2.5).

2.3.2 **Double-Stranded RNA**

Three species of dsRNA were detected from RhPV-infected aphids by gel electrophoresis (Fig. 2.6a). As no high molecular weight dsRNA markers are readily available, dsDNA markers were used. Double-stranded RNAs of known molecular weights such as those of TMV and CMV dsRNAs (1.2×10^6 to 4.1×10^6) could be accurately determined on non-denaturing gels using lambda DNA digested with *Hin*dIII endonuclease as a M_r marker. Using DNA markers, the sizes of RNA fragments were initially calculated as molecular weights and then converted to kilobases. The size estimation of the presumptive replicative form (RF) RhPV RNA was 10.6 ± 0.4 kb (three determinations) which is not significantly different to the ssRNA size of 10 kb estimated from denaturing gels using ssRNA markers. The second major dsRNA species had a size of 2.2 ± 0.1 kb (three determinations) and the third dsRNA species, which is present in much lower relative amounts, was 4.5 ± 0.1 kb (two determinations) in size. In dsRNA purifications of uninfected aphids (negative control) done in parallel, no dsRNA was detected.

The double-stranded nature of the RNA species was confirmed by their resistance to 0.01 mg/ml RNase in 0.3 M NaCl and digestion with 0.01 mg/ml RNAse A in water. ^{32}P-labelled RhPV virion RNA hybridized to all three bands confirming their viral origin (Fig. 2.6b).

2.3.4 Identification and Characterisation of RhPV clones

RhPV cDNA was cloned into pBR322 and transformed into *E. coli*. Sixty-five transformants were resistant to tetracycline and sensitive to ampicillin. As DNA was cloned by C-tailing into the G-tailed *Pst*1 endonuclease digested pBR322, digestion with *Pst*I endonuclease excised the inserts, thus allowing direct estimation of insert sizes. The largest insert size was 1.5 kb (Fig. 2.7).

Four clones with insert sizes between 1 kb and 1.5 kb were selected (pRHPV2, 11, 13 and 104) and screened for RhPV sequences by dot blot hybridization with 5′ end-labelled virion RNA. Virion RNA hybridized to all four clones, indicating that these were RhPV-specific inserts (Fig. 2.8). Double-stranded RNA was fractionated by electrophoresis on a 1% agarose gel, transferred to nylon membrane and probed with ^{32}P-labelled pRHPV2 (insert size of 1.5 kb). The pRHPV2 probe hybridized to the 10.6 kb, RF RNA. No hybridization was detected with the lower M_r dsRNAs or to TMV RNA included as a negative control (results not shown).

The largest plasmid, pRHPV2, was characterised by restriction endonuclease mapping (Fig. 2.9). No restriction endonuclease sites were detected in the insert for the following enzymes: *Ava*I *Ava*LI, *Bam*HI, *Bst*EII, *Bgl*I, *Bgl*II, *Bcl*I, *Bst*XI, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Pst*1, *Hin*dIII, *Kpn*I,

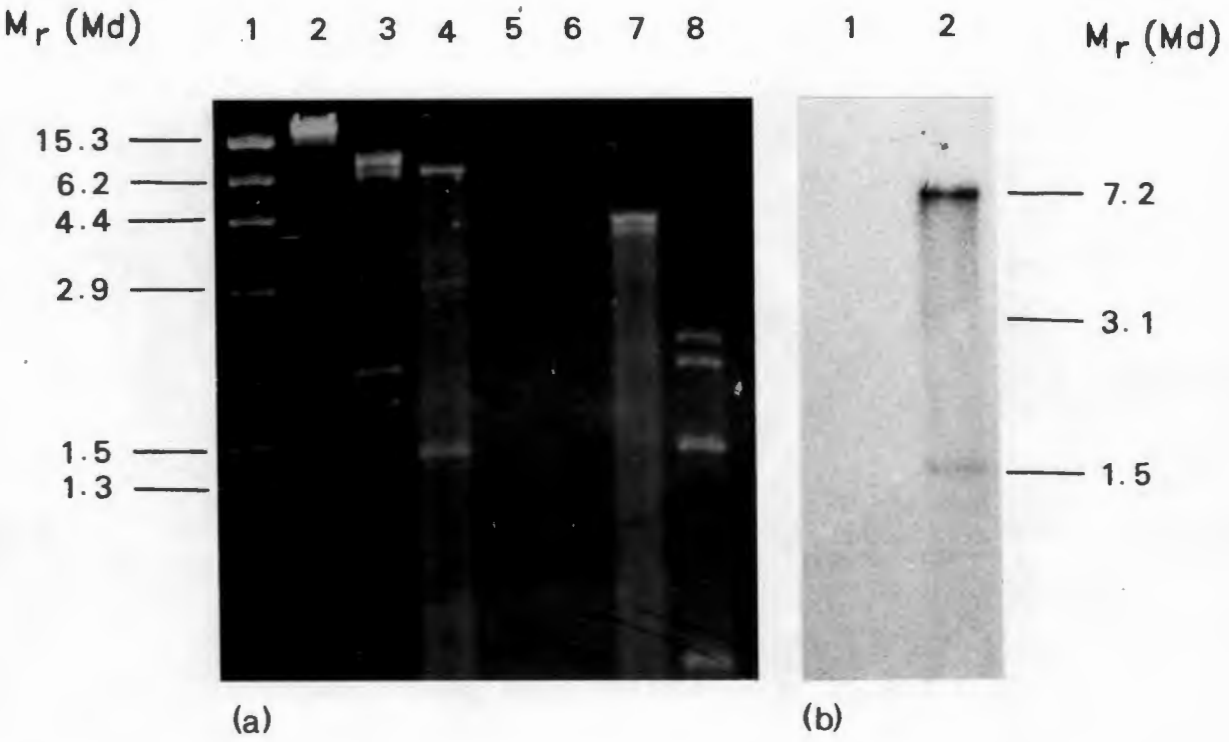


Figure 2.6

a) 0.8% agarose gel stained with ethidium bromide.
Lane 1, Lambda DNA digested with restriction endonuclease *Hin*dIII; Lane 2, Lambda DNA digested with restriction endonuclease *Sma*I (incomplete digest); Lane 3, Lambda DNA digested with restriction endonuclease *Sph*I (incomplete digest);
Lane 4, $RhPV_{OFS}$ dsRNA isolated from aphids; Lane 5, dsRNA isolated from virus-free aphids; Lane 6, dsRNA isolated from barley; Lane 7, TMV dsRNA; Lane 8, CMV dsRNA.

b) Autoradiograph of a Northern blot of dsRNA fractionated in 6% polyacrylamide gel and probed with ^{32}P 5′ end-labelled $RhPV_{OFS}$ virion RNA.
Lane 1, TMV dsRNA; Lane 2, $RhPV_{OFS}$ dsRNA.

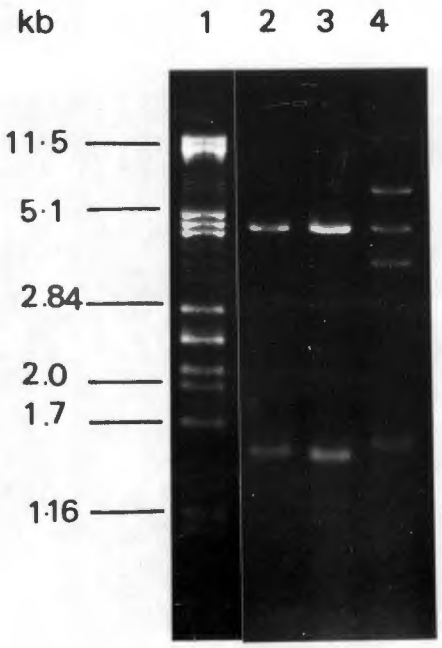


Figure 2.7
1% agarose gel stained with ethidium bromide. DNA was digested with restriction endonuclease *Pst*I.
Lane 1, Lambda; Lane 2, plasmid pRHPV2;
Lane 3, plasmid pRHPV11; Lane 4, plasmid pRHPV13.

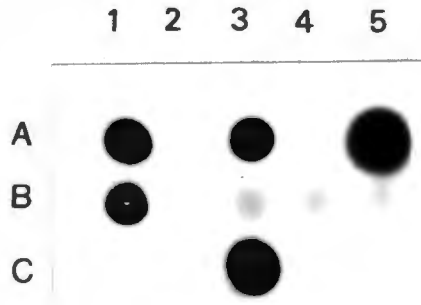


Figure 2.8
Dot-blot probed with ^{32}P-5′ end-labelled $RhPV_{OFS}$ virion RNA.
Row A: column 1, plasmid pRHPV2; Column 3, plasmid pRHPV11; Column 5, plasmid pRHPV13.
Row B: Column 1, plasmid pRHPV104; Column 3,4 and 5, uncharacterised recombinant plasmids in pEcoR251.
Row C: Column 1, plasmid pBR322; Column 3, $RhPV_{OFS}$ dsRNA, denatured *in vitro* and applied to the membrane after the DNA denaturation steps (Section 2.2.19).

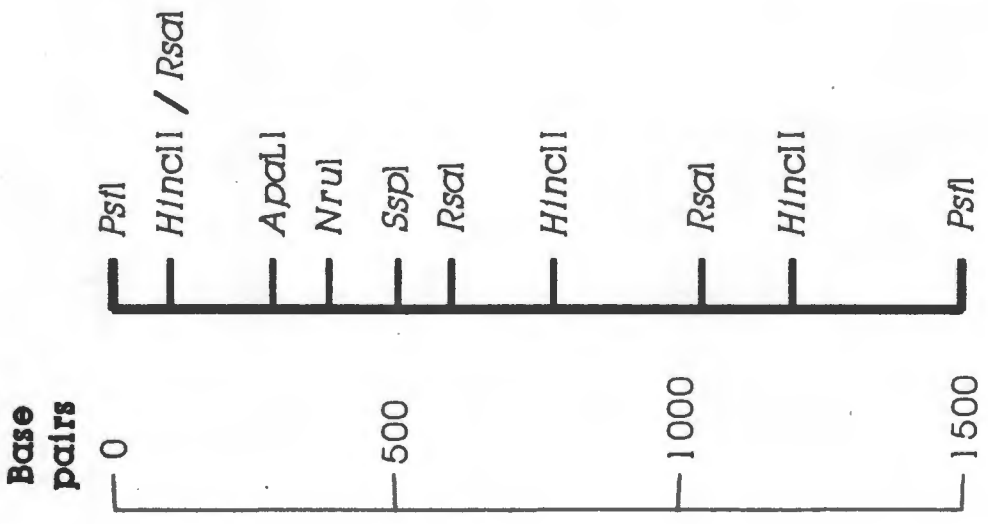


Figure 2.9
Restriction endonuclease map of plasmid pRHPV2.

Table 2.1. A comparison of the physical properties of $RhPV_{OFS}$, $RhPV_{ILL}$, CrPV and poliovirus.

Property	$RhPV_{OFS}$	$RhPV_{ILL}$	CrPV	Polio
Diameter (nm)	28	27	27	28
RNA ($Mr \times 10^{-6}$)	3.4	(31S)	2.9	2.4
Capsid Proteins ($Mr \times 10^{-3}$)	(40)*, 32,30,28,11	31,30,28	(43)*, 35,34,30	(41.0)*, 35,28,24,6
Buoyant Density (g/ml)	1.37	1.37	1.34	1.34
Sedimentation Coefficient (S)	172	162	167	155
Poly(A) RNA	+	ND	+	+

ND = not determined
*Minor amounts of proteins present, assumed to correspond to VP0.
References: Moore *et al.* (1985), Scotti (1985), King *et al.*, (1987).

*Pvu*II, *Sac*I, *Sca*I, *Sma*I, *Sph*I, *Sal*I, *Sty*I, *Xmn*I, *Xba*I and *Xho*I.

2.4 DISCUSSION

Although $RhPV_{ILL}$ and $RhPV_{OFS}$ are obviously very similar, differences in capsid protein profile and particle bouyant density indicate that they are not identical viruses. Double-stranded RNA profiles have been previously been used to differentiate between different virus strains (Gildow *et al.*, 1983). $RhPV_{OFS}$ dsRNA sizes of 10.6 kb and 2.2 kb are not significantly different to those reported for $RhPV_{ILL}$ dsRNAs of 11.2 kb and 2.5 kb (Gildow and D'Arcy, 1988). However a third, minor dsRNA species found for $RhPV_{OFS}$ was not detected in $RhPV_{ILL}$-infected aphids. This could be due to poor sensitivity of detection rather than an intrinsic difference between isolates.

It has previously been proposed that RhPV be classified in the family *Picornaviridae* (D'Arcy *et al.* 1981a). New physical characteristics reported in this study reinforce this assignment: the RNA is now known to be polyadenylated and single-stranded; the three major capsid proteins are present in essentially equimolar proportions; and a fourth low M_r 11 K protein and a 40 K protein were detected which could be analogous to the VP4 and VP0 found in the mammalian picornaviruses and *Drosophila* C virus (DCV) (Moore *et al.* 1985). Table 2.1 shows a comparison of the physical properties of $RhPV_{OFS}$ with $RhPV_{ILL}$ and other picornaviruses. The RhPV RNA size of 10 kb is significantly larger than mammalian and insect picornavirus RNAs, which range in size from 7 kb to 8.5 kb. Difficulties experienced in obtaining

sufficient amounts of pure RhPV hampered further investigations on the structure of the RNA (see Chapter 6). However, it should be noted that the genomic RNA could not be labelled using T4 polynucleotide kinase which, in the conditions used, catalyses the transfer of the phosphate of ATP to the 5'-OH terminus (Maniatis *et al.*, 1982). This would indicate that the 5'end of the RNA may be blocked by a genome-linked protein or a 5'cap structure.

RhPV is similar to poliovirus in that it is stable above pH 3 and is heat labile. The properties of the capsids after heating are very similar to the behaviour of rhino- and polioviruses (M^{C}Gregor and Mayor, 1971), and are consistent with a heat-induced structural alteration in capsids leading to loss of the genomic RNA from the virion. The changes in antigenic reactivity in DAS-ELISA of virions treated at different pH values reflects structural changes of the surface proteins (Rybicki and Coyne, 1983). However, unlike polio- and rhinoviruses in which VP1 occupies the most exposed position on the icosahedral capsid (Lonberg-Holm and Butterworth, 1976; Hogle *et al.*, 1985), the relative extent of ^{125}I-labelling of capsid proteins in whole RhPV virions indicates an exposed capsid surface position for VP3, a less exposed position for VP1, and an internal position for VP2. These conclusions are supported by the observations that RhPV VP3 is the most immunogenic of the proteins of intact capsids, followed by VP1 and VP2 , while all three isolated capsid proteins are equally immunogenic. In terms of relative surface exposure, RhPV

VP3 appears to be equivalent to poliovirus VP1, and RhPV VP1 to poliovirus VP3.

The restriction map of pRHPV2 did not have any restriction enzyme sites in common with CrPV, the only other insect virus that has been cloned and mapped (King *et al.*, 1987). Sequencing, which would have enabled a better comparison of homology of these viruses, was not attempted. It would be of interest to map other RhPV clones to determine if they originate from a similar region on the genome. Double-stranded RNA was used as a starting material for cDNA cloning in an attempt to obtain full-length clones. In retrospect, the chances of obtaining large clones would be greatly enhanced if a DNA polymerisation step was included after the DNA annealing step, to "fill-in" the single-stranded regions. The alternative method, commonly used to obtain full-length clones of picornaviruses, is direct transformation of *E. coli* with RNA-cDNA hybrids (van der Werf *et al.*, 1981). The RhPV probes characterised in this chapter were useful for virus detection. This is discussed in Chapter 6.

CHAPTER 3

CHARACTERISATION OF APHID LETHAL PARALYSIS VIRUS

SUMMARY

A new picorna-like virus, provisionally named aphid lethal paralysis virus (ALPV), was isolated from the aphid *Rhopalosiphum padi*. The virus particles are isometric with a diameter of 26-28 nm, a sedimentation coefficient of 164 S and a density in CsCl of 1.34 g/ml. Virions contain a 9.7 kb polyadenylated, single-stranded RNA and four polypeptides: three major polypeptides of molecular weights 34 400, 32 000 and 31 200 and one minor polypeptide of molecular weight 40 800.

CHARACTERISATION OF APHID LETHAL PARALYSIS VIRUS

3.1 INTRODUCTION

During the initial investigations on RhPV for this thesis, aphids were frequently introduced into the laboratory from the field. On one occasion, parasitic wasps were inadvertently introduced into the colony of *R. padi* aphids in which RhPV was propagated. These aphids were culled and a new, "virus-free" *R. padi* colony was established and infected with RhPV by leaf surface contamination (see Chapter 6). These aphids were thought to be free of RhPV as tested by DAS-ELISA and purification. Infected aphids developed an unusual behavioral syndrome. Aphids moved away from their food source and became uncoordinated in their movements. An apparent paralysis developed which was shortly followed by death. Extractions of affected aphids yielded ten to fifteen times the amount of virus expected for RhPV infections. Preparations were later shown by SDS-PAGE to apparently contain two different sets of proteins: RhPV proteins and another unrelated set of proteins. Infections with RhPV alone did not account for the observed symptoms (D'Arcy *et al.*, 1981b; Rybicki, 1984). These were, therefore, assumed to be caused by the second virus, which was accordingly named aphid lethal paralysis virus (ALPV).

The apparent pathogenicity of ALPV prompted further interest in the potential development of this virus as a biological control agent. For this, a thorough knowledge of

the virus is necessary. At the time of this study, the only natural infections of ALPV detected, occurred as mixed infections with RhPV. This section reports the effective separation of these two physically similar viruses on the basis of surface charge and the subsequent biophysical and biochemical characterisation of ALPV.

3.2 MATERIALS AND METHODS

3.2.1 Propagation and Purification of ALPV

ALPV was propagated in *R. padi* aphids co-infected with RhPV. Aphids were reared on barley (*Hordeum vulgare* L. cv. Clipper) as described (Appendix C.2.2). The virus mixture was extracted from aphids or from plants bearing aphids (Appendix B.1). The two viruses were separated on the basis of surface charge by sucrose density gradient zone electrophoresis (Appendix B.2). Where necessary, virions were further purified by rate-zonal sucrose gradient centrifugation (Appendix B.3) and/or isopycnic CsCl density gradient centrifugation (Appendix B.4).

3.2.2 Other Viruses

Purified ornithogalum mosaic virus (OMV) (a potyvirus) was obtained from J. Burger (Department of Microbiology, University of Cape Town, South Africa). Cricket paralysis virus (CrPV) was originally obtained from NERC Institute of Virology (Oxford, UK) and purified in this laboratory by K. Struthers (Department of Community Health, University of

Witwatersrand, South Africa). Purified infectious flacherie virus (IFV) was obtained from Y. Hashimoto (Laboratory of Sericultural Science, Nagoya University, Japan).

3.2.3 Electron Microscopy

Electrophoretically-purified preparations of ALPV were negatively stained in either 2% (w/v) ammonium molybdate, pH 5.5 or 2% (w/v) uranyl acetate, pH 4.1, and examined using a Philips 201C electron microcope. The size of the virus was calculated by measuring particle diameters on the negative using a Mitutoyo toolmaker's microscope. Freshly purified tobacco mosaic virus (TMV-vulgare) (van Regenmortel and von Wechmar, 1970) was used as an internal calibration standard.

3.2.4 Analytical Ultracentrifugation

Sedimentation analyses were performed using a Beckman Model E analytical ultracentrifuge (Appendix B.5) and sedimentation coefficients calculated according to Chervenka (1969).

3.2.5 Isopycnic Density Gradient Centrifugation

Buoyant densities were determined on caesium chloride gradients (Appendix B.4).

3.2.6 SDS-polyacrylamide Gel Electrophoresis

Viral proteins were analysed by SDS-PAGE on 12% and 15% polyacrylamide gels (Appendix B.6).

3.2.7 Iodination of Capsid Proteins

Virions were iodinated with Chloramine-T as described (Section 2.2.8).

3.2.8 Nucleic Acid Analysis

Isolation and enzyme digestion

Nucleic acid was extracted by SDS-disruption and phenol extraction (Appendix B.8). The type and form of the nucleic acid was established by digestion with 0.1 mg/ml proteinase K-treated DNase (Sigma) (Tullis and Rubin, 1980) in 20 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 20 mM $MgCl_2$ for 60 min at 37^0C and with 10 μg/ml RNase A in 0.3 M NaCl for 60 min at 37^0C.

Molecular weight estimation

The RNA size was estimated by electrophoresis in formaldehyde denaturing agarose gels (1%) (Appendix B.10).

Oligo(dT)-cellulose chromatography and reverse transcription

Oligo(dT)-cellulose chromatography and reverse transcription were performed as described (Sections 2.2.7 and 2.2.13 respectively).

3.2.9 Serology

Antisera were made as described (Appendix B.11). DAS-ELISA and immunoelectroblotting were performed as described (Appendices B.14 and B.15 respectively).

3.3 RESULTS

3.3.1 Purification of ALPV

Attempts to separate ALPV and RhPV by rate-zonal and isopycnic density gradient centrifugation were unsuccessful. A single light-scattering band was visualised after sucrose gradient centrifugation of purified preparations and absorption scans of fractionated gradients showed a single, asymmetrical UV absorbance peak. After CsCl centrifugation, ALPV appeared as an opalescent band and RhPV as a "shadow" band, differing slightly in diffraction properties. The absorption scan was similar to those obtained from sucrose gradients. Although preparations could be enriched for one or the other virus by these methods, pure virus preparations could not be obtained.

After zone electrophoresis, ALPV virions could be visualized by light scattering as a discrete blue opalescent band. Screening of 1 ml fractions by DAS-ELISA confirmed that RhPV and ALPV were effectively separated (Fig. 3.1). The electrophoretic mobility of ALPV relative to phenol red marker (R_ϕ) (van Regenmortel, 1972) was 0.238 $\pm$ 0.033 (ten determinations). In some preparations, an additional light-scattering band was present with an R_ϕ of approximately 0.35: this was a diffuse, milky-white zone. Analysis by SDS-PAGE and DAS-ELISA showed that this zone contained ALPV proteins; however, only a relatively small proportion of particles banded on CsCl density gradients, indicating that this zone consisted largely of unstable or denatured virions. The occurrence of this band appeared to be related

to the temperature at which the virus was propagated. Virus purified from aphids kept at 22^0C migrated almost exclusively as blue opalescent band with an R_ϕ of 0.238, whereas a large proportion of virus purified from aphids kept at 7 to 10^0C (12h day/night) migrated as a milky-white zone with an R_ϕ of 0.35. All characterisation studies were performed on virus harvested from the blue opalescent band of lower R_ϕ.

RhPV concentrations of virus purified from aphids co-infected with ALPV were too low to be detected visually by light scattering after electrophoresis. The zonal position was located by screening fractions by DAS-ELISA. An R_ϕ of 0.495 $\pm$ 0.049 was calculated for RhPV (six determinations).

As there was never enough ALPV to directly estimate the extinction coefficient ($E^{0.1\%}$), an $E^{0.1\%}$ at 260 nm of 6.0 was estimated from graphs relating $E^{0.1\%}$ to density and percentage RNA content (Gibbs and Harrison, 1976; Rybicki, 1984). Yields of ALPV propagated at 22^0C were approximately 6 to 10 mg/kg of plant material and 0.7 mg/g of aphids . This is an approximately three-fold greater yield than obtained for ALPV propagated at 7 to 10^0C.

Yields of RhPV propagated at 22^0C, were by contrast, approximately 0.2 mg/kg of plant material ($E^{0.1\%}$ at 260 nm of 7; Rybicki, 1984).

3.3.2 **Criteria of Purity of ALPV Preparations**

No RhPV proteins were detected in zone electrophoresis-purified ALPV by PAGE blue staining of polyacrylamide gels (Fig. 3.3) and by screening preparations for RhPV by

DAS-ELISA. In addition, purified ALPV preparations did not elicit an antibody response in rabbits to RhPV, even for eight weeks after the initial inoculation, as screened by indirect ELISA, immunoelectroblotting and immunosorbent electron microscopy (see Chapter 4). ALPV preparations were therefore considered to be free from RhPV contamination.

3.3.3 **Virion Morphology**

ALPV particles were isometric and of uniform size. A significant number of particles penetrated by stain were seen in purified preparations. Particles stained with ammonium molybdate had a mean diameter of 26 $\pm$ 1 nm (50 measurements) (Fig. 3.2a), which was not significantly different from RhPV particles which measured 25 $\pm$ 1 nm (50 measurements). ALPV and RhPV could not be distinguished morphologically; however, ALPV swelled to a diameter of 28 $\pm$ 1 nm (25 measurements) when stained in uranyl acetate, pH 4.1 (Fig. 3.2b), while the diameter of RhPV remained constant in both stains.

3.3.4 **Virion Proteins**

Zone electrophoresis-purified ALPV preparations contained three major proteins and one minor protein, as visualised by PAGE blue staining of SDS-PAGE fractionated virions. The major proteins had M_rs of 34 400 $\pm$ 500, 32 000 $\pm$ 800 and 31 200 $\pm$ 800 (eight determinations) (Fig. 3.3). In accordance with picornavirus nomenclature, these proteins were referred to as VP1, VP2 and VP3 respectively (Rueckert and Wimmer, 1984). Relatively

smaller amounts of a protein of M_r 40 800 ± 500 (four determinations) were regularly seen, even with virions purified by banding in CsCl gradients. The protein profile is similar to other insect picornaviruses in which this higher M_r protein is assumed to be the picornavirus VP2/VP4 precursor, VP0 (Moore *et al.* 1985). The 41 K protein will accordingly be referred to as VP0. Occasionally, a faint band was visualised with an approximate M_r of 30 000.

In addition to the proteins visualised by PAGE blue staining, proteins of M_r 64 000 ± 100, 17 000 ± 500 and 13 500 ± 500 (three determinations) were reproducibly detected by radioiodination of several different virion preparations purified by two cycles of zone electrophoresis and by CsCl gradient fractionation (data not shown). However, these proteins were present in very low amounts relative to the capsid proteins and could not conclusively be identified as virion proteins. Parallel extractions were done from plants bearing non-infected aphids (negative control) and labelled similarly to virus preparations. A 19 K and an 80 K protein were occasionally identified in both the negative control samples and the virion preparations, and were assumed to be of host origin.

3.3.5 **Sedimentation Coefficient**

The $S_{20,w}$ at pH 7.0 of ALPV was determined to be 164 S ± 1 S (four determinations).

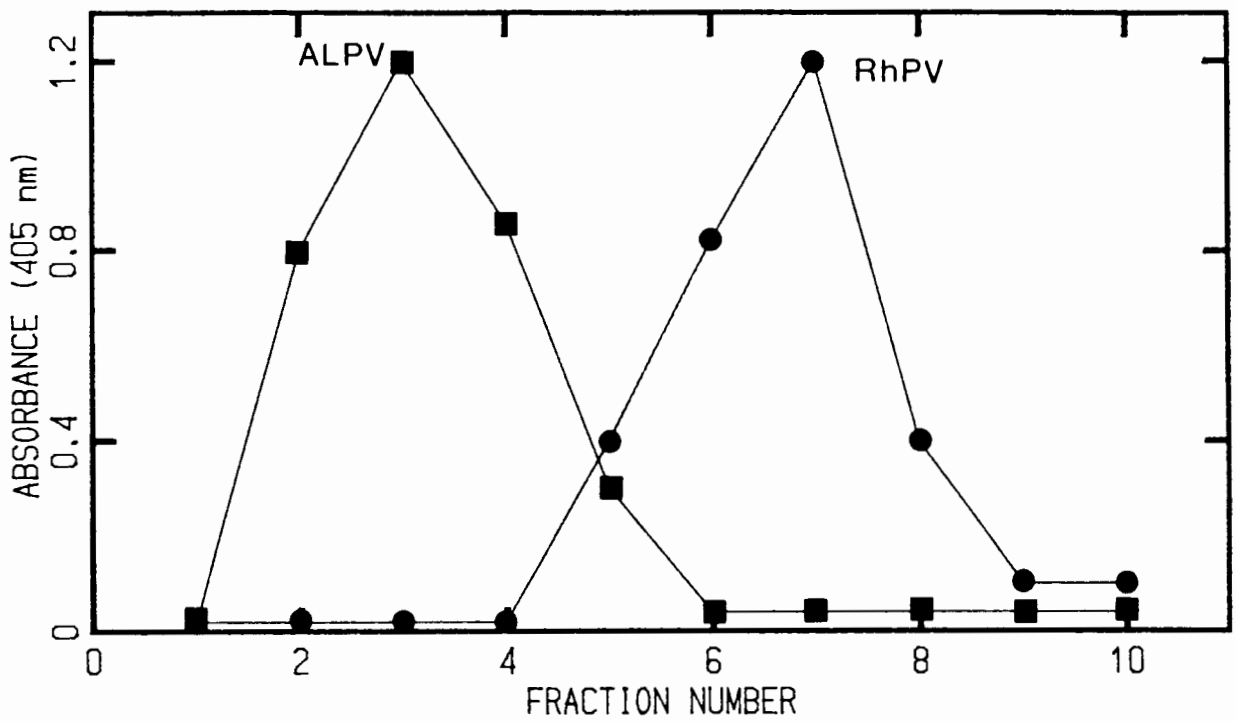


Figure 3.1
Separation of ALPV and RhPV by sucrose gradient zone electrophoresis. Fractions were screened by DAS-ELISA using antisera against ALPV (■) and RhPV (●).

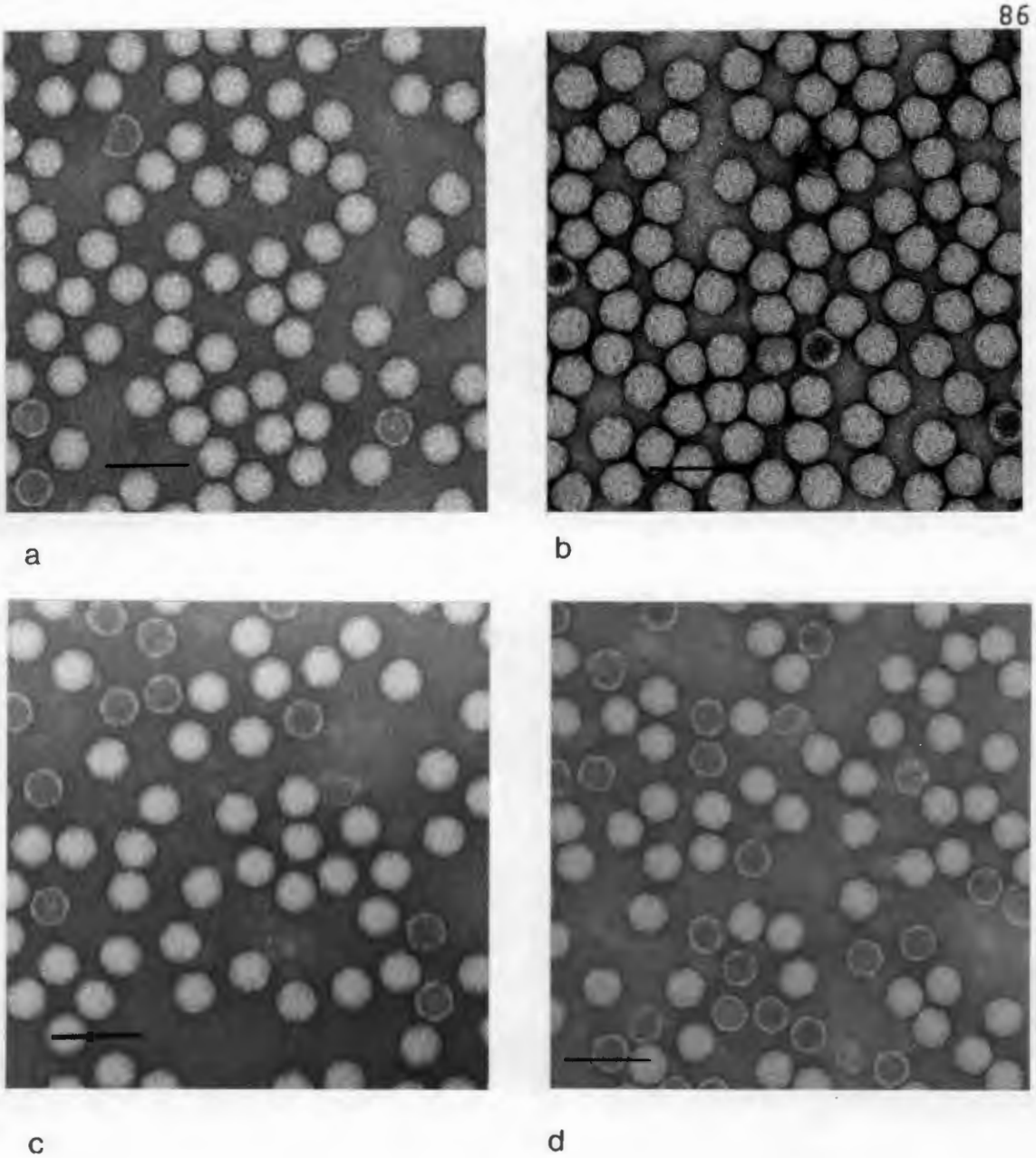


Figure 3.2
Electron micrographs of ALPV. Size bar represents 50 nm.

a) ALPV negatively stained in 2% (w/v) ammonium molybdate, pH 5.5.

b) ALPV negatively stained in 2% (w/v) uranyl acetate, pH 4.1.

c) ALPV incubated in pH 3.0 buffer for 3 days at 22^0C. Virions negatively stained with 2% (w/v) ammonium molybdate, pH 5.5.

d) ALPV incubated in pH 8.0 buffer for 3 days at 22^0C. Virions negatively stained with 2% (w/v) ammonium molybdate, pH 5.5.

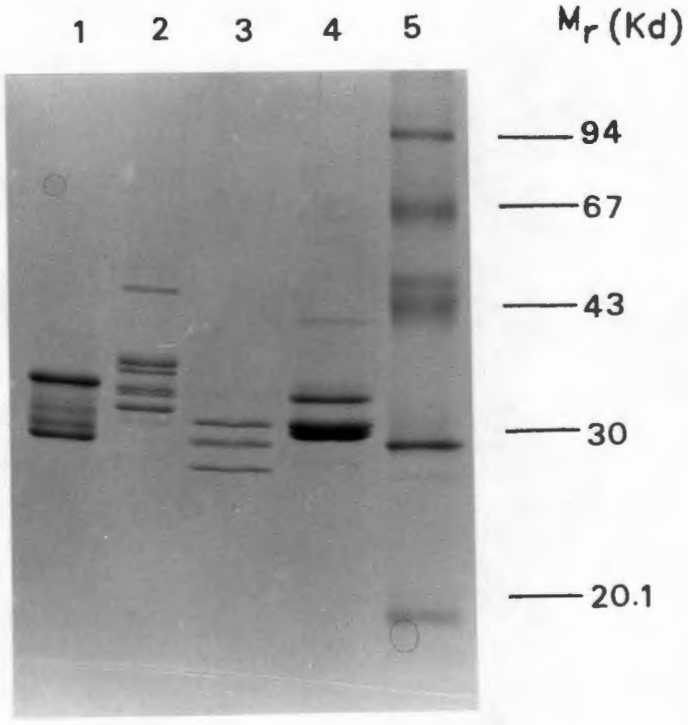


Figure 3.3
a) Comparison of the polypeptides of different insect viruses by SDS-PAGE in 15% gels stained with PAGE blue. Lane 1, CrPV; lane 2, IFV; lane 3, RhPV; lane 4, ALPV; lane 5, M_r markers (Pharmacia).

3.3.6 Buoyant Density

One major and two minor light-scattering bands were regularly obtained with zone-electrophoresis purified ALPV preparations fractionated on isopycnic CsCl gradients. Particles from the opalescent major band had a buoyant density of 1.343 ± 0.005 g/ml (seven determinations) and were identified by SDS-PAGE and DAS-ELISA as ALPV. CrPV virions (Section 2.2.1) centrifuged in parallel had an approximate buoyant density of 1.360 g/ml. This is in good agreement with the value of 1.368 g/ml obtained by Scotti (1985). Two light-scattering bands were visualised when ALPV and CrPV were co-centrifuged (Fig. 3.4), indicating that the buoyant densities of the two viruses were significantly different under these conditions. The top band of the doublet was shown by DAS-ELISA to be ALPV.

The denser of the two minor bands was opalescent, occurred at a buoyant density of 1.450 ± 0.012 g/ml (five determinations), and had the same protein profile as the major band as assessed by SDS-PAGE . The spectrophotometric scan was typical of nucleoprotein, with an absorption maximum of 260 nm (results not shown).

The second minor band was milky-white, had a density of 1.277 ± 0.010 g/ml (two determinations), and had the same protein profile in SDS polyacrylamide gels as the major band. The spectrophotometric scan was typical of protein, with an absorption maximum of 280 nm. This band probably contained empty capsids.

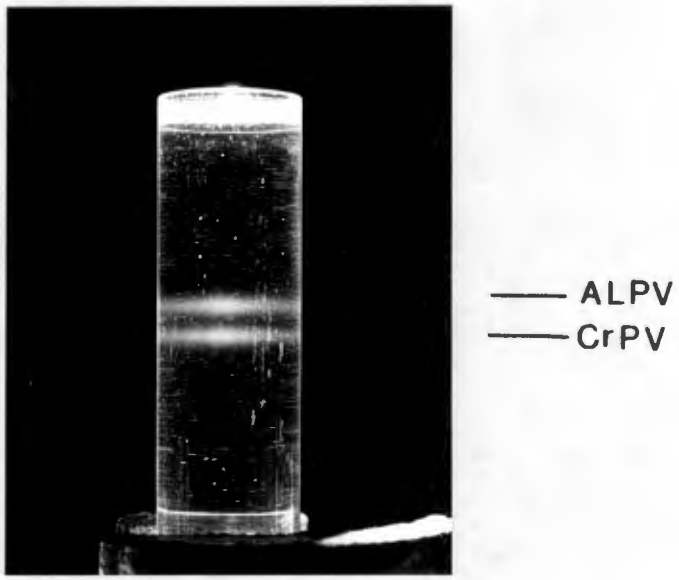


Figure 3.4
Photograph of a CsCl density gradient tube containing a mixed preparation of CrPV and ALPV. Light-scattering fractions identified by DAS-ELISA as ALPV (top band) and CrPV (bottom band). The starting CsCl density was 1.377g/ml in 0.1M phosphate buffer and the tube was centrifuged in a Beckman SW50.1 rotor at 46 000 rpm for 18 h at 20^0C.

3.3.7 Virion Nucleic Acid

The nucleic acid extracted from virions was resistant to DNAse, and was degraded by RNAse in high salt, indicating that it is single-stranded RNA. The average size determined in denaturing agarose gels was 9.7 $\pm$ 0.2 kb (five determinations) (see Chapter 2, Fig. 2.3). The percentage of applied RNA which bound to oligo(dT) cellulose was 65 $\pm$ 5% (4 determinations). Approximately 65% of the poly(A)$^+$ RNA (OMV, a potyvirus) and less than 5% of the poly(A)$^-$ RNA (TMV) bound to oligo(dT) cellulose columns. ALP virion RNA was efficiently transcribed when primed with oligo(dT) in the presence of reverse transcriptase. These results indicated the presence of a RNA poly(A) tract.

3.3.8 The Effect of pH on Antigenicity and Morphology

The effects of changes of pH on ALPV virions was monitored by the changes in antigenicity and morphology. Virions were resuspended to an equivalent A_{260} in 0.2 ionic strength buffers of pH 2, 3, 5, 7 and 8 (Appendix A.2.2) and incubated at 22^{0}C. Virions were checked for changes in antigenicity by DAS-ELISA after 2 h and subsequently every 48 h for 5 days. Preparations were checked after 3 days for changes in morphology by electron microscopy.

Antigenicity

Changes in antigenicity were measured by the change in A_{405} of viral solutions at a concentration of 10 μg/ml at a particular pH relative to virus resuspended in buffer at pH 7.0. Virions incubated for 2 h in buffers of pH 3, 5 and

Table 3.1. A comparison of the properties of ALPV, RhPV, CrPV, DCV and mammalian enteroviruses.

Property	ALPV	RhPV	CrPV	DCV	entero.
Diameter (nm)	26	25	27	28	28
RNA ($M_r \times 10^{-6}$)	3.3	3.4	2.9	2.5-3	2.4
Capsid Proteins ($M_r \times 10^{-3}$)	(40.8)*, 34,32,31	(40)*, 32,30,28,11	(43)*, 35,34,30	(37)*, 31,30,28,9	(41.0)* 35,28,24
Buoyant Density (g/ml)	1.34	1.37	1.37	1.34	1.34
Sedimentation Coefficient (S)	164	167	167	153	155
Poly(A) RNA	+	+	+	+	+

*Minor amounts of proteins present, assumed to correspond to mammalian picornavirus VP0.
References: Moore *et al.* (1985), Scotti (1985).

8 were antigenically similar, while virions incubated in buffer at pH 2 did not react in this system. Virions incubated in buffers at pH 5, 7 and 8 remained antigenically similar for 5 days, however virions incubated in buffer at pH 3 became antigenically less reactive after 48 h.

Morphology

The effect of 3 days exposure to buffers of various pHs on virion morphology was assessed visually by EM (Fig. 3.2). No intact particles were seen in samples incubated in buffer at pH 2.0, although approximately equal numbers of particles were seen in all preparations incubated in buffers from pHs 3 to 8. There was an apparent increase in the number of particles penetrated by stain from less than 1% at pH 7 and pH5 to approximately 10% at pH 3 and 44% at pH 8. This indicates that ALPV is most stable at pH 5 to 7 and less stable outside this pH range.

3.4. DISCUSSION

The separation of viruses on the basis of surface charge by zone electrophoresis provided an effective method of purifying ALPV. In the past, this technique has been used for the separation of plant virus strains and in the purification of unstable viruses (van Regenmortel, 1964). Although other methods are available for the charge separation of particles, zone electrophoresis proved to be efficient, simple and very economical.

ALPV, like the accepted members of the *Picornaviridae*, has small virions containing three major proteins with M_rs of approximately 30 K, as well as small amounts of a higher M_r protein which could be analogous to the VP0 found in mammalian picornaviruses, infectious flacherie virus, CrPV and *Drosophila* C virus (DCV) (Hashimoto and Kawase, 1983; Moore *et al.* 1985). The genome consists of a single 9.7 kb ssRNA (M_r 3.3 x 10^6) which is polyadenylated. These characteristics indicate that ALPV should be classified in the picornavirus group. In addition, virion preparations contain particles of high density, similar to the dense components observed for DCV (Jousset *et al.*, 1977), poliovirus (Yamagushi-Koll *et al.*, 1975), and several other enteroviruses of vertebrates (Rowlands *et al.*, 1975).

A fourth, low M_r capsid protein (VP4), characteristic of mammalian picornaviruses, DCV and IFV (Table 1.1), has not been detected for CrPV (Moore *et al.* 1980, Moore *et al.*, 1981c). Although 13.5 K and 17.0 K proteins were identified by radioiodination in highly purified ALPV preparations, they cannot be identified with certainty as virion proteins. They were present in very low amounts and have a higher M_r than typical picornavirus VP4s, DCV VP4 and the presumptive VP4 of RhPV (see Chapter 2). Failure to detect an ALPV VP4 could be due to the protein being lost due to exposure to alkaline pH during the purification procedure as has been found with other picornaviruses (Mak *et al.*, 1970).

It would also be of interest to know whether ALPV has a small protein covalently bound to the 5′ end of the genome (genome-linked protein or VPg) like other mammalian

picornaviruses, CrPV, DCV (King *et al.*, 1986) and IFV (Hashimoto *et al.*, 1986). Preliminary experiments showed that 3' end-labelled virion RNA banded at a density of 1.67 g/ml in trifluoroacetate gradients (Pharmacia). Virion RNA labelled by a protein-specific radioiodination reaction, also banded at this density. This suggests that ALPV RNA has a genome-linked protein. Trifluoracetate is a powerful denaturing reagent which strips off proteins non-specifically associated with nucleic acid. ALPV RNA-associated proteins banded at a density of approximately 1.56 g/ml (data not shown).

ALPV virions stained with uranyl acetate, pH 4.1 swell relative to their sizing in ammonium molybdate, pH 5.5, whereas particles of RhPV do not. It would be of interest to test other picornaviruses to see if this reflects basic differences in structure or stabilising forces between virus subgroups. The effect of pH on virion morphology indicated that ALPV is most stable at pH 7.0, becoming increasingly unstable with increase or decrease in pH. The large number of empty capsids present in preparations incubated at pH 8.0 suggest that 0.1 M borate buffer, pH 8.6 is not an ideal buffer for purification by zone electrophoresis and an alternative buffer such as 0.03M phosphate pH 7.5 (van Regenmortel, 1972) should be used for increased virion stability and virus yields.

ALPV is a new aphid virus, physically distinct from RhPV (Table 3.1), which is the only other aphid virus which has been physically characterised. The physical properties

of ALPV also differentiate it from other small RNA-containing viruses of insects such as DCV, IFV (Fig. 3.3), *Gonometa* virus, Kawino virus, *Lymantria ninayi* virus, sacbrood virus, Kashmir bee virus, bee slow paralysis virus, Egypt bee virus, bee acute paralysis virus, and black queen-cell virus (Table 1.1). Although the sedimentation coefficients of ALPV and CrPV are similar (Table 3.1), CrPV and ALPV were found to have different buoyant densities in CsCl and differences were seen between the protein profiles when CrPV and ALPV capsid proteins were co-electrophoresed (Fig. 3.3 and 3.4). The reported value of 8.5 kb for CrPV RNA (King *et al.*, 1987) is approximately 1.5 kb smaller than that of ALPV. Further investigations on the serological and nucleic acid relationship between ALPV and other insect viruses is provided later (Chapter 4).

In nature, the occurrence of insect populations infected with more than one small RNA virus appears to be commonplace; at least two viruses were detected in emperor gum moths (*Antheraea eucalypti*) including CrPV and *Antheraea* virus (Reinganum, 1975) and there have been numerous reports of multiple virus infections of bees (Bailey and Milne, 1969; Bailey and Woods, 1977; Bailey *et al.*, 1979; Bailey *et al.*, 1981, Anderson and Gibbs, 1988). When considering viruses as pesticides, these multiple infections and their interactions with each other and their host become particularly relevant. The difficulties in obtaining quantitative amounts of RhPV from aphids co-infected with ALPV suggests that ALPV interferes with RhPV replication.

CHAPTER 4

SEROLOGICAL AND NUCLEIC ACID RELATIONSHIPS BETWEEN ALPV, RHPV AND OTHER SMALL RNA VIRUSES

SUMMARY

Antigenic relationships between ALPV, RhPV and other small RNA viruses were studied by immunosorbent electron microscopy, DAS-ELISA, indirect ELISA and immunoelectroblotting. ALPV was found to be serologically related to cricket paralysis virus and *Drosophila* C virus (insect picornaviruses); and unrelated to RhPV and *Sitobion avenae* virus (aphid picorna-like viruses), infectious flacherie virus (a picorna-like virus of silkworms), five picorna-like bee viruses and echovirus 4 (a mammalian picornavirus). No serological relationship was detected between RhPV and any of the above mentioned viruses. Nucleic acid homology was studied by dot blot hybridization. No homology was detected between ALPV cDNA, and CrPV and RhPV RNAs.

SEROLOGICAL AND NUCLEIC ACID RELATIONSHIPS BETWEEN ALPV, RHPV AND OTHER SMALL RNA VIRUSES

4.1 INTRODUCTION

Traditionally, assessment of antigenic relatedness between viruses has been based on antigen-antibody precipitation reactions in liquid or in gel (van Regenmortel and von Wechmar, 1970; Scotti and Wigley, 1982). However, in recent years these techniques have been largely superseded by tests such as enzyme-linked immunosorbent assay (ELISA) and immunoelectroblotting (Western blotting). These techniques are far more sensitive and require less material than precipitin assays. In addition, immunoelectroblotting provides information on the molecular weights of the antigenic protein(s) (Towbin *et al.*, 1979). Several different variations of ELISA exist. DAS-ELISA has been widely applied in viral diagnosis; however, it is extremely strain specific and therefore has limited use for the detection of distantly related viruses (Koenig, 1978; Ford *et al.*, 1978). Indirect ELISA is less specific than DAS-ELISA (Rybicki and von Wechmar, 1981) and has been used to estimate the degree of serological cross-reactivity between viruses (Jaegle and van Regenmortel, 1985; Clark and Barbara, 1987). It has the advantage over DAS-ELISA in that it does not require separate antibody-enzyme conjugates for each distinct virus or serotype to be tested.

Assessments of virus relatedness by serological techniques using antisera to virions are limited in that only the antigenic relationship of the structural proteins

(representing roughly 10% of the total coding capacity of the viral genome) are compared and not the more conserved, non-structural proteins such as the RNA-dependent RNA polymerases or the proteases (Goldbach, 1987). Several studies on the serological relationship between non-structural proteins of picornaviruses have been done using antisera to non-structural proteins produced *in vitro* (Emini *et al.*, 1985; Grubman *et al.*, 1987). However, as the virion genome dictates the structure and function of viruses, it is obvious that gene sequence and predicted protein sequence comparisons would provide the ultimate data on virus relationships. Although many of the mammalian picornaviruses have been sequenced, to date the genome of only one insect picornavirus - CrPV - has been cloned and even partially sequenced (King *et al.*, 1987). This approach is beyond the scope of this project but promises to provide new and exciting information on the relationships between the insect viruses and small RNA viruses and the evolutionary position of insect viruses in the "picorna-like virus super-group" (Goldbach, 1987).

Finally, double-stranded (ds) RNA profiles provide a useful tool for identification and differentiation of viruses. Single-stranded, monopartite, plus-strand RNA viruses often produce a range of distinct virus-specific dsRNA species (Valverde *et al.*, 1986). These dsRNA profiles may be useful in distinguishing virus strains that are otherwise physically indistinguishable (Gildow *et al.*, 1983).

In this chapter the relationship between $RhPV_{OFS}$ and $RhPV_{ILL}$, and between ALPV, $RhPV_{OFS}$ and several picorna- and picorna-like viruses, was investigated by DAS-ELISA, indirect ELISA, immunosorbent electron microscopy and/or immunoelectroblotting. The ALPV dsRNAs was compared with RhPV dsRNAs reported previously (Chapter 2). Attempts to detect nucleic acid homology between ALPV cDNA and $RhPV_{OFS}$ and CrPV were made by dot-blot nucleic acid hybridization.

4.2 MATERIALS AND METHODS

4.2.1 Viruses

$RhPV_{OFS}$ was propagated in *R. padi* infected with RhPV only, extracted as described (Appendix B.1), and purified further by sucrose density gradient centrifugation (Appendix B.3). Purified $RhPV_{ILL}$ was obtained from C.J. D'Arcy (Dept. of Plant Pathology, University of Illinois, USA). ALPV was propagated in *R. padi* infected with both RhPV and ALPV, extracted as described (Appendix B.1) and purified further by zone electrophoresis (Appendix B.2). Tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV) were propagated in tobacco plants (*Nicotiana tabacum* cv. Soulouk). CrPV originated from the NERC Institute of Virology, Oxford, and was propagated and purified in this laboratory by K. Struthers (Department of Community Health, University of the Witwatersrand, South Africa). Purified Ornithogalum

mosaic virus (OMV) (a potyvirus) was obtained from J. Burger (Department of Microbiology, University of Cape Town, South Africa).

4.2.2 **Antisera**

Antisera to RhPV and ALPV were raised in rabbits as described (Appendix B.11). Antisera to the following viruses were used in serological studies: TMV (M.B. von Wechmar, Department of Microbiology, University of Cape Town, South Africa), $RhPV_{ILL}$ (C.J. D'Arcy); DCV and CrPV (N.F.Moore, NERC Institute of Virology, Oxford, UK); infectious flacherie virus (IFV) (Y. Hashimoto, Laboratory of Sericultural Science, Nagoya University, Japan); echovirus 4 (G.A. Keen, Medical Microbiology, University of Cape Town, South Africa); and bee acute paralysis virus, black queen cell virus, Kashmir bee virus, sacbrood virus, slow paralysis virus of bees; and $RhPV_R$ and *Sitobion avenae* virus of aphids (M. Allen, Rothamsted Experimental Station, Harpenden, U.K.).

4.2.3 **Serology**

ELISA (DAS- and indirect) and immunoelectroblotting (Western blotting) were performed as described (Appendices B.14 and B.15 respectively).

Immunosorbent electron microscopy (ISEM) was performed as described by the method of Milne and Lesemann (1984). Virus particles were 'trapped' using grids coated at an antiserum dilution of 1/100 or 1/1000 and 'decorated' with antibodies at an antiserum dilution of 1/10 or 1/100.

Samples were negatively stained with 2% (w/v) uranyl acetate, pH 4.1, and examined in a Philips 201C electron microscope.

4.2.4 Dot-Blotting of Virus Protein and RNA

Purified preparations of ALPV, CrPV, RhPV and OMV were adjusted to equivalent concentrations in 0.1 M phosphate buffer pH 7.0, adjusted to a final concentration of 1% (w/v) SDS and heated for 5 min at 95^0C. Samples (50 μl) were spotted onto nitrocellulose (BA 85, Schleicher and Schuell) and onto nylon membrane (Hybond-N, Amersham) using a 96-well micro-sample filtration manifold (Minifold, Schleicher and Schuell).

Nitrocellulose membranes were probed with CrPV antiserum and ALPV antiserum as described for immunoelectroblotting (Appendix B.15). Nylon membranes were probed with ALPV cDNA transcribed using random hexanucleotide primers (Section 2.2.13) and hybridized under conditions described (Section 2.2.22).

4.2.5 Double-Stranded RNA Isolation and Hybridization

Double-stranded (ds) RNA was isolated from 1 to 5 g of *R. padi* infected with RhPV only and from *R. padi* infected with both RhPV and ALPV. Double-stranded RNA was purified by phenol extraction and cellulose (Whatman CF-11) affinity chromatography (Section 2.2.5). The RNA was fractionated by electrophoresis in 0.8 to 1% agarose gels (Appendix B.10).

Gel-fractionated dsRNAs was transferred to nylon membrane by electroblotting (Section 2.2.21) and probed with

5′ end-labelled virion RNA (Section 2.2.18) under hybridization conditions described (Section 2.2.22).

4.3 RESULTS

4.3.1 The Serological Relationship Between $RhPV_{OFS}$ and $RhPV_{ILL}$

No qualitative or quantitative differences were noted for the DAS-ELISA titration curves of identical dilution series of $RhPV_{OFS}$ and $RhPV_{ILL}$. Both had a dilution end-point of 1.25 ng when screened with anti-$RhPV_{OFS}$ antibodies by DAS-ELISA, and curves could be superimposed without significant differences, indicating that the viruses were effectively serologically identical (results not shown). In Western blot assays, $RhPV_{ILL}$ antiserum reacted predominantly with $RhPV_{ILL}$ and $RhPV_{OFS}$ VP3 and VP1 and to a much lesser extent with VP2 (Fig. 4.1). $RhPV_{OFS}$ antisera reacted primarily with $RHPV_{OFS}$ VP3, to a lesser extent with VP1 and very weakly with VP2 (Chapter 2, Fig. 2.1b)

4.3.2 Tests for Serological Relationship between ALPV and RhPV

4.3.2.1 *ISEM*

Virus preparations containing both RhPV and ALPV (prior to zone electrophoresis fractionation) were used for ISEM studies. When virions were trapped with ALPV antiserum no virions were 'decorated' by RhPV antibodies and vice versa (Fig. 4.2a and b). When virions were trapped with ALPV

antiserum, these particles were also 'decorated' by ALPV antiserum (Fig. 4.2c). When virus preparations were adsorbed directly onto a grid and treated with ALPV antiserum, approximately 95% of the particles were 'decorated'. If the same preparation was treated with anti-RhPV antiserum, only 5% of the particles were 'decorated'. Figure 4.2d shows preparations which have been enriched with RhPV and treated with RhPV antiserum.

4.3.2.2 *ELISA and immunoelectroblotting*

Antisera to ALPV did not react with RhPV (Fig. 4.3a) - and *vice versa* (data not shown) - in indirect ELISA tests with antisera and by immunoelectroblotting .

4.3.3 **Tests for Serological Relationship between RhPV and Other Small RNA viruses**

$RhPV_R$ antisera reacted with $RhPV_{OFS}$ proteins in indirect ELISA and immunoelectroblotting (results not shown). No serological reaction could be detected, by indirect ELISA and immunoelectroblotting between $RhPV_{OFS}$ and antisera to the following viruses (Table 4.1): CrPV, DCV (Fig. 4.3b), IFV, acute paralysis virus, black queen cell virus, Kashmir bee virus, sacbrood virus, slow paralysis virus and *Sitobion avenae* virus (insect picorna-like viruses) and echovirus 4 (mammalian picornavirus). None of the antisera tested reacted with TMV, included as a negative control. RhPV antisera reacted strongly with RhPV only. CrPV antiserum reacted positively with CrPV.

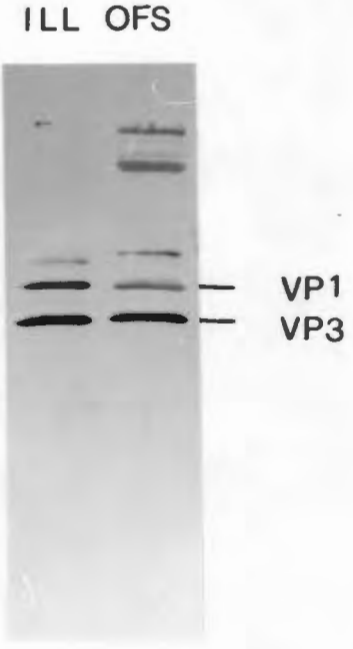


Figure 4.1
Immunoelectroblot of $RhPV_{ILL}$ (ILL) and $RhPV_{OFS}$ (OFS) polypeptides fractionated by SDS-PAGE in a 15% gel and probed with $RhPV_{ILL}$ antiserum (1/200 dilution).

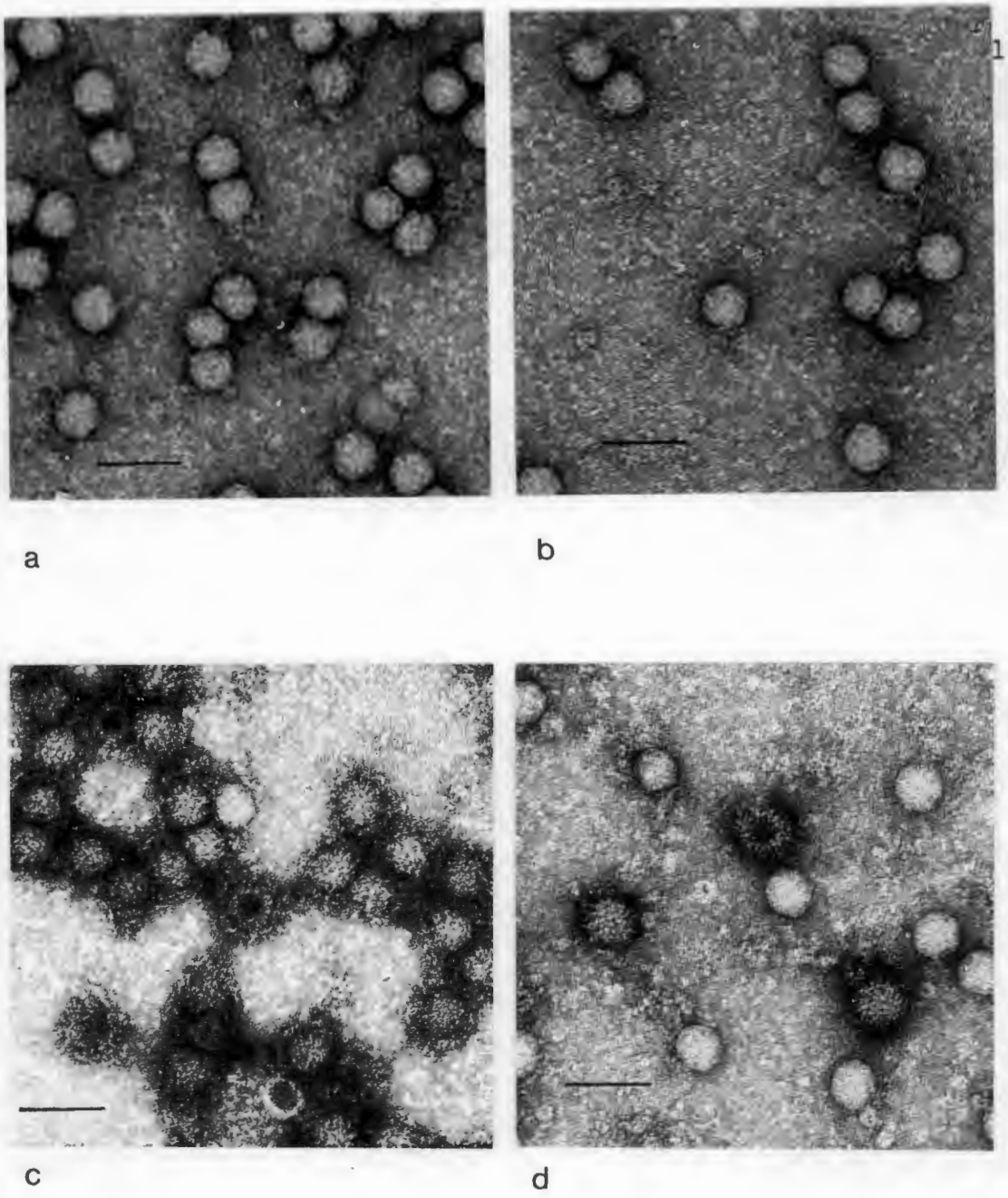


Figure 4.2

Immunoelectron micrographs of virions stained with 2% (w/v) uranyl acetate, pH 4.1. Bar marker represents 50 nm.

a) Virions from a mixed preparation of ALPV and RhPV were trapped with anti-RhPV antiserum (1/1000 dilution) and decorated with anti-ALPV antiserum (1/10 dilution).

b) Virions from a mixed preparation of ALPV and RhPV were trapped with anti-ALPV antiserum (1/100 dilution) and decorated with anti-RhPV antiserum (1/10 dilution).

c) Virions from a mixed preparation of ALPV and RhPV were trapped with anti-ALPV antiserum (1/1000) and decorated with anti-ALPV antiserum (1/100).

d) A mixed preparation of ALPV and RhPV was adsorbed directly onto the grid, and treated with anti-RhPV antiserum.

Table 4.1. Serological reactions between ALPV, RhPV and other small RNA viruses.

Antisera to	Viruses ALPV	RhPV	CrPV
ALPV (early)*	+++	-	-
ALPV (late)*	+++	-	+
$RhPV_{OFS}$	-	+++	-
$RhPV_{R}$	-	+++	ND
CrPV	++	-	+++
DCV	++	-	ND
IFV	-	-	ND
Acute bee paralysis	-	-	ND
Black queen cell	-	-	ND
Kashmir bee	-	-	ND
Sacbrood	-	-	ND
Slow paralysis	-	-	ND
Sitobion avenae	-	-	ND
Echovirus 4	-	-	ND

+++ = strong positive reaction
+ = positive reaction
- = no reaction
ND = not determined
*early = 6 weeks after initial immunization,
late = 16 weeks after initial immunization.

4.3.4 Tests for Serological Relationship Between ALPV and Other Small RNA Viruses of Insects

No serological reaction was detected by indirect ELISA and Western blotting between ALPV and the following virus antisera (Table 4.1): IFV, acute paralysis virus, black queen cell virus, Kashmir bee virus, sacbrood virus, slow

paralysis virus and *Sitobion avenae* virus (insect picorna-like viruses) and echovirus 4 (mammalian picornavirus).

In Western blots, early bleedings (up to six weeks after initial immunization) of ALPV antiserum reacted with ALPV but not with CrPV or RhPV (Fig. 4.3a). Higher titred serum from later bleedings (16 weeks) gave a weak positive reaction with CrPV, reacted strongly with ALPV, and not at all with TMV or RhPV. In a blot done in parallel, CrPV antiserum reacted strongly with CrPV, to a lesser extent with ALPV and did not react with RhPV (Fig. 4.3b). ALPV antisera reacted predominantly with ALPV VP3, to a lesser extent with VP1 and not all with VP2. Both CrPV and DCV antisera reacted predominantly with VP1 and VP2. CrPV antiserum also reacted with VP3.

In immunodot-blots, CrPV antiserum reacted with CrPV to an endpoint of 10 ng/dot compared with an endpoint of 100 ng/dot of ALPV. CrPV antiserum did not react with RhPV, or the OMV used as a negative control (Fig. 4.4a). ALPV antiserum (early bleed) reacted with ALPV to an endpoint of 1 μg/dot and did not react with CrPV, RhPV or OMV. These results indicate that ALPV and CrPV are distantly related.

In indirect ELISAs, CrPV and DCV antisera reacted with ALPV. As the antiserum titre was unknown, it was not possible to quantify these results.

4.3.5 Nucleic Acid Dot-Blot Hybridization

ALPV cDNA hybridized with ALPV RNA to an endpoint of 1 ng/dot (approx. 300 pg RNA assuming an RNA content of 30% by weight of virions). ALPV cDNA did not detectably

hybridize with equivalent amounts of CrPV RNA, RhPV RNA or OMV RNA, included as a negative control (Fig. 4.4b).

4.3.6 A Comparison of the Double-Stranded RNAs of ALPV and RhPV

One major species and at least two minor dsRNA species were identified in aphids infected with both RhPV and ALPV (Fig. 4.5a). These RNAs were 10.1 $\pm$ 0.2, 2.1 $\pm$ 0.1 and 1.7 $\pm$ 0.1 kb in size respectively (M_r of 6.8 x 10^6, 1.4 x 10^6 and 1.2 x 10^6 respectively) (three determinations). The major 10.1 kb RNA species was assumed to be the replicative form (RF) RNA. Despite several cycles of cellulose chromatography and purification by repeated precipitation with LiCl, an increase in the amount of sample loaded into the gel resulted in a smear down the lane after electrophoresis. ^{32}P-end-labelled ALPV RNA hybridized to all three bands, although identification of the minor bands was difficult as the RNA also hybridized to the smear down the lane (Fig. 4.5b). This confirmed that all three bands were ALPV in origin. These bands were resistant to digestion by RNAse A in 0.3 M NaCl but were sensitive to digestion by RNAse A in water, indicating that they were double-stranded in nature. Several additional bands were detected in low amounts in some dsRNA preparations. These had sizes ranging from 4.1 kb to the 10.1 kb.

The ALPV dsRNA profile differed from the RhPV dsRNA profile in that one major and two minor ALPV RNAs species were detected as opposed to two major and one minor RhPV RNA

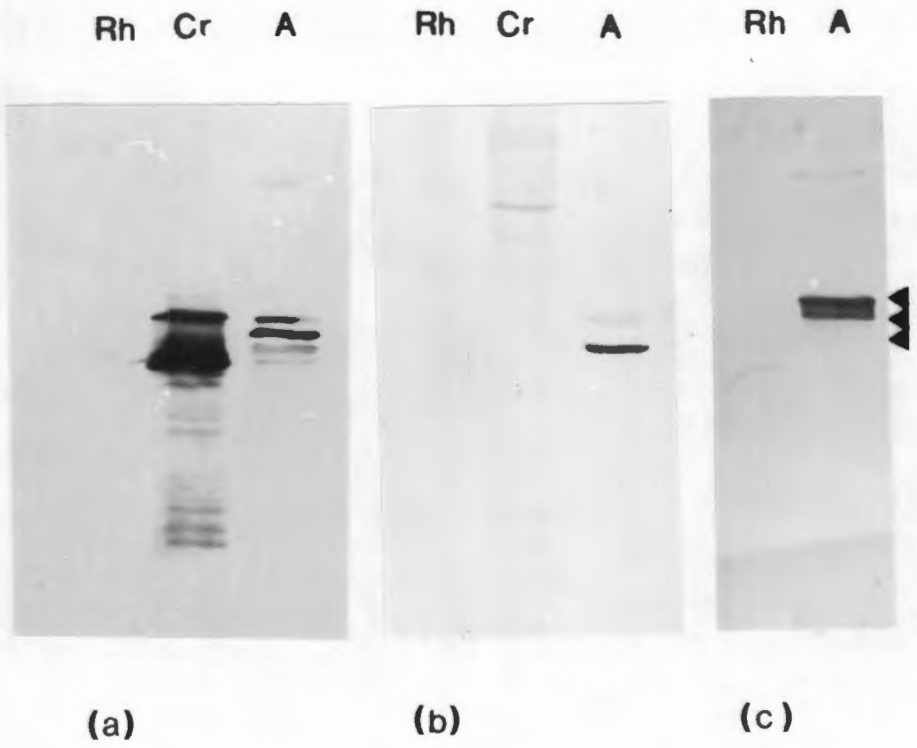


Figure 4.3
Immunoelectroblot of RhPV (Rh), CrPV (Cr) and ALPV (A) polypeptides fractionated by SDS-PAGE in a 15% gel.
a) Probed with anti-ALPV antiserum (1/200 dilution).
b) Probed with anti-CrPV antiserum (1/250 dilution).
c) Probed with anti-DCV antiserum (1/250 dilution).

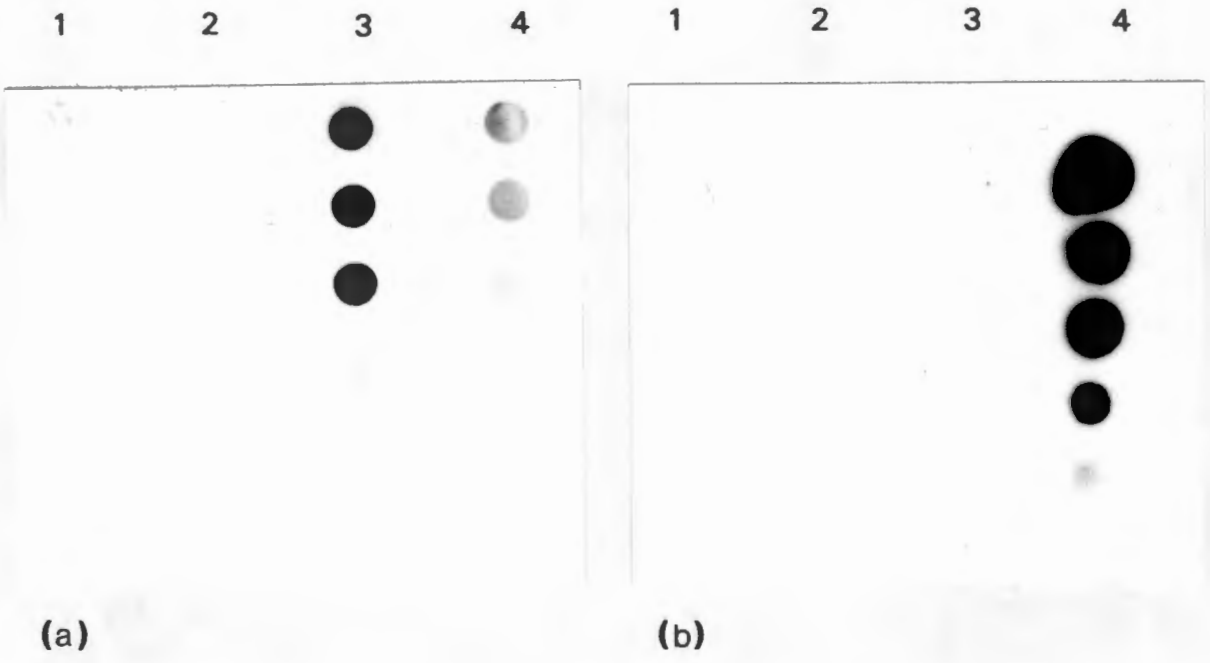


Figure 4.4
Dot-blot of a 10-fold virion dilution series with a starting concentration of 10 μg/dot.
Column 1, RhPV; column 2, OMV; column 3, CrPV, column 4 ALPV.
Probed with (a) CrPV antiserum (1/200 dilution) and (b) ALPV cDNA.

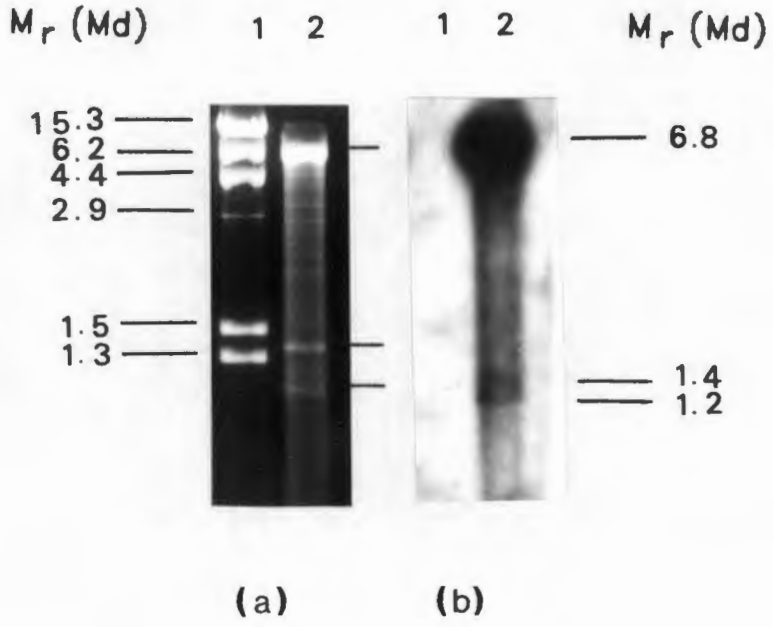


Figure 4.5

a) 1% agarose gel stained with ethidium bromide. Lane 1, Lambda DNA digested with endonuclease *Hin*dIII; Lane 2, ds RNA isolated from aphids infected with RhPV and ALPV. Markers on the RHS represent M_r of 6.8 Md, 1.4 Md and 1.2 Md.

b) Autoradiograph of a Northern-blotted 1% agarose gel probed with 5′ end-labelled ALPV virion RNA (10^6cpm). Lane 1, TMV dsRNA; Lane 2, ds RNA isolated from aphids infected with ALPV and RhPV.

species (Chapter 2, Fig. 2.). RhPV dsRNAs were 10.6 kb and 2.2 kb in size which is only slightly different from two of the ALPV dsRNA species (10.1 kb and 2.1 kb respectively). There was no evidence of RhPV dsRNAs in preparations isolated from aphids infected with both viruses. RhPV dsRNA bands may have been masked by ALPV dsRNA bands due to their M_r similarity. The inability to detect RhPV dsRNA may also be due to ALPV interfering with RhPV replication, resulting in lower levels of dsRNA synthesised. It has already been established that RhPV virions yields were lower when purified from aphids infected with both ALPV and RhPV compared with virus purified from aphids infected with only RhPV (see Chapters 2 and 3).

4.4 DISCUSSION

Three different aphid viruses were compared in this study: *Sitobion avenae* virus found in England (Allen and Ball, 1986); ALPV found in South Africa, and RhPV found in South Africa, USA and England (B. Ball, pers. comm.). No serological relationship was detected between these three aphid viruses. A comparison of the African ($RhPV_{OFS}$) and American ($RhPV_{ILL}$) isolates of RhPV indicate that, although they are physically distinct (Chapter 2), they are serologically indistinguishable. It is interesting that two viruses from such geographically well-separated locations should be so closely related, given that RhPV does not appear to be related to any other known insect virus. RhPV possibly spread through aphid populations some time after

its introduction into aphid populations. This spread may have been facilitated by man; preliminary results (von Wechmar, pers. comm.) have shown that RhPV can be detected in seeds collected from plants on which aphids had fed. RhPV could have been spread through the world by dissemination of contaminated seeds. It is however, possible, that RhPV spread, with aphids, from an original ancestral aphid stock. In this case, the two isolates would have been evolutionarily separated for a long period of time. Given the high mutation frequency generally observed for RNA genomes, together with the fact that the Aphidoidea evolved 280 million years ago (Dixon, 1985), this explanation seems highly unlikely.

The results obtained indicate that ALPV is distantly related to CrPV. Early bleeding of ALPV antisera did not react with CrPV, and later bleedings reacted only weakly; CrPV antiserum reacted weakly with ALPV; and both CrPV and DCV antisera recognised different ALPV proteins compared to the homologous antiserum in Western blots. The serological relationship between ALPV and CrPV - though distant - provides further evidence for the classification of ALPV into the picornavirus group. There is little or no direct sequence homology between ALPV and CrPV RNAs as ALPV cDNA did not detectably hybridize to CrPV RNA under moderately stringent hybridization conditions.

In addition to the assumed RF RNA, lower molecular weight dsRNAs (sub-RF) were also identified in both ALPV and RhPV infected aphids. Hybridization showed that these sub-RFs were viral in origin. Their exact function is not

known. It would be of interest to determine if they are translated *in vitro* and where they are located on the viral genome. A detailed study on other insect virus dsRNAs has not been done. However, a single CrPV dsRNA species was reported by Eaton and Steacie (1980). Sub-RF RNAs are often found in plants infected with monopartite viruses (Valverde *et al.*, 1986). It has generally been speculated that they are a result of premature termination of transcription or of defective interfering particles, although some of them have been associated with sub-genomic mRNA (Dawson, 1983).

CHAPTER 5

A COMPARATIVE STUDY ON THE TRANSLATION OF ALPV AND RHPV RNAs

SUMMARY

The genomic RNAs of ALPV and RhPV were both efficiently translated in rabbit reticulocyte lysates into predominantly high molecular weight products. ALPV RNA translated into primary translation products with molecular weights ranging from 92 K to 170 K. These underwent post-translational cleavage to form polypeptides with molecular weights comparable to those of the viral structural proteins. The 92 K polypeptide was antigenically related to the capsid proteins, indicating that it is a capsid protein precursor. RhPV RNA translated into products of molecular weights ranging from 45 K to 175 K. There was no evidence for post-translation cleavage of RhPV translation products. However, a 60 K polypeptide was precipitated with antiserum to RhPV virions, indicating that RhPV RNA translates a capsid precursor protein.

A COMPARATIVE STUDY ON THE TRANSLATION OF ALPV AND RHPV RNAs

5.1 INTRODUCTION

In recent years, the technique of *in vitro* translation in conjunction with SDS-PAGE has emerged as a powerful tool for the characterisation of viral RNA translation. Commercially available, cell-free translation kits provide an easy and efficient means of testing the translation of RNA preparations.

All picornaviruses have a similar translation strategy. Picornavirus RNA is translated into a polypeptide ("polyprotein"), from a single, strong initiation site, the size of which is approximately equal to the coding capacity of the genome. This polyprotein is subsequently cleaved into non-structural and structural proteins. Poliovirus precursor proteins are, for example, cleaved by at least two different viral proteases and one autocatalytic event (Toyoda *et al.*, 1986).

In this study, the *in vitro* translation of ALPV and RhPV RNA in rabbit reticulocyte lysates was compared. The time taken for the appearance of the major translation products was determined and processing of these products was investigated by pulse-chase experiments. These experiments involved the radiolabelling of translation products with ^{35}S-methionine for a short period of time, after which an excess of unlabelled methionine was added. Radiolabelled polypeptides produced after the initial labelling period would represent products of proteolysis and not of

translation. Further investigations on post-translation cleavage were done using the translation inhibitor cycloheximide, which inhibits the elongation of peptide chains. Polypeptides produced after the addition of cycloheximide therefore represent products of post-translation cleavage.

To determine whether the cleavage of viral polypeptides was protease mediated or autocatalytic, serial dilution experiments were done. The rate of a bimolecular protease-type reaction was assumed to be dependent on the concentration of the substrate and the enzyme, whereas an intramolecular, autocatalytic reaction would be concentration independent (Palmenberg and Rueckert, 1982).

Finally, the identification of polypeptides that were antigenically related to ALPV and RhPV capsid proteins was investigated by immunoprecipitation. The translation strategies of RhPV and ALPV and those of mammalian insect picornaviruses are discussed.

5.2 MATERIALS AND METHODS

5.2.1 Viral RNA Preparations

ALPV and RhPV were propagated as a mixed virus infection in *R. padi* and were purified (Appendix B.1) and separated by zone electrophoresis (Appendix B.2). RNA was isolated by SDS-disruption and phenol extraction (Appendix B.8).

5.2.2 Cell-Free Translation of Viral RNA

In vitro translation was performed in amino acid-depleted, nuclease-treated rabbit reticulocyte lysate (Amersham N150). The synthesis of radiolabelled polypeptides required the addition of messenger-sense RNA and a mixture of 19 unlabelled amino acids lacking L-methionine (Amersham, N133), supplemented with L-^{35}S-methionine (15 mCi/ml, 1443 Ci/mmol, Amersham SJ.1515). Prior to translation experiments, the integrity of the RNA was checked by agarose gel electrophoresis (Appendix B.10).

The optimisation of translation conditions

The optimum translation conditions were determined by varying a single parameter in a standard mix and then measuring the maximum trichloroacetic acid (TCA)-precipitable counts in 1.5 μl aliquots of translation mix after 60 min incubation (Section 5.2.3).

The optimum concentration of potassium ions was determined at a constant magnesium acetate concentration of 20 mM. Incorporation of ^{35}S-methionine, in translation mixes containing potassium acetate concentrations of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM, was determined.

Similarly, the optimum magnesium acetate concentration was determined at the optimum concentration of potassium ions for the specific RNA, as determined above. Incorporation of ^{35}S-methionine, in translation mixes containing magnesium acetate concentration of 1 mM, 1.25 mM, 1.5 mM, 1.75 mM and 2 mM, was determined.

The optimum RNA concentration was determined under conditions of optimum potassium and magnesium ion concentrations for the specific RNA. Translation mixes containing RNA concentrations of 1 μg/ml, 0.5 μg/ml, 0.25 μg/ml, 0.10 μg/ml, 0.05 μg/ml and 0.025 μg/ml were compared.

Standard translation mixture

Typically, the standard protein synthesis mixture contained 70% (v/v) lysate, 0.05 mM amino acid mixture minus L-methionine and 50 μCi L-^{35}S-methionine. The optimum concentrations of potassium, magnesium and RNA were added and the mixture was incubated at 30^{0}C.

5.2.3 Trichloroacetic Acid (TCA) Precipitation

Incorporation of ^{35}S-methionine into polypeptides was monitored by TCA precipitation. Three x 1.5 μl samples of the translation mixture were spotted onto separate GFC filter discs (Whatman), boiled for 5 min in 10% (w/v) TCA (Appendix A.2.13), and rapidly cooled by the addition of ice. The filters were rinsed twice with each of 5% (w/v) TCA and water, and once with each of 70% (v/v) ethanol and acetone. The filters were air-dried, placed in vials and 5 ml toluene-based scintillation fluid (Beckman Instruments) was added per disc prior to counting for ^{35}S for 5 or 10 min in a scintillation counter.

5.2.4 Kinetics

In a time-course analyses of the protein synthesis reactions, 10 μl samples were removed from a 200 μl translation mix at various times and disrupted immediately for analysis by SDS-PAGE. Comparable amounts of each sampled were loaded onto the gel.

5.2.5 Pulse Chase Experiments

Translation products were radiolabelled for 40 min with L-^{35}S-methionine (pulse translation mix). Unlabelled L-methionine was added to a final concentration of 5 mM and the translation mix incubated for a further 5 h (approx. 10^6 moles unlabelled L-methionine per mole L-^{35}S-methionine). The polypeptide profiles before and after addition of unlabelled L-methionine were compared by SDS-PAGE.

5.2.6 The Inhibition of Translation

The efficiency of the inhibition of translation by cycloheximide was determined as follows. Cycloheximide (Sigma) was used at a final concentration of 60 μg/ml. A standard translation mix containing ALPV RNA was prepared and divided into 3 samples. Cycloheximide was added to the first sample 15 min after the initiation of translation and to the second sample 20 min after the initiation of translation. An equivalent volume of water was added to the third sample 15 min after initiation of translation (control). The three reactions were monitored by TCA-

precipitable counts 0, 5, 10, 15, 20, 25, 30, 45 and 60 min after the initiation of translation.

The effect of cycloheximide on ALPV and RhPV RNA translation products was determined. These products were labelled for 35 min prior to the addition of cycloheximide and the reactions were subsequently incubated for a further 5 h. The polypeptide profile before and after the addition of cycloheximide were compared by SDS-PAGE.

5.2.7 The Effect of Concentration of Translation Products on Processing

In order to determine the effect of concentration of translation products on processing of ALPV polypeptides, translation mixes were diluted 1/5, 1/50 and 1/100 in NET buffer (Grubman, 1984; Appendix A.2.9), 45 min after the start of translation (Blackhurst, 1987). The reactions were incubated for a further 5 h, dialysed against water for 60 min on dialysis discs (0.025 μm pore, Millipore), lyophilised, resuspended in equal volumes of NET buffer and heated in SDS-PAGE disruption buffer (Appendix B.6). Equal volumes of each sample were analysed by SDS-PAGE

5.2.8 Immunoprecipitation

Protein A bacterial adsorbent (Miles-Yeda Ltd., Israel) refers to inactivated and hardened cells of *Staphylococcus aureus* Cowan I strain, bearing protein A on their surface. Protein A has a high specific adsorption capacity for IgG molecules.

Unless otherwise stated, all of the following manipulations and incubations were done at room temperature. The protein A bacterial adsorbent was washed by centrifugation and resuspended to 10% (w/v) in NETS-BSA-NP40 (0.15 M NaCl, 0.005 M EDTA, 1% w/v BSA, 0.05% v/v NP40 and 0.05 M Tris.HCl, pH 7.5). To 150 μl samples of a 10% (w/v) suspension of protein A bacterial adsorbent, was added either 40 μl of preimmune rabbit serum (preimmune-protein A bacterial adsorbent complex) or 40 μl of rabbit antiserum against viral capsids (immune-protein A bacterial adsorbent complex). After incubation for 60 min with shaking, the mixture was washed by centrifugation, 3 times, with 1 ml NETS-BSA-NP40 and resuspended in the original volume.

To eliminate non-specific immunoprecipitation, 60 μl of the *in vitro* translation products were diluted to 200 μl with NETS-BSA-NP40. A 10% (w/v) suspension (100 μl) of the preimmune-protein A bacterial adsorbent complex was added. After 60 min incubation with shaking, the bacterial adsorbent was removed by centrifugation for 1 min in an Eppendorf microfuge and the supernatant divided.

Viral proteins were immunoprecipitated from 100 μl of the supernatant by the addition of 100 μl immune-protein A bacterial adsorbent complex. To the second half of the supernatant, 100 μl of preimmune-protein A adsorbent complex was added (negative control). After 60 min incubation with shaking the bacterial adsorbent complex was washed four times by centrifugation with 1 ml NETS-BSA-NP40 buffer and the proteins eluted by dissociation at 95^0C in SDS-PAGE disruption mix (Appendix B.6). The bacterial ghosts were

removed by centrifugation and the immunoprecipitated products analysed by SDS-PAGE.

All immunoprecipitation reactions used an antiserum which reacted equally well with all major polypeptides, as determined in electroblot tests. Negative control reactions were done in parallel for each precipitation experiment using preimmune antiserum, these were analysed identically to the experimental samples.

5.2.9 SDS-PAGE

Translation products and immunoprecipitation products were analysed by SDS-PAGE (Appendix B.6) and autoradiography (Appendix B.7).

5.3 RESULTS

5.3.1 The Optimisation of Translation Conditions

The optimum Mg^{2+}, K^{+} and RNA concentrations for ALPV RNA translation were 1.75 mM, 200 mM and 50 μg/ml respectively.

The optimum Mg^{2+}, K^{+} and RNA concentrations for RhPV RNA translation were 1.5 mM, 80 mM and 100 μg/ml respectively.

Under optimum reaction conditions, TCA-precipitable counts were monitored at times 0, 15, 30, 45 and 60 min after the start of translation. For both ALPV and RhPV RNA translation, the incorporation of L-^{35}S-methionine was

linear for approximately 45 min of incubation, reaching a plateau by 60 min of incubation (Fig. 5.1). Typically, ^{35}S-methionine incorporation into precipitable products was approximately 60-fold above background for ALPV and 25-fold above background for RhPV, where "background reactions" were identical to experimental reactions except that the RNA was replaced by water.

5.3.2 Kinetics of the Synthesis of ALPV and RhPV RNA Translation Products

In a time-course analysis of ALPV RNA translation products, a 92 ± 4 K polypeptide (four determinations) appeared within 20 min after initiation of translation, and translation of the high molecular weight products was complete by 45 min. At this time the major translation products were the 92 K polypeptide and polypeptides with a M_r range from 110 to 170 K. Minor amounts of a 58-60 K doublet, a 48-50 K doublet, and a 30 K polypeptide were also present. Longer incubations (6 h) resulted in the appearance of four additional polypeptides. These were a 70-71 K doublet, 41.5 ± 0.5 K, 35 ± 1 K and 32 ± 1 K polypeptides (four determinations). The appearance of these polypeptides was accompanied by the synchronous decrease in band intensities of the high M_r polypeptides (110 K to 170 K and the 92 K polypeptide) (Fig. 5.2). No polypeptides were present in the negative control lysate in which water was substituted for RNA.

In a time-course analysis of RhPV RNA translation products, a 45 ± 0.5 K polypeptide (three determinations)

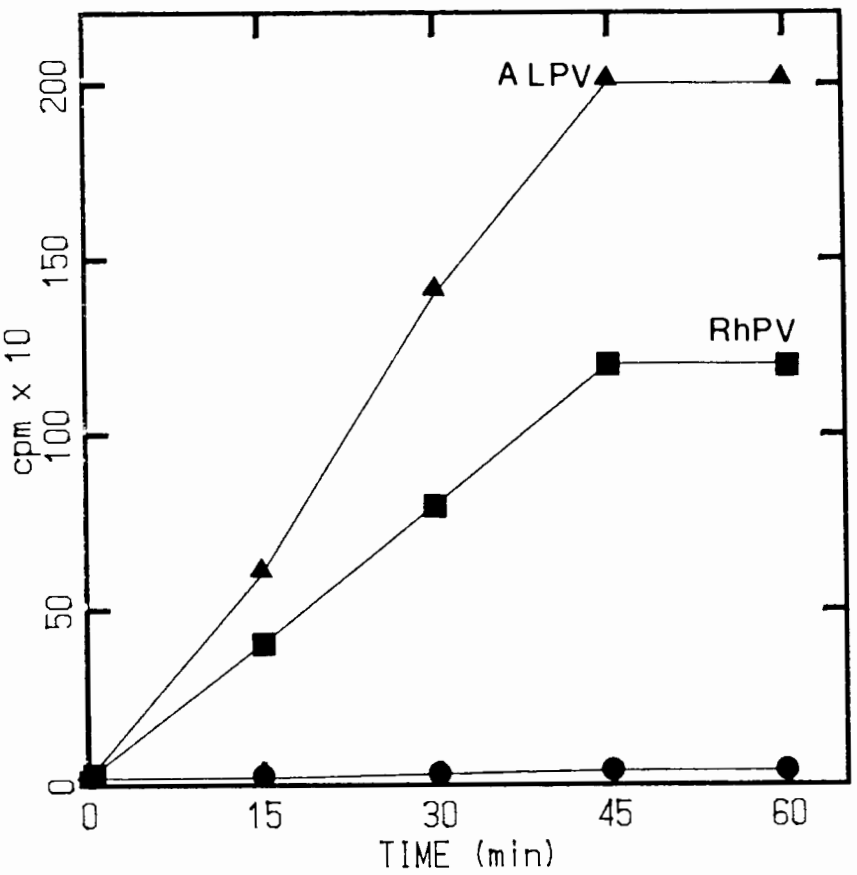


Figure 5.1

The efficiency of translation in a standard translation mix, monitored by the incorporation of ^{35}S-L-methionine into TCA-precipitable protein. Samples (3 x 1.5 µl) taken at times 0, 15, 30, 45 and 60 min. (▲) ALPV translation mix containing 1.75 mM Mg^{2+}, 200 mM K^{+} and 50 µg/ml RNA; (■) RhPV translation mix containing 1.5 mM Mg^{2+}, 80 mM K^{+} and 100 µg/ml RNA; (●) negative control translation mix containing 1.5 mM Mg^{2+}, 80 mM K^{+} and no RNA.

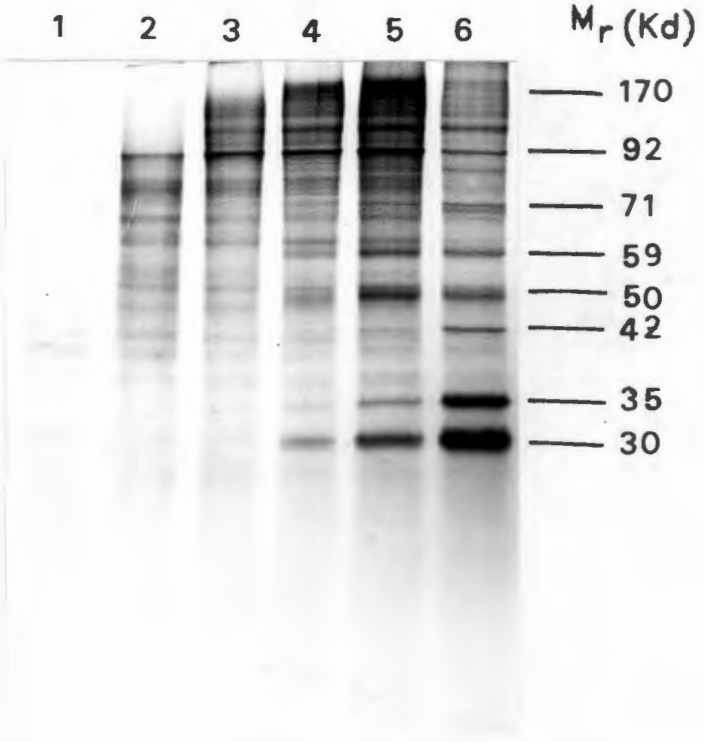


Figure 5.2
Autoradiograph showing the kinetics of appearance of ALPV translation products. Polypeptides were fractionated by SDS-PAGE in a 12.5% gel. RNA was translated under optimum conditions and samples were taken after 10, 20, 30, 40, 60 and 360 min, corresponding to lanes 1 to 6.

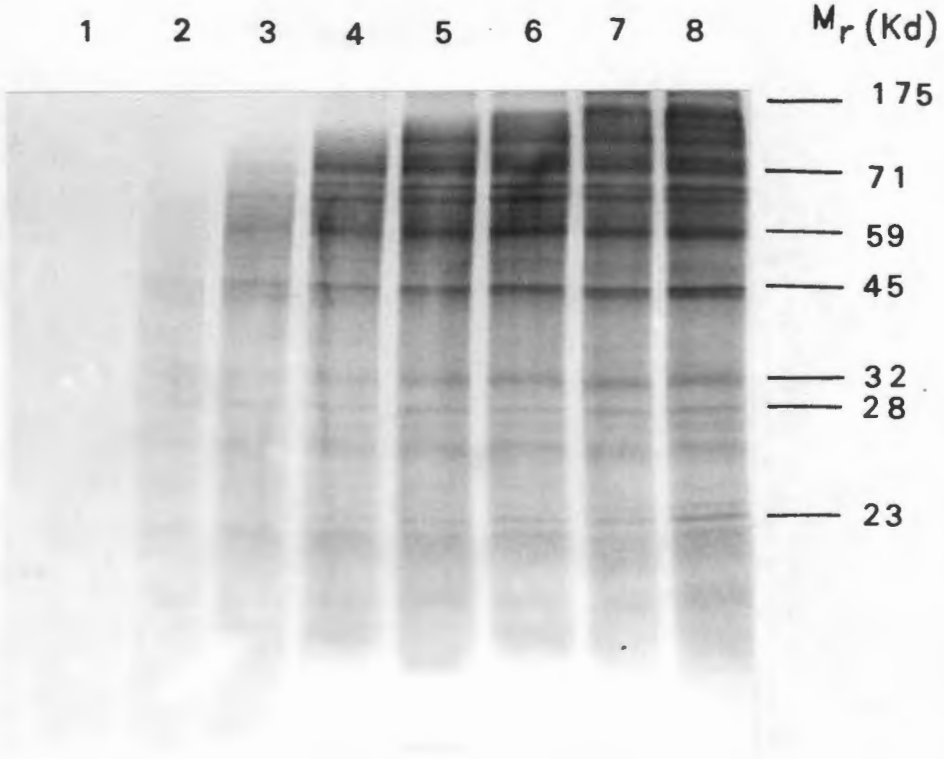


Figure 5.3
Autoradiograph showing the kinetics of appearance of RhPV translation products. Polypeptides were fractionated by SDS-PAGE in a 15% gel. RNA was translated under optimum conditions. Samples were taken at 5, 10, 15, 20, 25, 30 45 and 60 min, corresponding to lanes 1 - 8.

and products of M_r ranging from 59 ± 2 K to 81 ± 3 K (three determinations) were seen 20 min after initiation of translation. Translation of high M_r products was complete by 45 min with the highest M_r translation product being 175 ± 5 K (3 determinations). At this time the major products had M_rs of 45 K; 47 ± 1 K; 59 K; 64 ± 1 K, 68 ± 1 K, 71 ± 2K, 77 ± 2 K (three determinations). There was also a range of products with M_rs values between 81 K and 175 K. Minor amounts of 23 K, 28 K and 32 K polypeptides were also present, the latter two having a similar M_r to the viral capsid proteins (Fig. 5.3). This polypeptide profile remained unchanged after prolonged incubation (6 h and 16 h) (results not shown). No polypeptides were present in the negative control lysate in which water was substituted for RNA.

5.3.3 **Pulse-Chase Experiments**

The ALPV polypeptide profiles were different prior to and after the addition of the unlabelled L-methionine (Fig. 5.4). Five hours after the addition of unlabelled methionine, the amount of the 110 K to 170 K polypeptides and the 92 K polypeptides had dramatically decreased with a concomitant appearance of polypeptides with M_r 71 K, 42 K, 36 K, 35 K and 32 K, and an increase in the relative concentration of the 30 K polypeptide. The relative polypeptide concentrations remained unchanged from 6 h to 16 h incubation, indicating that they were stable products (results not shown).

The general trend from high molecular weight polypeptides to lower molecular weight polypeptides is demonstrated by densitometer scans of the polypeptide profiles (Fig. 5.5). The changes in the amounts of polypeptides were quantitatively analysed from the areas of individual polypeptide peaks from densitometer traces (Table 5.1).

A comparison of the polypeptide profiles of the RhPV before and after the addition of unlabelled L-methionine to the translation mix showed no apparent change in banding patterns (Fig. 5.6).

5.3.4 Inhibition of Translation with Cycloheximide

Reaction mixes in which cycloheximide was added 15 min and 20 min after the start of translation showed linear incorporation for 15 min and 20 min respectively and reached a plateau at 25 and 30 min respectively. Cycloheximide, therefore, completely inhibited translation of ALPV RNA within 10 min of addition, whereas the control reaction showed linear incorporation of L-^{35}S-methionine for approximately 45 min (Fig. 5.7).

The ALPV polypeptide profiles of a translation mix incubated for 45 min differed from the polypeptide profile of the translation mix to which cycloheximide was added at 35 min and incubated further. After 5 h incubation, the amounts of 110 K to 170 K polypeptides and the 92 K polypeptides had dramatically decreased in amount (Table 5.1). This decrease of high M_r products was associated with a concomitant appearance of polypeptides with M_r 71 K, 42 K, 36 K, 35 K

and 32 K and an increase in the 30 K polypeptide (Fig. 5.4). This trend is demonstrated on densitometer scans of the polypeptides profiles (Fig. 5.5). These results are effectively identical to those obtained in the pulse-chase experiments.

In a similar experiment on RhPV, no difference in polypeptide profiles were observed before and after the addition of cycloheximide (Fig. 5.6).

Table 5.1. A comparison of the relative amounts of ALPV polypeptides present before and after the addition of unlabelled L-methionine or cycloheximide. The relative amounts of polypeptides was determined by measurements of the peak areas from densitometer scans

Polypeptide M_r (Kd)	Percentage area		
	45 min pulse	45 min pulse/ 5 h chase	45 min pulse/ 5 h inhibition
110-170	49	4	8
92	13	3	7
71	<4	6	7
60	4	9	6
50	6	10	14
42	-	6	6
36	-	6	4
35	<4	18	10
32	-	6	5
30	8	35	23

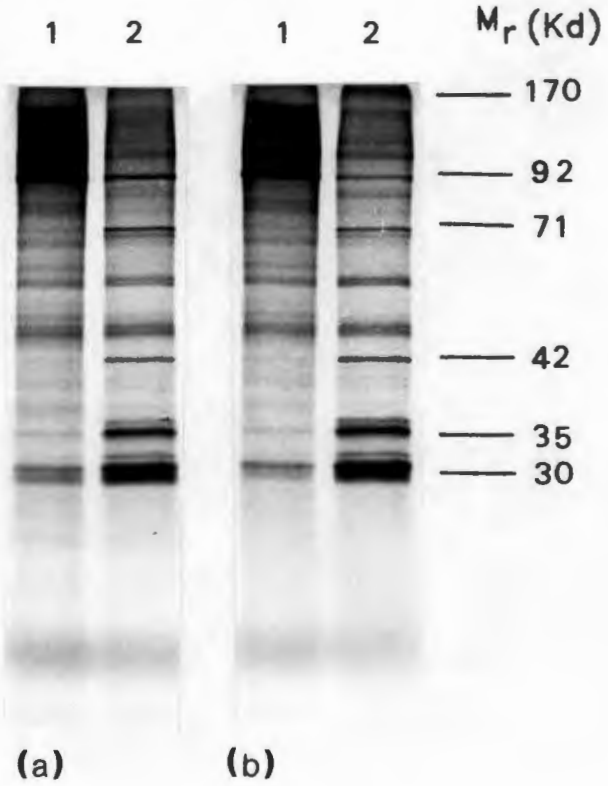


Figure 5.4

Autoradiograph showing the effects of pulse-chase and cycloheximide on ALPV translation products. Equal volumes of each sample were analysed by SDS-PAGE in 12.5% gels.

a) Lane 1, RNA was translated under standard conditions for 45 min with ^{35}S-methionine; lane 2, RNA was translated for 40 min with ^{35}S-methionine, adjusted to a final concentration of 5 mM unlabelled methionine and analysed after 5 h incubation.

b) Lane 1, RNA translated for 45 min under standard conditions; lane 2, RNA translated for 35 min, adjusted to a final concentration of 60 μg/ml cycloheximide and incubated for a further 5 h before analysis.

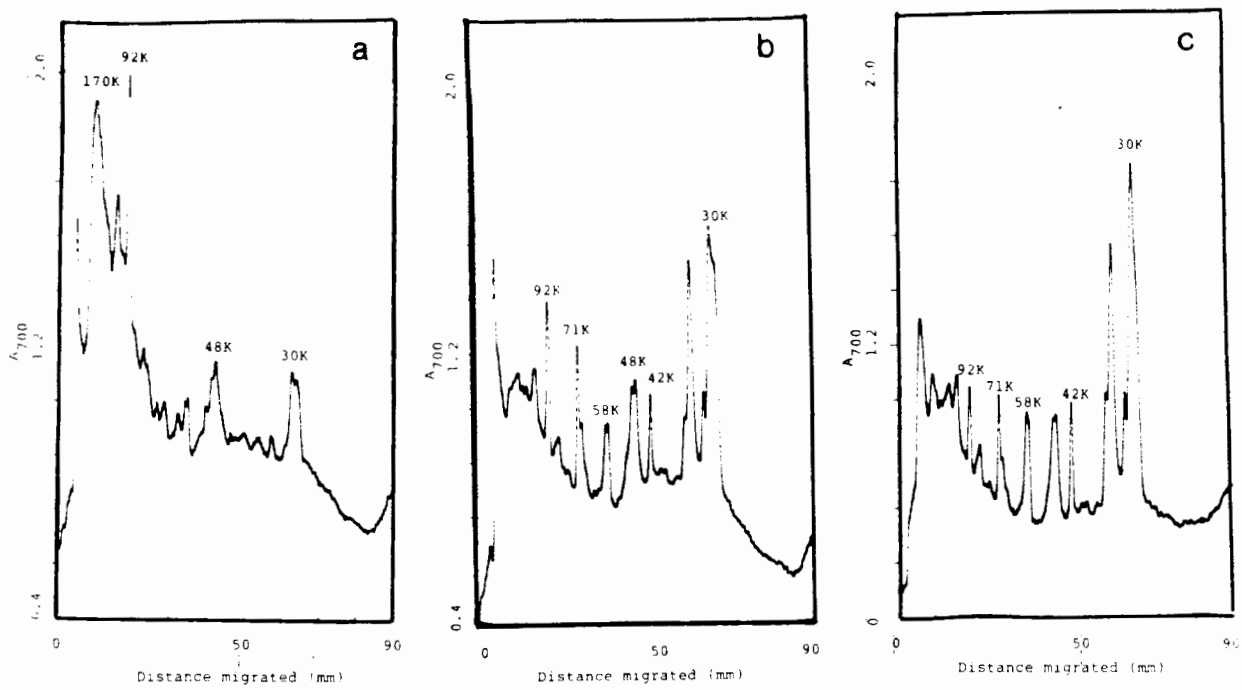


Figure 5.5
Densitometer scan of autoradiographs shown in Fig 5.4
a) ALPV RNA translated for 45 min with ^{35}S-methionine.
b) ALPV RNA translated for 40 min with ^{35}S-methionine, adjusted to a final concentration of 5 mM unlabelled methionine and analysed after 5 h incubation.
c) ALPV RNA translated for 35 min with ^{35}S-methionine, adjusted to a final concentration of 60 μg/ml cycloheximide and analysed after 5 h incubation.

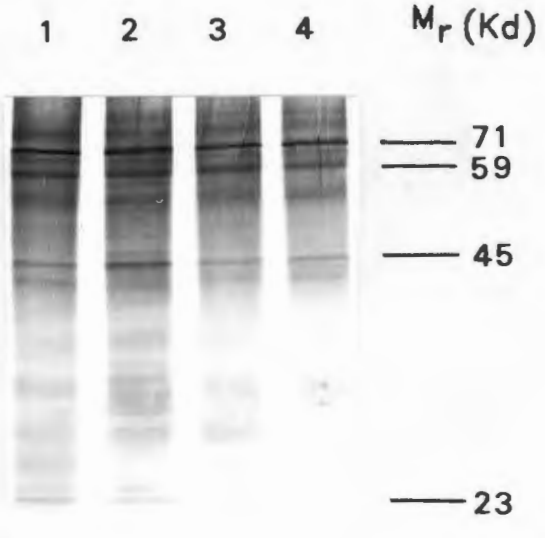


Figure 5.6

Autoradiograph showing the effects of pulse-chase and cycloheximide on RhPV translation products. Equal volumes of each samples were analysed by SDS-PAGE in 12.5% gels.

Lane 1, RNA was translated under standard conditions for 45 min with ^{35}S-methionine; lane 2, RNA was translated for 6 h with ^{35}S-methionine; lane 3, RNA was translated for 40 min with ^{35}S-methionine, adjusted to a final concentration of 5 mM unlabelled methionine and analysed after 5 h incubation; lane 4, RNA translated for 35 min, adjusted to a final concentration of 60 μg/ml cycloheximide and incubated for a further 5 h before analysis.

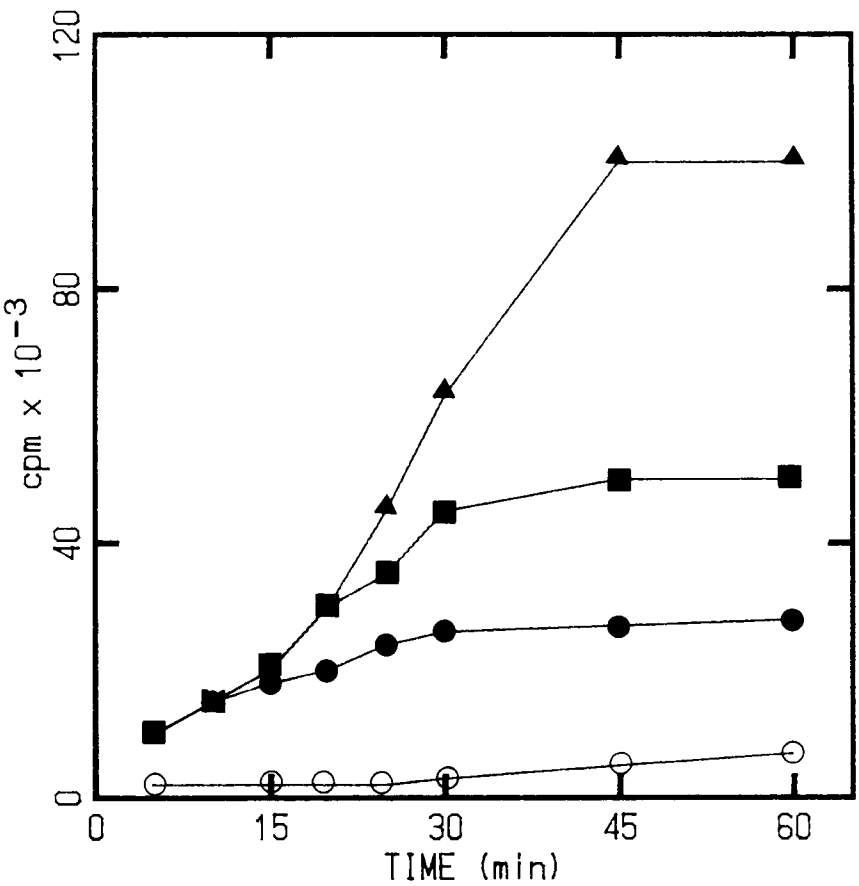


Figure 5.7
The effect of addition of 60 μg/ml cycloheximide on the translation of ALPV RNA, monitored by TCA precipitation. Three x 1.5 μl samples taken at times indicated. Cycloheximide added at 15 min (●) and 20 min (■). Positive control to which no cycloheximide added (▲). (○) Negative control to which no RNA was added.

5.3.5 The Effect of the Concentration of Translation Products on Processing

The ALPV polypeptide profiles of a range of concentration of translation products were compared to that of an undiluted control reaction. Decreasing concentration of translation products resulted in a concomitant decrease in the amount of the 42 K, 35 K and 31 K polypeptides (Fig. 5.8).

As no evidence of post-translational cleavage was obtained for RhPV RNA translation products in the pulse chase or translation inhibition experiments, the effect of concentration of translation products was not investigated for this virus.

5.3.6 Immunoprecipitation of Translation Products

Antibodies to ALPV particles precipitated the 92 K polypeptide from a translation that had been incubated for 60 min. Minor amounts of the 32 K polypeptide were precipitated with both the anti-ALPV antiserum and preimmune serum (Fig. 5.9a).

Antibodies to ALPV particles precipitated the 92 K and 36 K polypeptides and minor amounts of the 42 K and 32 K polypeptides from a translation mix that had been incubated for 6 h (Fig. 5.9b). No polypeptides were precipitated with preimmune antiserum.

Antibodies to RhPV particles precipitated a 60 K polypeptide from a translation mix which had been incubated for 60 min (Fig. 5.10). No polypeptides were precipitated with preimmune antiserum.

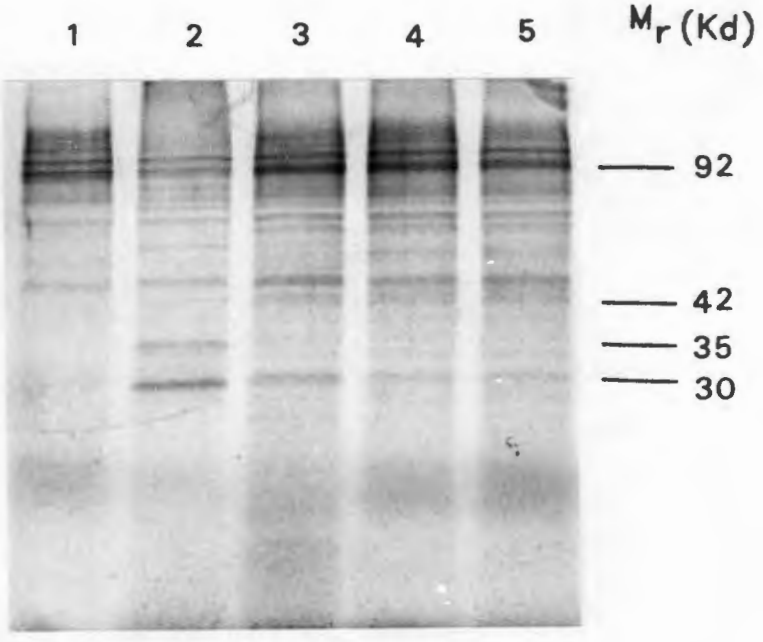


Figure 5.8

Autoradiograph to show the effect of the concentration of translation products on processing. Polypeptides fractionated by SDS-PAGE in a 12.5% gel.
Lane 1, ALPV RNA translation mix after 45 min incubation; lane 2, ALPV RNA translation mix after 5 h incubation.
After 45 min the translation mix was diluted in NET buffer (A.2.9). Lane 3, 1/5 dilution; lane 4, 1/50 dilution; lane 5, 1/100 dilution.

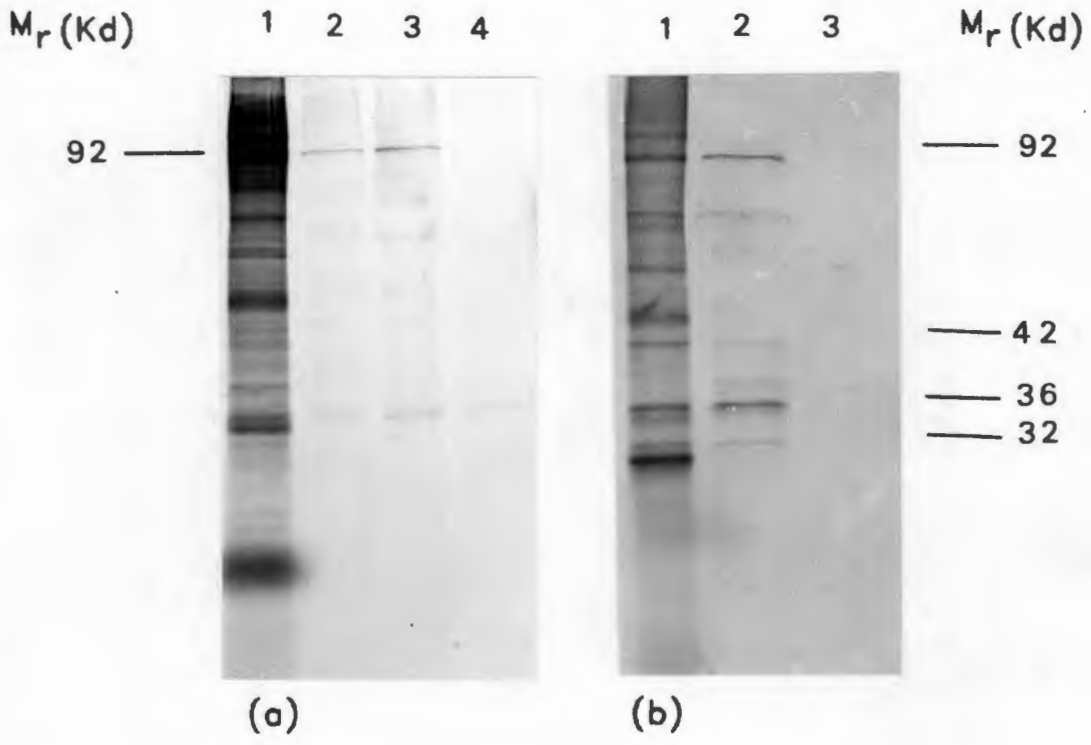


Figure 5.9

Autoradiograph to illustrate immunoprecipitation of ALPV translation products. Polypeptides were analyzed by SDS-PAGE in 12.5% gels.

a) Lane 1, Translation mix incubated for 60 min; lanes 2 and 3, immunoprecipitation with anti-ALPV antiserum from a translation mix incubated for 60 min; lane 4, immunoprecipitation with preimmune serum from a translation mix incubated for 60 min.

b) Lane 1, translation mix incubated for 6 h; lane 2, immunoprecipitation with anti-ALPV antiserum from a translation mix incubated for 6h; lane 3, immunoprecipitation with preimmune serum from a translation mix incubated for 6 h.

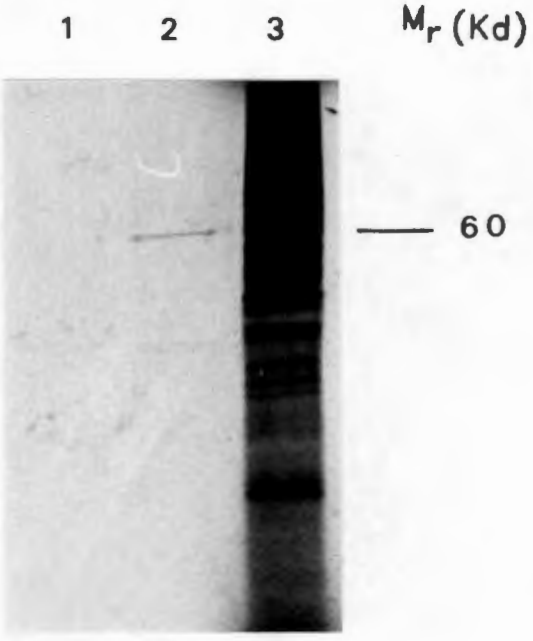


Figure 5.10
Autoradiograph to illustrate immunoprecipitation of RhPV translation products. Polypeptides were analyzed by SDS-PAGE in a 12.5% gel.
Lane 1, immunoprecipitation with preimmune serum from a translation mix incubated for 60 min; lane 2, immunoprecipitation with anti-RhPV antiserum from a translation mix incubated for 60 min; lane 3, translation mix incubated for 60 min.

5.4 DISCUSSION

ALPV and RhPV have many biophysical and biochemical properties in common with mammalian picornaviruses, and ALPV is serologically related to the insect picornaviruses - DCV and CrPV (Table 3.1 and 4.1). However, allocation to this group can be confirmed "...only if their RNA content and strategy for multiplication accord with those features described as characteristic of the *Picornaviridae*" (Fenner, 1976).

Pulse-chase experiments indicate that high M_r products of ALPV RNA, including the 92K putative capsid precursor, were post-translationally cleaved to lower molecular weight products. These included polypeptides of M_r 42 K, 36 K and 32 K. Cycloheximide had no effect on the appearance of these polypeptides, supporting the conclusion that these polypeptides are products of post-translational cleavage. These polypeptides are presumably equivalent to the capsid proteins VP0, VP3 and VP1 as they have similar M_rs to these proteins and small amounts were immunoprecipitated with antiserum to ALPV virions. The 92K polypeptide is not large enough to be equivalent to the picornavirus P1 capsid protein precursor and is too large to be the VP1/VP3 precursor protein. It is possible that the post-translational cleavage of ALPV polypeptides follows an alternative pathway to those of picornaviruses. A comparison of peptide maps of the capsid proteins and the polypeptides synthesised *in vitro* would confirm these

speculations. As the processing of ALPV polypeptides was concentration-dependent, cleavage probably required at least one protease. It was not determined whether this putative protease activity was of viral or lysate origin.

Although the ALPV 35K and 30K polypeptides were present in much higher concentration than the 32K and 36K proteins, they were not precipitated with antiserum to ALPV particles. These are either non-structural proteins, or products of premature termination of translation and/or aberrant proteolysis.

Cell-free translation of RhPV RNA obviously differs from that of ALPV, as no post-translational cleavage products were identified in pulse-chase and translation inhibition experiments done on this virus. However, the identification of a 60 K putative VP1/VP3 capsid precursor protein indicated that processing does occur. There are three possible reasons why processing was not detected in this system. Firstly, proteolysis of RhPV precursor proteins *in vitro* could be very efficient and occur very rapidly. Detection of proteolysis would therefore require the use of one of the following: protease inhibitors; amino acid analogues that are incorporated into newly synthesized polypeptides but inhibit proteolysis; or a label such as N-formyl(^{35}S)methionyl-tRNA^{MET}(f-Met) in which the tRNA donates its amino acid specifically to the amino terminus of initiated polypeptides, resulting in an unlabelled cleaved polypeptide containing the carboxy terminus. Secondly, as there is no apparent accumulation of capsid proteins, it is possible that the normal processing pathway which gives rise

to these proteins did not occur *in vitro*. It is possible that unlike mammalian picornaviruses, but similar to CrPV (Reavy and Moore, 1983a), a cellular protease plays a role in the processing. Thirdly, RhPV RNA may not be faithfully translated *in vitro* because it requires the supplementation of additional factors such as tRNA, dithiothreitol or other unidentified factors which are present in aphids. Aberrant internal initiation was shown to occur with *in vitro* translation of poliovirus RNA, for example, which could be corrected by factors present in HeLa cell extract (Dorner *et al.*, 1984). Supplementing lysate with tRNA or dithiothrietol is also well known to assist translation and stimulate proteolysis (Gupta *et al.*, 1985 and Pelham, 1979 respectively).

A putative VP0 was identified as an ALPV translation product. No VP0 has been identified in RhPV, CrPV or DCV *in vitro* translation mixes (Reavy and Moore, 1981b; Reavy and Moore, 1983b). Like CrPV (Reavy and Moore, 1981a,b), ALPV translated only two putative capsid proteins. Picornaviruses also translate only two of the four capsid proteins *in vitro*. The remaining two are generated when VP0 is processed during the final stages of virion morphogenesis (Arnold *et al.*, 1987). It would be of interest to establish if ALPV and RhPV virion morphogenesis occurs *in vitro* and if assembly is a prerequisite for the cleavage of ALPV VP0. It would also be of interest to determine if proteolytic cleavage of precursor proteins occurs at glutamine-glycine and tyrosine-glycine sites as has been determined for poliovirus (Semler *et al.*, 1987).

Similar to CrPV, DCV and other picornaviruses (Moore *et al.*, 1985; Palmenberg, 1987), the RNA of ALPV and RhPV is positive sense and translates primarily into high M_r products. The M_r aggregate of ALPV and RhPV polypeptides exceeded the coding capacity of the genomes indicating that proteolysis plays a role in the production of some polypeptides. The occurrence of post-translation cleavage is supported by the detection of presumed capsid precursor proteins which were identified for both viruses by immunoprecipitation. These results strengthens the case for the inclusion of both ALPV and RhPV in the family *Picornaviridae*.

CHAPTER 6

GENERAL OBSERVATIONS ON THE BIOLOGY OF ALPV AND RHPV

SUMMARY

ALPV and RhPV were detected in natural aphid populations in the major small grain-producing regions in South Africa. Both viruses were detected in the aphids *R. padi*, *Schizaphis graminum*, *Metapolophium dirhodum*, *Sitobion avenae* and *Diuraphis noxia*. ALPV and RhPV double-infections caused a significant decrease in longevity and fecundity of infected *R. padi*. The viruses were horizontally and vertically transmitted through aphid populations, and plants and predators were implicated in virus dissemination. Investigations showed that both viruses could cause inapparent or "latent" infections. A comparison of nucleic acid hybridization and ELISA methods for virus detection showed hybridization to be more sensitive for the detection of virus in single aphids.

GENERAL OBSERVATIONS ON THE BIOLOGY OF ALPV AND RHPV

6.1 INTRODUCTION

Although RhPV and ALPV have been characterised physically, their biology has not been as well studied. It is known that RhPV is horizontally transmitted between aphids and vertically transmitted to 15-28% of the next generation (D'Arcy *et al.*, 1981b; Williamson, 1983; Gildow and D'Arcy, 1988). RhPV has been detected in South Africa (Rybicki, 1984), the United States (D'Arcy *et al.*, 1981b), England (B. Ball, Rothamsted Experimental Station, UK pers. comm.) and possibly Sweden (Eweida and Oxelfelt, 1985). It infects several aphid species including *R. padi*, *Schizaphis graminum*, *Metapolophium dirhodum*, *R. rufiabdominalis* (D'Arcy *et al.* 1981b) and *R. maidis* and *Diuraphis noxia* (von Wechmar and Rybicki, 1981; Rybicki and von Wechmar, 1984). RhPV infected *R. padi* aphids were shown to have reduced longevity and fecundity (D'Arcy *et al.* 1981b, Rybicki, 1984). However, in this laboratory, general observations indicate that RhPV has become less pathogenic after prolonged propagation under laboratory conditions (Rybicki, 1984; von Wechmar and Williamson, unpublished data). This could be due to the development of "tolerant aphid clones", or the selection of a mild strain.

Problems encountered in obtaining aphids infected with RhPV only resulted in investigations on the possibility that either or both viruses occur as inapparent or latent virus infections. Latency plays an important role in the

persistence of viral diseases, a typical example being that of herpesvirus in humans (Baichwal and Sugden, 1988). The latent state is characterised by the ability of a virus to persist in the host or host generations without causing recognisable symptoms. Latent viruses are known to occasionally switch from a latent state to pathological activity. Podgwaite and Mazzone (1986), in a recent review on latency of insect viruses, used the term "latency" as synonymous with "inapparent infection". However, the size of aphids makes it difficult to assess symptoms unless they are severe. For this reason the term "inapparent infection" will be used when referring to viral infections that could not be detected symptomatically or by the current viral detection methods. A number of factors are known to activate these infections, including temperature changes, food source, overcrowding, UV irradiation, and exposure to foreign proteins and chemicals (Podgwaite and Mazzone, 1986).

The apparent lethal effect of ALPV and RhPV infections stimulated interest in these viruses as possible viral pesticides (Chapter 3.1). A complete investigation of factors influencing the use of these viruses as biological control agents was beyond the scope of this project. However, as these viruses were propagated in aphids, it was imperative to have some knowledge of their effect on the aphids and the factors influencing virus propagation. This chapter presents information on the pathogenicity, host range, distribution, incidence and mode of transmission of RhPV and ALPV. Finally, different methods of virus

detection in aphids were compared. Due to difficulties experienced in obtaining aphids infected with only one virus, all investigations were performed with aphids co-infected with both ALPV and RhPV. Subsequent to this study, a *R. padi* clone was isolated which was infected with ALPV only (von Wechmar, Department of Microbiology, University of Cape Town, South Africa, unpublished data). These aphids developed similar symptoms to those described for RhPV and ALPV infections (Section 3.1).

6.2 METHODS

6.2.1 Aphid Maintenance and Propagation

Details of the origin of the aphid clones, and of aphid maintenance and propagation, are given in Appendix C.

6.2.2 Virus Detection

Unless otherwise stated, virus detection was by DAS-ELISA (Appendix B.14) using antisera specific for either RhPV or ALPV (Appendix B.11). The minimum level of purified antigen reliably detected by DAS-ELISA was approximately 10 ng/well (200 μl).

6.2.3 Distribution

In a collaborative study, aphids were collected by M.B. von Wechmar (Department of Microbiology, University of Cape Town, South Africa) from the major small grain-producing

regions around South Africa. Aphid samples were either tested immediately or stored at -20^{0}C. Air-dried or live aphids were also provided by K. Aalbersberg (Small Grain Center, Bethlehem, Orange Free State) and one sample was supplied by P. Cronje (University of the Orange Free State, South Africa).

In a retrospective study, aphids stored at -20^{0}C since 1979 were screened for both viruses (aphids supplied by M.B. von Wechmar). Where possible, samples were assayed in duplicate with a single sample consisting of more than 20 aphids.

Another retrospective study involved testing for the presence of virus indirectly, by screening for ALPV and RhPV antibodies in antisera prepared against aphid extracts and against extracts of plants that had been associated with aphids during 1978-84 (antisera prepared by M.B. von Wechmar). Antisera were screened by immunoelectroblotting (Western blotting) (Appendix B.15).

6.2.4 **Incidence**

In order to monitor the incidence of both viruses in laboratory-propagated and field-collected aphids, single aphids were crushed in 400 μl of PBS-T-MP (Appendix A.2.6); 170 μl was screened by DAS-ELISA for ALPV, and 170 μl was screened for RhPV.

6.2.5 **Vertical Transmission**

The vertical transmission of ALPV was monitored using mature *R. padi* infected with ALPV and RhPV. Nymphs were

removed from the mother on birth prior to feeding. Individual nymphs were either screened by DAS-ELISA for virus immediately or were maintained on barley leaves in Petri dishes and screened after one week. No attempts were made to dissect aphids and screen for virus in embryos.

6.2.6 **Horizontal Transmission**

Virus transmission by leaf surface contamination

Infected aphids were homogenised in a 10% (w/v) sucrose solution made in 0.01 M phosphate buffer, pH 7.0 (Appendix A.2.1) and the homogenate was painted onto plants. Alternatively, purified virus (Appendix B.1) was resuspended (1:1 v/v) in sucrose solution and sprayed onto plants with an atomiser. Aphids were fed on the surface-contaminated plants for approximately one week, transferred to virus-free plants and propagated for approximately 10 days before testing for virus by DAS-ELISA.

Virus transmission via plants

Plants maintained in growth chambers at 7-10^{0}C (Appendix C.2) were colonised with infected aphids for two weeks, sprayed with insecticide, placed in the plant growth room and rinsed daily until used. Harvested leaves were rinsed under running water for 30 min and confirmed to be free of aphid remains by stereo-microscopy. Healthy aphids were either fed on harvested and washed leaves that had been free of virus-infected aphids for 24 h or fed on plants that had been free of virus-infected aphids for three weeks.

Aphids were tested for virus after approximately 10 days by DAS-ELISA.

Virus transmission via aphid predators

Praying mantids (Mantodea: *Sphodromantis gastrica*) were fed on a diet of infected *R. padi* for one to four weeks before being transferred to a diet of either virus-free *R. padi* or virus-free *M. persicae*. Aphids and praying mantids were tested for virus after approximately 10 days by DAS-ELISA.

6.2.7 **Host Range**

Aphids

Different aphid species were artificially infected with virus by feeding them on plants that had been surface contaminated with virus as described above (Section 6.2.6). Aphids were transferred to virus-free plants and propagated for up to three weeks prior to virus detection by DAS-ELISA.

Plants

Plants on which infected aphids had fed (Section 6.2.6) three weeks previously, were processed for purification of virions (Appendix B.1) or for dsRNA (Chapter 2.2.5). The dsRNA extract was analysed by agarose gel electrophoresis (Appendix B.10).

6.2.8 Effect of Virus Infection on Longevity and Fecundity

Aphids

To reduce the potential effect of aphid response to virus infection due to genetic differences, all pathogenesis studies were performed using aphids from a colony established from a single adult by parthenogenesis. *R. padi* Stellenbosch clone (Appendix C.1) was used in all pathogenesis experiments. Aphids were maintained in Petri dishes on leaves (Appendix C.2.3), under temperature and light conditions of 22-24^{0}C and 12h day/night respectively. Aphids of the same age were collected by removing mature females from the stock colony and placing them on barley leaves in a gauze-topped container. The barley leaves were either virus-free or sprayed (Section 6.2.6) with a purified virus preparation of RhPV and ALPV (Appendix B.1). After approximately 16 h, the mature females were removed and the nymphs left to develop. After 3 days the nymphs were placed individually in Petri dishes (Appendix C.2.3).

Longevity and Fecundity

Individual aphids were monitored daily and the number of live aphids and the number of nymphs per adult aphid were counted and scored. Thereafter, nymphs were removed from the Petri dish. Dead aphids were tested for virus by DAS-ELISA (Appendix B.14). No attempts were made to dissect adults and determine the number of nymphs in aphids.

6.2.9 Radioisotope Labelling of Nucleic Acid Probes

ALPV RNA was primed with hexanucleotide random primers and cDNA was synthesized in the presence of $^{\alpha-32}$P-dCTP as described previously (Section 2.2.13).

Single-stranded RNA preparations were partially hydrolysed and 5′ end-labelled with ^{32}P as described (Section 2.2.18).

6.2.10 Dot-Blot Hybridization

Aphids (one or two aphids per sample) were crushed in 100 µl phosphate buffer, pH 7.0. Where applicable, half of each sample (50 µl) was screened for virus by DAS-ELISA (Appendix B.14). The remaining 50 µl of aphid extract was adjusted to 50 mM NaOH and incubated at room temperature for 10 min before neutralisation of the samples with 1 M Tris.HCl, pH 7.4. The total volume was spotted onto nylon membrane (Hybond-N, Amersham) using a 96-well micro-sample filtration manifold (Minifold, Schleicher and Schuell). The membrane was rinsed (2 x 2 min) in chloroform and air-dried, and the RNA was cross-linked to the membrane by exposure to a 254 nm UV transilluminator for 4 min. Membranes were probed with either radiolabelled cDNA, or recombinant plasmid probes as described (Section 2.2.22). Virus samples were adjusted to a final concentration of 1% (w/v) SDS and disrupted by heating for 5 min at 95^{0}C. Serial dilution of disrupted virus or purified genomic RNA were usually included on blots as a positive control and to monitor sensitivity of detection. All blots contained a

nucleic acid positive control (virion RNA and/or virus-infected aphids) and a nucleic acid negative control (heterologous RNA and/or virus-free aphids). In latency tests *Myzus persicae* aphids were used as negative controls as this aphid species does not host ALPV or RhPV.

6.2.11 **Investigations of Inapparent Infections**

Inapparent infections of field-collected aphids

Aphids collected from the field were screened in bulk (more than 20 aphids per sample) for virus by DAS-ELISA before and after laboratory propagation of aphids on barley plants in cages kept at 24-26^{0}C as described in Appendix C.2.

Inapparent infections at Low Temperatures

Aphids maintained at 4^{0}C were transferred to 22-24^{0}C and maintained in cages as described in Appendix C.2. Aphids were tested before transfer to higher temperatures and plants and aphids were harvested weekly thereafter and purified preparations (Appendix B.1) screened for virus by DAS-ELISA.

Investigations on activation of inapparent infections

RhPV-infected aphids stored at -20^{0}C from 1980-1985 were used to infect "virus-free" aphids by leaf-surface contamination (Section 6.2.6). Both aphid samples were checked for virus by DAS-ELISA and hybridization. Aphids were maintained in growth chambers at 10^{0}C - 15^{0}C as described in Appendix C.2. Aphids were screened for

inapparent infections by DAS-ELISA and nucleic acid hybridization.

6.3 RESULTS

6.3.1 Virus Distribution

Both ALPV and RhPV were detected in aphids collected from widely separated geographical regions (Fig. 6.1). Both viruses were detected as single- and double-infections in specimens of *R. padi*, *Metapolophium dirhodum*, *Sitobion avenae* and *D. noxia* aphids. RhPV was detected in *R. padi* stored for seven years at -20^{0}C. ALPV was detected in a single *R. padi* colony propagated in this laboratory four years previously (1983). ALPV was not detected in stock aphid colonies in which RhPV was propagated from 1980-1986.

Antibodies to ALPV were detected, by immunoelectroblotting, in antiserum made in 1980 to a partially-purified preparation of field-collected *D. noxia* aphids (Fig. 6.2). No ALPV antibodies were detected in antisera to RhPV made from 1980-86, indicating that ALPV was not present in the RhPV stock colonies prior to its re-introduction into the laboratory in 1986.

6.3.2 Host range

Under laboratory conditions, most of the cereal aphids found in South Africa acquired and maintained ALPV and RhPV for several generations (Table 6.1). Natural virus infections are presented above (section 6.3.1).

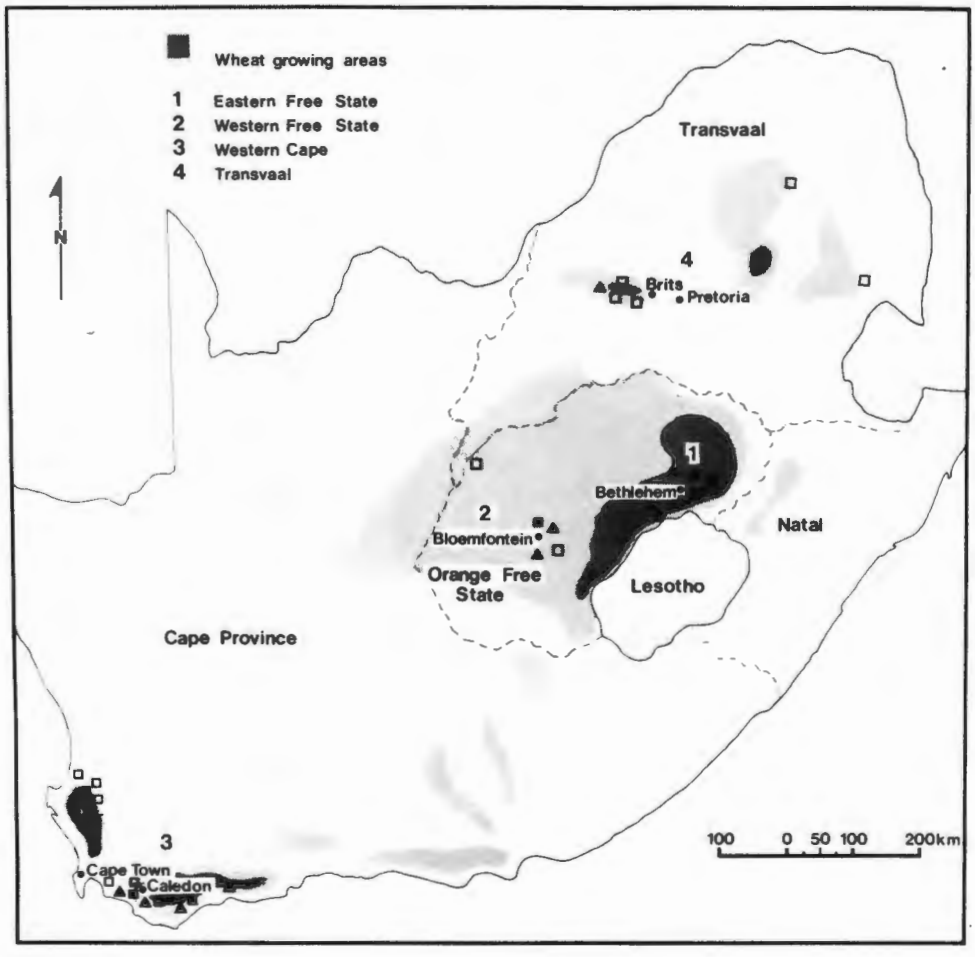


Figure 6.1
Distribution of ALPV and RhPV in the major wheat-producing areas in South Africa. Aphids were tested in bulk (more than 20 aphids per sample) by DAS-ELISA. (■), ALPV detected; (▲) RhPV detected; (□) neither RhPV nor ALPV detected. Aphids collected from 1984-86 were screened for RhPV only and aphids collected from 1986-88 were screened for both RhPV and ALPV.

Figure 6.2
Immunoelectroblot of RhPV (Rh) and ALPV (A) polypeptides fractionated by SDS-PAGE in a 15% gel. Blots were probed with antiserum (1/200 dilution) made to partially purified preparation of *D. noxia* aphids collected from Bethlehem, Orange Free State in 1980.

Three attempts to infect a clonal population of *D. noxia* aphids with RhPV and ALPV failed. This clone (UCT clone, see Appendix C.1.) had been maintained in the laboratory for 8 years (von Wechmar, pers. comm.) and had previously been used to propagate RhPV. In an experiment done in parallel, using a recently field-collected *D. noxia* clonal population (originating from Brits, Transvaal), aphids acquired and maintained ALPV but not RhPV. This indicates that aphids of the same species vary in their response to virus. In addition, it is possible that the host range of RhPV has become restricted with time. No attempts were made to determine if this phenomenon occurred in the field.

Table 6.1. Aphid host range of ALPV and RhPV.

APHID[#]	ALPV	RHPV
R. padi (Els)	+	+*
R. maidis (Els)	-	-
D. noxia (UCT)	-	-
D. noxia (Brits)	+	-
S. graminum (Brits)	+	+*
M. dirhodum (Els)	+	+*
Sitobion avenae (Els)	+	+
Myzus persicae	-	-

*Similar to results reported by Rybicki and von Wechmar, 1984.
#See Appendix C.1

RhPV virions could be detected in low amounts in concentrated extracts of plants on which infected aphids had fed three weeks previously. The virus was detected by electron microscopy, immunoelectroblotting and DAS-ELISA (results not shown). Approximate virus yields were 3 μg/kg as assayed by DAS-ELISA. This confirms the preliminary results reported by Rybicki (1984).

Double-stranded RNA is characteristic of replication of single-stranded RNA viruses. In three different attempts, no replicative form (RF) RNA was isolated from 50 to 100 g of plants on which RhPV infected aphids had fed (as determined by electrophoresis and ethidium bromide staining, see Chapter 2, Fig. 2.6). In parallel experiments, RF RNA could be isolated from low dsRNA-yielding barley yellow dwarf virus infected plants (results not shown) and from RhPV infected aphids as described previously (Section 2.3.3).

6.3.3 **Vertical Transmission**

ALPV was detected in 2/25 new born nymphs and 6/25 nymphs reared for one week after birth. The incidence of virus in the parent generation was 14/25. No RhPV was detected in new born nymphs.

6.3.4 **Horizontal Transmission**

Transmission by leaf surface contamination

Aphids acquired RhPV and ALPV after feeding on plants, surface-contaminated with virus-infected aphid homogenate or

purified virus preparations. Aphids were routinely infected in this manner.

Transmission via plants

Virus-free aphids acquired RhPV and ALPV from leaves colonised 24 h previously by virus-infected aphids (experiment repeated three times).

Virus-free aphids acquired RhPV from plants colonised three weeks previously by virus-infected aphids, indicating that RhPV is "persistently transmitted" by plants (experiment repeated three times). ALPV transmission was not tested.

Transmission via aphid predators

Praying mantids which had fed on virus infected aphids, transmitted ALPV and RhPV to uninfected *R. padi*. ALPV and RhPV could be detected in both aphids and predators. However, virus was not detected in praying mantids that had fed on virus-infected aphids prior to a diet of *M. persicae* (not a host for RhPV or ALPV). Thus indicating that neither virus replicates in praying mantids. Transmission could be due to plant surface contamination by faeces.

6.3.5 **Virus Incidence**

Maintenance *R. padi* colonies kept at 22-24^{0}C (Appendix C.2) were routinely screened for the incidence of ALPV and RhPV. Usually 50 single aphids were screened. The incidence of ALPV and RhPV in single *R. padi* aphids fluctuated between 20 to 80% for ALPV and 50 to 90% for

RhPV. Virus incidence in natural aphid populations showed similar variations. Aphids were infected with both viruses simultaneously or with only one virus.

6.3.6 **Pathogenicity**

The effects of ALPV and RhPV on longevity and fecundity were monitored on aphids infected via two different routes (Table 6.2). Uninfected *R. padi* were monitored similarly. In all experiments the aphids were of comparable age and were maintained under identical temperature and light conditions.

Table 6.2. The different routes whereby nymphs were infected with virus

Expt	Parent infected/uninfected	Food source contaminated/uncontaminated
A	Infected	Uncontaminated
B	Infected	Contaminated
Negative control	Uninfected	Uncontaminated

6.3.6.1 Longevity

ALPV and RhPV infections resulted in a reduction in longevity, independent of the route of infection (Fig. 6.3). Aphids that had acquired virus before or during birth and had subsequently been fed on virus-contaminated leaves from birth (Expt B), had the greatest reduction in life span. The time taken for the aphid population to halve was 12 days

which was approximately half the time taken by uninfected aphids (25 days) (Table 6.3). Infected aphids appeared to either die very quickly or survive for nearly the expected life span of uninfected aphids. This is reflected in the large standard deviation in the mean life span (Table 6.3). Prior to death, most infected aphids left their food source and became dehydrated and paralysed.

Table 6.3. The effect of ALPV and RhPV on longevity and fecundity of *R. padi* aphids

Expt.[1]	Mean life span (days)	Time to 50% mortality (days)	Mean no. nymphs/ adult	%Pos.[2] (ELISA) RhPV	ALPV
A[3]	19 ± 10	19	42 ± 25	35	48
B[4]	15 ± 9	12	37 ± 30	88	91
Negative[5] control	25 ± 5	25	63 ± 15	0	0

[1]See Table 6.2
[2]No. dead aphids screened: Expt.A = 24, Expt. B = 36, negative control = 36.
[3]50 aphids monitored.
[4]48 aphids monitored.
[5]48 aphids monitored.
± = standard deviation.

6.3.6.2 Fecundity

Aphids infected with ALPV and RhPV produced fewer nymphs than uninfected adults (Fig. 6.4). Aphids born of infected females that were fed on virus-contaminated leaves from birth had the greatest decrease in fecundity (Expt. B) (Table 6.3). The overall reduction in the number of offspring was due to both a decrease in the number of nymphs

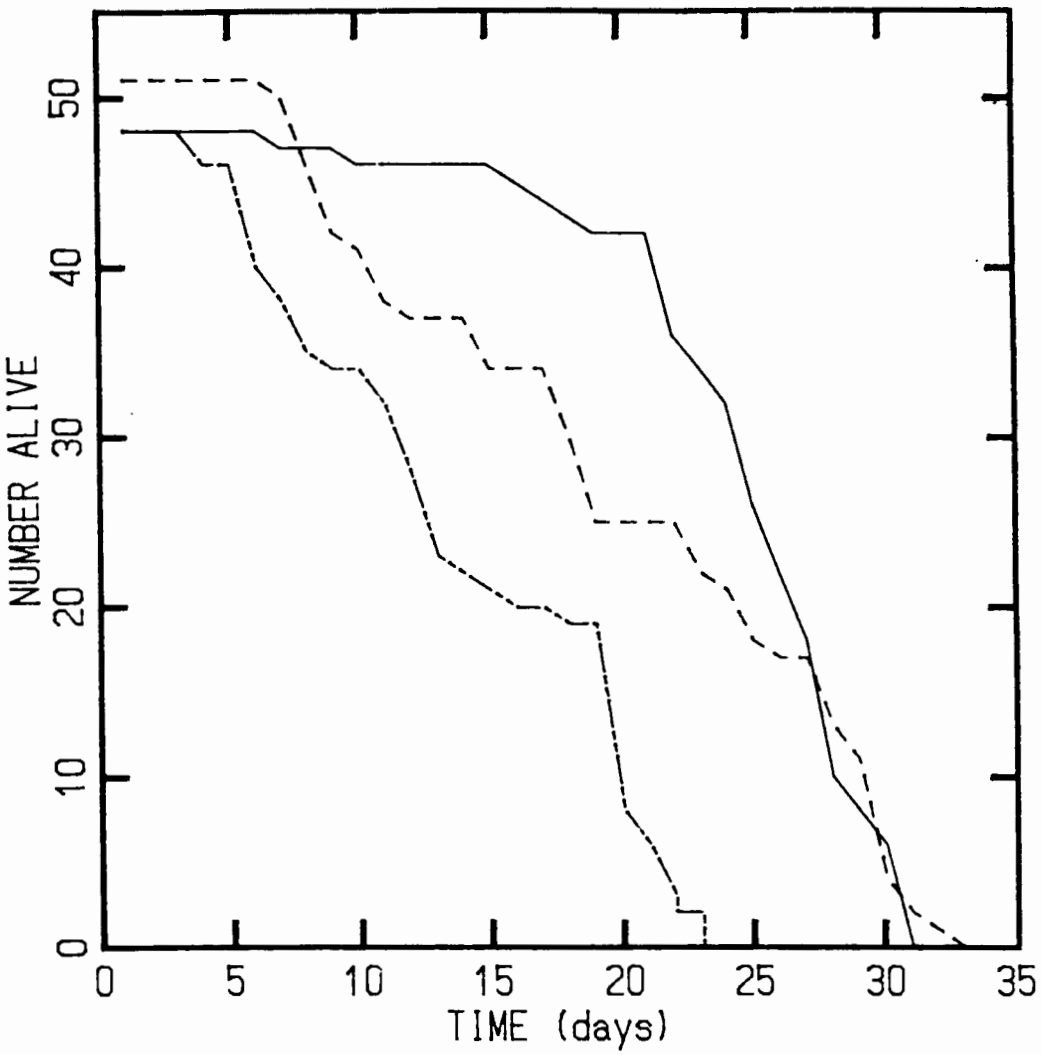


Figure 6.3

The effect of ALPV and RhPV on the longevity of *R. padi* aphids.
(– –) expt. A: aphids acquired virus from the parent generation, 52 aphid monitored.
(– · –) expt. B: aphids acquired virus from the parent generation and by leaf surface contamination, 48 aphids monitored;
(——) Negative control: uninfected aphids, 48 aphids monitored.

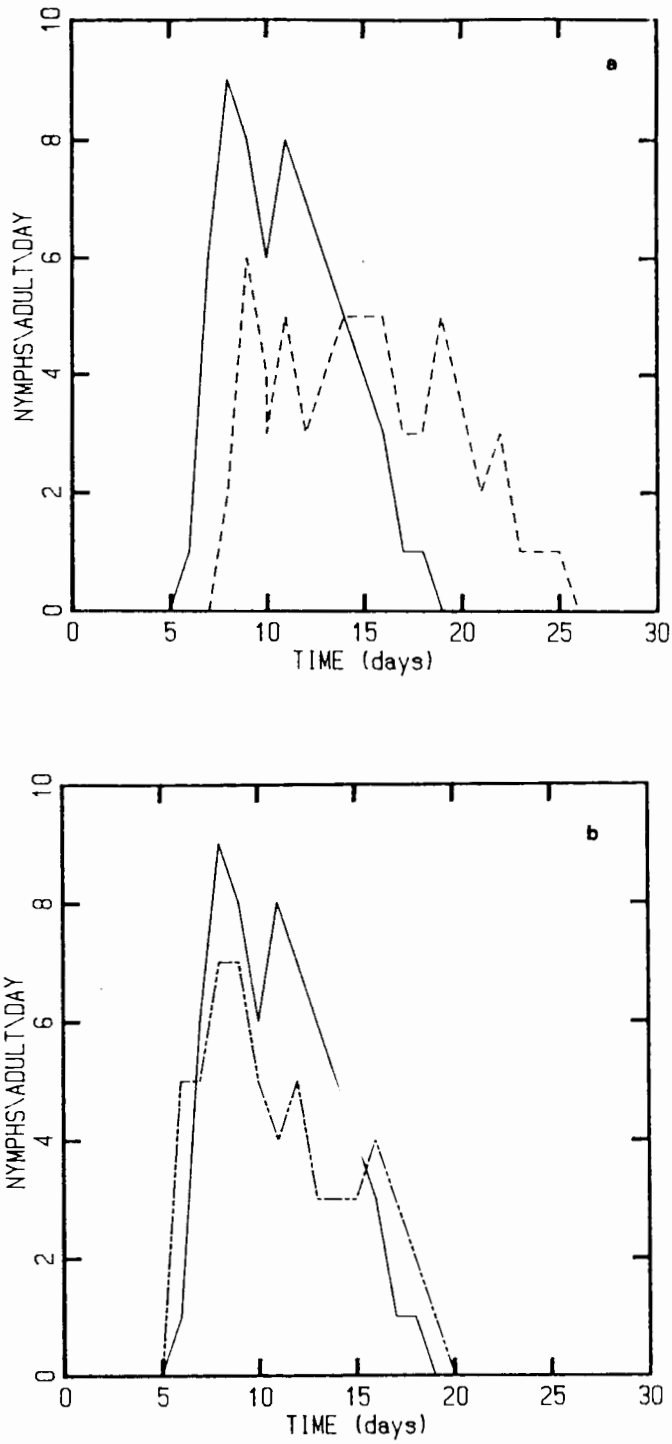


Figure 6.4

The effect of ALPV and RhPV on total number of nymphs produced per day by *R. padi* aphids.

a) (- -) Expt. A: aphids acquired virus from the parent generation, 52 aphid monitored.
(——) Negative control: uninfected aphids, 48 aphids monitored.

b) (—·-) Expt. B: aphids acquired virus from the parent generation and by leaf surface contamination, 48 aphids monitored.
(——) Negative control: uninfected aphids, 48 aphids monitored.

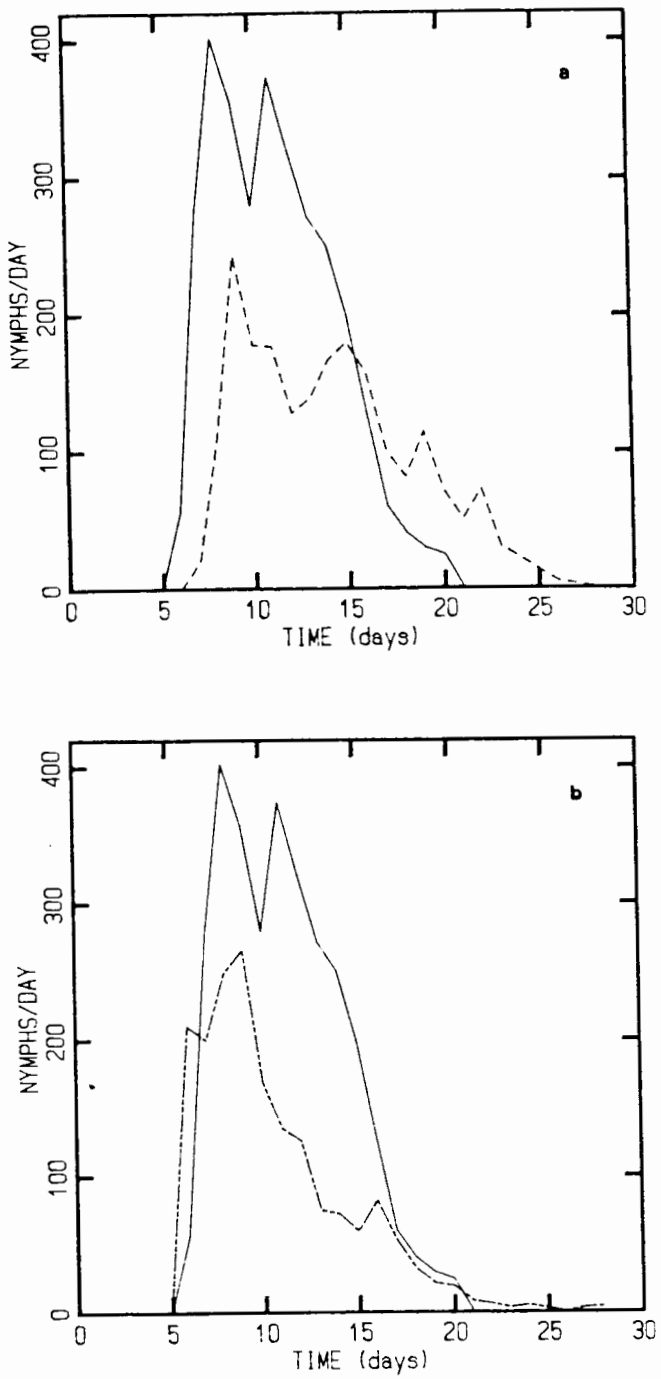


Figure 6.5

The effect of ALPV and RhPV infection on the mean number of nymphs produced per aphid per day.

a) (- -) Expt. A: aphids acquired virus from the parent generation, 52 aphids monitored.
(——) Negative control: uninfected aphids, 48 aphids monitored.

b) (—-) Expt. B: aphids acquired virus from the parent generation and by leaf surface contamination, 48 aphids monitored.
(——) Negative control: uninfected aphids, 48 aphids monitored.

produced per adult (Fig. 6.5) and premature death of potential producers (Fig. 6.3). Aphids which had the shortest mean life span and lowest number of off-spring, also had the highest incidence of virus (Table 6.3). Uninfected aphids (negative control) remained virus-free throughout the experiment.

6.3.7 Comparison of ELISA and Hybridization for Virus Detection

The sensitivity of DAS-ELISA and nucleic acid hybridization methods for detecting purified ALPV and RhPV were directly compared. A sensitivity of detection for both ALPV and RhPV was 0.1 ng/dot with hybridization and 10 ng/well by ELISA. A RhPV clone (plasmid pRHPV2) developed previously (Chapter 2) and ALPV cDNA were used in hybridization tests.

A direct comparison of the incidence of virus in crude *R. padi* aphid extract (one aphid per sample) was made, in which half of the extract was screened by DAS-ELISA and half by hybridization. Of 40 samples screened, ALPV cDNA probes detected 14 definite positives compared to 10 positives detected by DAS-ELISA (Fig. 6.6). Similarly, of the 40 samples screened, pRHPV2 hybrid plasmid probe detected 16 definite positive samples compared to 11 positives obtained by DAS-ELISA (Fig. 6.7). There was, therefore, an approximately 30% increase in the number of positive samples detected by hybridization over those detected by ELISA. In most instances, samples that were antigenically positive were also positive in hybridization studies. However, occasionally samples were strongly positive for the presence

of nucleic acid and not for antigen and vice versa. Given that nucleic acid hybridization is more sensitive than ELISA, there is no apparent reason as to why samples were positive for antigen and not for nucleic acid. The background hybridization observed in Figures 6.6 and 6.7 was eliminated in subsequent blots by washing the membrane in chloroform prior to hybridization (Section 6.2.10).

The use of ALPV cDNA probes to screen *D. noxia* aphids produced a marked improvement in virus detection compared with ELISA. Of 24 *D. noxia* samples (Brits, Appendix C.1) screened, DAS-ELISA detected virus in three samples, compared to six positive samples detected by cDNA hybridization (Fig. 6.8). Of 24 *D. noxia* samples (Beth., Appendix C.1.) screened, none appeared positive by DAS-ELISA, although, 19 virus containing samples were detected by cDNA hybridization (Fig. 6.8). Virus was not detected in any of the 24 negative control samples (*M. persicae*) by either hybridization or ELISA tests.

6.3.8 **Inapparent Infections in *R. padi***

Inapparent infections in field-collected aphids

Field-collected *R. padi* aphids from 3 separate regions in the Western Cape were tested for RhPV and ALPV by DAS-ELISA (Section 6.2.2). Neither RhPV nor ALPV were detected in these aphids. However, after 10 days propagation in the laboratory (temperature of 22-24^{0}C and a 12h day/night cycle, Appendix C.2), all aphid samples contained amounts of virus that were detectable by ELISA. No virus was detected in five different field-collected *R. padi* samples treated similarly.

Inapparent infections at low temperatures

RhPV, but not ALPV, was detected in aphids maintained at 4^0C for four months. If these aphids were transferred to 22-24^0C, both viruses were detectable by DAS-ELISA after four weeks propagation. RhPV-infected aphid clones have been successfully maintained at 4^0C for at least five years.

The investigation of activation of inapparent infections

"Virus-free" *R. padi* were infected by leaf surface contamination with crude extract of RhPV-infected *R. padi*. These RhPV-infected aphids had been propagated prior to 1986, when ALPV was introduced, and have previously been established to be ALPV-free by ELISA. In addition antisera made to RhPV during this period did not elicit an antibody response to ALPV (Section 6.3.1). No ALPV was detected in these aphids or in the "virus-free" aphids, when screened by dot-blot hybridization (Fig. 6.9). RhPV was detected by DAS-ELISA in aphids within 10 days after infection. However, ALPV became detectable after four weeks. Five attempts were made to obtain colonies infected with RhPV only. In parallel experiments, "virus-free" aphids were fed on plants surface contaminated with "virus-free" aphids (two repeats). These aphids did not become infected with ALPV or RhPV, indicating that ALPV infections were not due to contamination of the equipment and were not activated by factors in the aphid extract. ALPV was, therefore, either present in an inapparent form in *R. padi* or was present in undetectable amounts in the RhPV source.

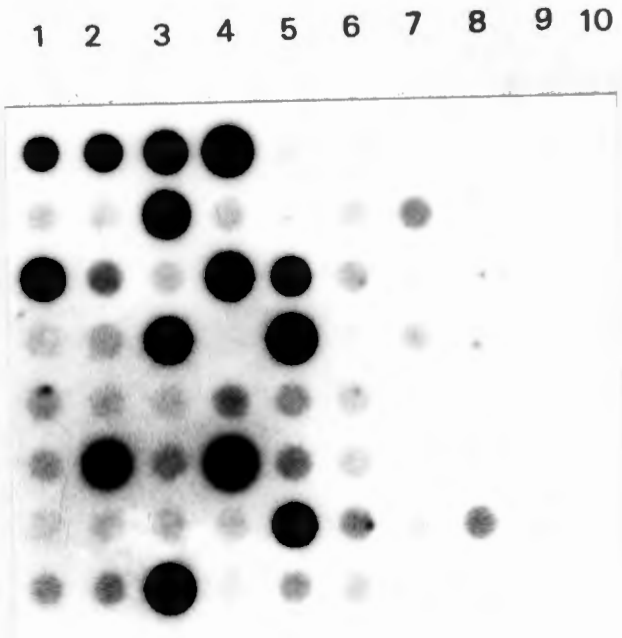


Figure 6.6
Dot-blot of crude aphid extract probed with ALPV ^{32}P-labelled cDNA. 1 dot is equivalent to half an aphid. Columns 1 to 5, *R. padi* infected with RhPV and ALPV. Columns 6 to 10, "uninfected" *R. padi* aphids.

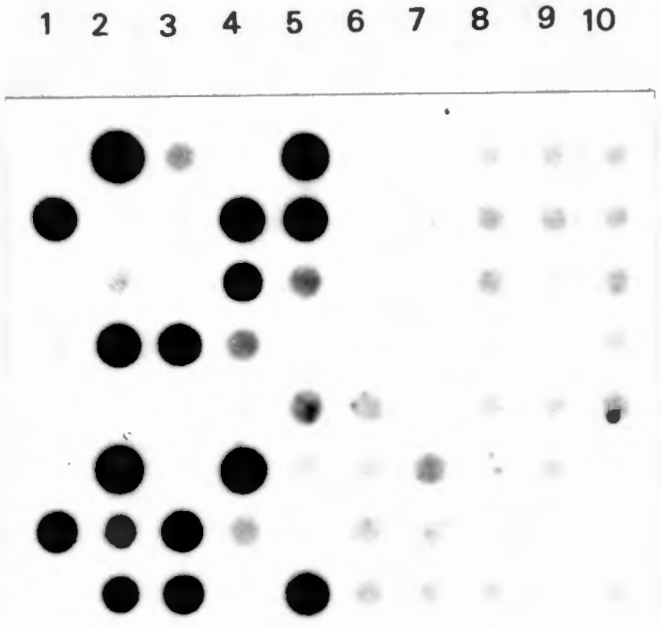


Figure 6.7
Dot-blot of crude aphid extract probed with pRHPV2 labelled with ^{32}P by nick-translation. 1 dot is equivalent to half an aphid.
Columns 1 to 5, *R. padi* infected with ALPV and RhPV.
Columns 6 to 10, "uninfected: *R. padi*.

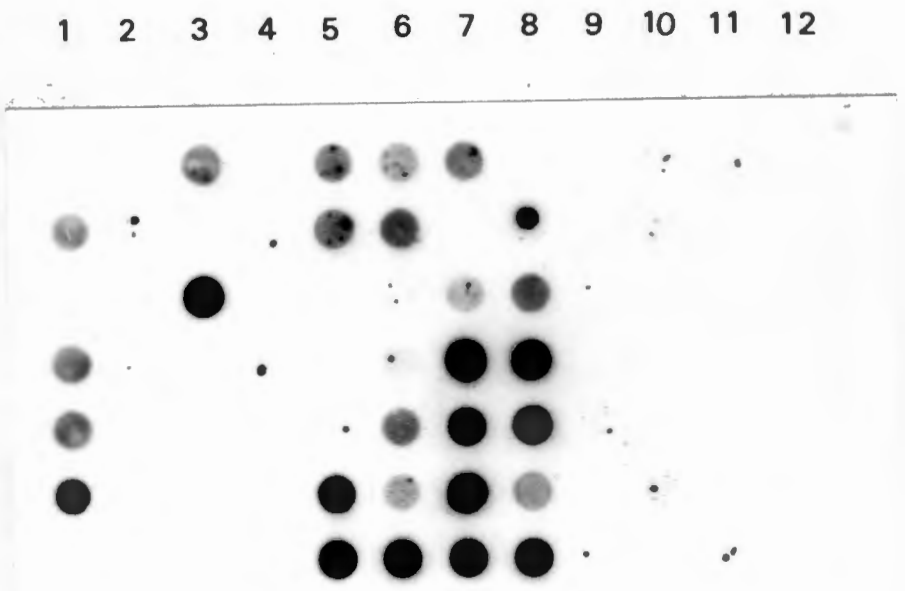


Figure 6.8
Dot-blot of crude aphid extracts probed with ^{32}P-labelled ALPV cDNA. 1 dot is equivalent to 1 aphid.
Columns 1 to 4, *D. noxia* (Brits) aphids.
Columns 2 to 8, *D. noxia* (Beth) aphids.
Columns 9 to 12, *Myzus persicae* aphids (negative control).

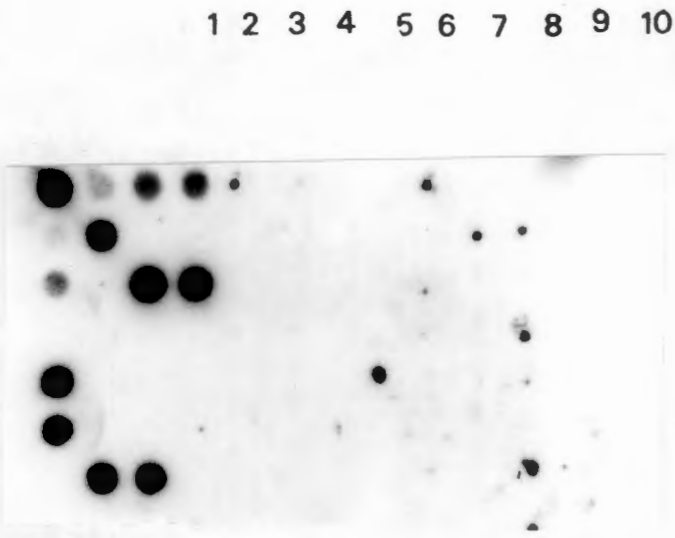


Figure 6.9
Dot-blot of crude aphid extracts probed with ^{32}P-labelled ALPV cDNA. 1 dot equivalent to 1 aphid.
Columns 1-4, RhPV and ALPV infected *R. padi*.
Columns 5-8, "virus-free" *R. padi*.
Columns 9-12, *Myzus persicae* aphids (negative controls).

6.4 DISCUSSION

ALPV and RhPV were detected in geographically widely separated regions in South Africa, indicating that they are not adventitious laboratory pathogens. Their success in nature may be attributed to their stability (see Chapters 2 and 3), efficiency of transmission, the number of aphid species they infect, their dissemination by predators and plants and their ability to exist as inapparent infections. In addition, under laboratory conditions, although longevity and fecundity were affected, most of the virus-infected aphids survived long enough to reproduce, thus ensuring the transmission of the virus to the next generation. A detectable amount of ALPV in nymphs on birth suggest ALPV replicates in embryos. This is supported by the detection of ALPV by *in situ* hybridization in thin sections of embryos of infected mothers (Hatfill, 1988).

ALPV antibodies in antiserum prepared in 1980 indicated that the virus has been present in South Africa for at least eight years. Although ALPV was detected in aphids propagated in the laboratory in 1983, there is no indication that it contaminated RhPV-infected aphid stock colonies prior to its discovery in 1986. The virus propagation schedule previously used in which aphids were routinely maintained at 4^0C for long periods of time (Rybicki, 1984; von Wechmar, pers. comm.) may have selected for RhPV infections only, as ALPV is not detectable in aphids maintained at this temperature.

Although RhPV virions could be isolated from plants on which infected aphids had fed, no replicative form (RF) RNA was detected in these plants. Therefore, it would seem unlikely that RhPV replicates in both insects and plants as previously speculated by Rybicki and von Wechmar (1982a). This is supported by the findings of Gildow and D'Arcy (1988), who showed that although RhPV was transmitted through barley plants, there was no increase in virus titer over 24 days. Similarly, they also did not detect RF RNA in plants. However, in contrast to our finding that infectious virus persisted in plants for three weeks (Rybicki, 1984; Williamson *et al.*, 1985), their investigations showed that RhPV remained infectious for less than 72 h. The ability of plants to act as "vectors" for an insect virus has been previously reported by Ofori and Francki (1985) for leafhopper A virus (LAV). However, unlike RhPV and ALPV, transmission of LAV through maize plants was dependent on co-infestation of plants by virus-infected and virus-free insects.

Difficulties experienced in obtaining aphids solely infected with RhPV may have been due to inapparent ALPV infections. Similar observations in which virus infections have been activated by foreign substances have been made with numerous other small RNA viruses. Activation of *Antheraea eucalypti* virus due to superinfection with CrPV was speculated by Reinganum (1975); and Dall (1985) found that inoculation of pupae of *Apis mellifera* with rabbit sera resulted in activation of sacbrood and Kashmir bee viruses.

Nucleic acid probes played an important role in detecting ALPV in *D. noxia* aphids in which the virus does not appear to be present in as high concentration as in *R. padi*. The relatively low concentration of ALPV in *D. noxia* would suggest that *D. noxia* is a poor virus host.

There are numerous drawbacks with propagating viruses in a biologically dynamic system such as aphids. Aphids may be infected with more than one virus strain or have inapparent viral infections. Experiments performed in the laboratory may not be a true reflection of the field situation as virus propagated under controlled environmental conditions may result in the development of viral resistance in aphids or the selection of virulent or mild virus strains. Propagation and maintenance of aphids is labour-intensive and aphid colonies have to be regularly monitored to ensure viruses or other pathogens or parasites have not been inadvertently introduced. Tissue culture would have been of great benefit in overcoming many of these problems. Unfortunately, attempts to infect *Drosophila* cells (Schneiders' cell line 1) with RhPV or ALPV were unsuccessful.

It is possible that RhPV and ALPV play a role in limiting aphid populations in nature. ALPV was detected in aphids during an otherwise inexplicable population decline of *R. padi* in the Riviersonderend area of the South-Western Cape (von Wechmar, unpublished data and S.S. Walters, personal communication).

The biological investigations of ALPV and RhPV are in the preliminary stages. Extensive investigations are

necessary before ALPV and/or RhPV can be considered as biological control agents of aphids. Factors such as aphid resistance to virus infection, changes in virus host range and extensive host range studies need to be investigated. The environmental factors influencing pathogenicity and activation of inapparent infections are presently under investigation in this laboratory (J. Laubscher, Department of Microbiology, University of Cape Town, South Africa).

CHAPTER 7

GENERAL CONCLUSIONS AND DISCUSSION

ALPV and RhPV appear to be endemic in some aphid populations in South Africa. ALPV is a newly discovered aphid virus found during epidemiological investigations on RhPV. Some of the properties of ALPV have been reported elsewhere (Williamson *et al.*, 1988). The only other aphid virus characterised, to date, is Sitobion avenae virus (Allen and Ball, 1986). However this virus has only been detected in laboratory populations of aphids and is not thought to be of relevance ecologically (B. Ball, Rothamsted Experimental Station, UK, pers. comm.).

The majority of the studies reported on insect picorna-like viruses are in the preliminary stages. Most of the data available being on the basic properties of virions, with some data on the virus pathology. The only viruses which have been characterised in any detail, on a molecular level, are CrPV, DCV, IFV, and now RhPV and ALPV. With the accumulation of data on these viruses, and the discovery of new picorna-like insect viruses, it is imperative that they be formally classified.

The physicochemical properties of ALPV and RhPV are very similar to those of insect and mammalian picornaviruses (Table 3.1). In addition, similar to all picornaviruses, ALPV and RhPV RNAs translate into high molecular weight proteins, at least one of which is probably a capsid precursor protein. From the similarities in virion

properties and translational expression between ALPV and RhPV, and their similarities to other picornaviruses, there is little doubt that these aphid viruses should be classified in the picornavirus group (family *Picornaviridae*). The inclusion of ALPV into this group is further substantiated by its serological relationship with CrPV and DCV.

Sequence data now available, and subsequent evolutionary speculations, indicate that the traditional plant/animal barrier for classification of viruses is redundant (Goldbach, 1987). This is further exemplified by the recent discovery of two plant viruses that have very similar properties to picornaviruses (Murant *et al.*, 1987). Nucleotide sequences between different picornaviruses are generally well conserved. However, a recent analysis of the 3′ end of CrPV genome showed little or no sequence homology with mammalian picornaviruses (King *et al.* 1987). A new genus, "entomopicornaviruses", should perhaps be created to encompass insect viruses such as CrPV. This genus would also include serologically related viruses ALPV and DCV, and probably also *Gonometa* virus, IFV and RhPV.

The problems encountered in purifying quantitative amounts of RhPV from mixed virus preparations suggest that ALPV infections interfere with RhPV replication. It would be of interest to investigate the interaction of these viruses further. The effect of ALPV on natural aphid populations is unknown. However, in the laboratory RhPV appears to cause persistent virus infections whereas ALPV appears to be more lethal to aphids. This topic is currently under

investigation in this laboratory (J. Laubscher, Department of Microbiology, University of Cape Town, South Africa).

APPENDIX A

CHEMICALS, BUFFERS, SOLUTIONS AND MEDIA

A.1 **Chemicals**

A.2 **Buffers and Solutions**

A.2.1 0.1 M Potassium phosphate buffer, pH 7.0

A.2.2 0.2 Ionic strength buffers

A.2.3 Saline

A.2.4 Phosphate buffered saline (PBS)

A.2.5 PBS-Tween (PBS-T)

A.2.6 PBS-Tween-Milk powder (PBS-T-MP)

A.2.7 Tris-Borate-EDTA (TBE)

A.2.8 Tris-EDTA (TE)

A.2.9 NaCl-EDTA-Tris (NET)

A.2.10 100 x Denhardt's

A.2.11 20 x SSPE

A.2.12 10 mg/ml denatured herring sperm DNA

A.2.13 100% (w/v) TCA

A.2.14 Knopps solution

A.3 **Media**

A.3.1 Luria-Bertani (LB) broth

A.3.2 LB agar

CHEMICALS, BUFFERS, SOLUTIONS AND MEDIA

A.1 Chemicals

Analytical grade chemicals were used for analytical work and technical grade chemicals were used for preparative work. Chemicals were supplied by Sigma (USA), Merck (Darmstadt), BDH Chemical Ltd. (Poole, England) or SAARCHEM Pty. Ltd. (South Africa). Unless otherwise stated, glass double-distilled water was used throughout.

A.2 Buffers and Solutions

A.2.1 0.1M Potassium phosphate buffer pH 7.0

(Williams and Chase, 1967)

0.5 M K_2HPO_4	122 ml
0.5 M KH_2PO_4	78 ml
water	800 ml

A.2.2 0.2 Ionic strength buffers (Miller and Golder, 1950)

The following stock solutions were made up: 5M NaCl (A); 1M glycine, 1M NaCl (B); 2M HCl (C); 2M sodium acetate (D); 3.5 M acetic acid (E); 0.5 M Na_2HPO_4 (F); 4M NaH_2PO_4 (G); 0.5 M sodium barbitone (H). To obtain the required pH, solutions were mixed as indicated and made up to a final volume of 1 liter with water.

pH 2: 36 ml solution A, 5.3 ml solution B and 7.35 ml solution C.

pH 3: 36 ml solution A, 15.8 ml solution B and 2.1 ml of solution C.

pH 5: 36 ml solution A, 10 ml solution D and 16.85 ml solution E.

pH 7: 36 ml solution A, 11.35 ml solution F and 3.3 ml solution G.

pH 8: 36 ml solution A, 5.2 ml solution C and 40 ml solution H.

A.2.3 **Saline**

NaCl 0.15 M

A.2.4 **Phosphate buffered saline (PBS)**

1 volume of 0.1 M potassium phosphate, pH 7.0 (Appendix A.2.1) was added to 1 volume of saline (A.2.3).

A.2.5 **PBS-Tween (PBS-T)**

PBS was adjusted to a final concentration of 0.05% (v/v) Tween-20.

A.2.6 **PBS-T-Milk powder (PBS-T-MP)**

PBS-T was adjusted to a final concentration of 0.2% (w/v) milk powder (non-fat) and stored at 4^0C.

A.2.7 **Tris-borate EDTA (TBE, Ph 8.0)** (Maniatis *et al.*, 1982)

Tris	0.089 M
Boric acid	0.089 M
EDTA	0.002 M

A.2.8 **Tris-EDTA (TE)**

Tris, pH 7.4	10 mM
EDTA	1 mM

A.2.9 **NET buffer** (Grubman, 1984)

NaCl	150 mM
EDTA	2 mM
Tris.HCl, pH 7.5	100 mM

A.2.10 **100 X Denhardt's** (Maniatis *et al.*, 1982)

BSA (Fraction V, Sigma)	2% (w/v)
Ficoll	2% (w/v)
Polyvinyl pyrollidone	2% (w/v)

Filter sterilised and stored in aliquots at -20^{0}C.

A.2.11 **20 x SSPE**

NaCl	3.6 M
Sodium phosphate, pH 7.7	0.2 M
EDTA	0.002 M

Autoclaved before use.

A.2.12 **Denatured Herring Sperm DNA** (Maniatis *et al.*,1982)

Herring sperm DNA (lyophilised sodium salt, Boehringer Mannheim) was dissolved in distilled water to a final concentration of 10 mg/ml. The DNA solution was sheared by passing the solution through an 18 gauge hypodermic needle several times. Prior to use, the DNA was denatured by boiling for 10 min and immediately placed on ice. Sheared, denatured DNA was stored in aliquots at -20^0C.

A.2.13 **100% Trichloroacetic acid (TCA)** (Maniatis *et al.*,1982)

227 ml of water was added to a 500 g bottle of TCA.

A.2.14 **Knopps Solution** (Inorganic nutrient solution) (D.P. Whittier and T.A. Sleaves, 1960; Can. J. Bot., 88, pp. 925.)

KNO_3	250 mg
$Ca(NO_3)_2$	1000 mg
$MgSO_4.7H_20$	250 mg
KH_2PO_4	250 mg

Made up to final volume of 1 liter with water and sterilised by autoclaving.

A.3 Media

A.3.1 Luria-Bertani (LB) Broth

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g
Water	1000ml

Sterilised by autoclaving.

A.3.2 LB Agar

Add 1.5% (w/v) bacto-agar to LB broth. Sterilised by autoclaving and allowed to cool to 55^{0}C before adding antibiotics and/or pouring plates.

APPENDIX B

STANDARD METHODS

STANDARD METHODS

B.1 Virus extraction and purification

Unless otherwise stated, virus was extracted at room temperature and virus preparations were stored in 0.1 M potassium phosphate buffer, pH 7.0 (A.2.1) at 4^0C. Low speed centrifugation refers to 10 000 x *g* for 10 min and high speed centrifugation refers to 50 000 x *g* for 120 min. Routine virus purification was from aphids propagated at 24-26^0C. Aphids were maintained as described in Appendix C.

Virus was extracted from up to 2 kg of plants bearing aphids as described by Rybicki and von Wechmar (1982a). The plants bearing aphids were homogenised with phosphate buffer (0.1 M phosphate buffer, pH 7.0; Appendix A.2.1) (1:1 mix w/v) in a Waring-type blender. The homogenate was strained through cheesecloth and adjusted to 20% (v/v) chloroform. The emulsion was stirred at 4^0C for 20 min and the phases separated by low-speed centrifugation. Virus was concentrated from the aqueous phase by precipitation with 9% (w/v) polyethylene glycol (PEG M_r 6 000) and 2.5% (w/v) NaCl. Precipitates were collected by low-speed centrifugation and dissolved in phosphate buffer. The insoluble debris was removed by low speed centrifugation. The virus suspension was further purified and concentrated by one or two cycles of differential ultracentrifugation.

Virus was extracted from aphids essentially as described by D'Arcy *et al.* (198la). One to 10 g of aphids were ground to a powder in liquid N_2 with a mortar and pestle. The powder was then homogenised in 50 to 100 ml of

0.1 M phosphate buffer, pH 7.0, strained through 4 layers of cheesecloth, adjusted to 1% (v/v) Triton X-100, and stirred for 10 min before clarification with 20% (v/v) chloroform as described above. Further purified was by one or two cycles of differential ultracentrifugation.

Virus preparations were further purified by zone electrophoresis (Appendix B.2.) and/or sucrose gradient rate-zonal centrifugation (Appendix B.3.) and/or isopycnic density gradient centrifugation (Appendix B.4.).

B.2 **Sucrose density gradient zone electrophoresis**

Zone electrophoresis was performed by the method described by van Regenmortel (1964) in the same apparatus. The electrophoresis apparatus was filled with electrophoresis buffer (0.035 M H_3BO_3, 0.0175 M NaOH, 0.0075 M HCl, 0.037 M NaCl, pH 8.6). A linear 10 - 40% (w/v) sucrose gradient was poured by bottom displacement onto a 50% (w/v) sucrose cushion, into the left arm of the apparatus. Gradients were poured with the aid of a two-flask gradient maker. The electrode vessels were filled with a saturated NaCl solution to cover two reversible Ag-AgCl electrodes. Approximately 1mg (0.2 - 0.5 ml) of a mixed virus suspension was adjusted to 45% (w/v) sucrose concentration and phenol red was added as a standard reference marker. The sample was loaded by bottom displacement to lie between the 50% sucrose cushion and the sucrose gradient. Electrophoresis was for approximately 18 h at 20 mA at room temperature. ALPV bands were located by light-scattering and columns were fractionated from the

bottom. RhPV bands were located by screening the fractions by DAS-ELISA (Appendix B.14). The fractions were immediately diluted in 0.1 M phosphate buffer, pH 7.0 and concentrated by ultracentrifugation (Appendix B.1).

B.3 Sucrose gradient rate-zonal centrifugation

Sucrose gradients were prepared immediately before use with the aid of a gradient maker which mixed 10% (w/v) and 40% (w/v) sucrose solutions to produce linear gradients. Alternatively, gradients were prepared by freezing 25% (w/v) sucrose solutions in centrifuge tubes (-20^{0}C), followed by thawing overnight at 4^{0}C. This cycle was repeated once before gradients were used. All sucrose solutions were made up in 0.1 M phosphate buffer, pH 7.0 and formed in Beckman ultraclear centrifuge tubes (9.0 cm x 2.5 cm). A maximum of 2 ml of sample (up to 2 mg virus) was layered onto the gradients and the tubes were centrifuged at 26 000 rpm for 150 min in a Beckman SW28 rotor. Virus bands were located by light scattering using an overhead point light source. Gradients were scanned at 254 nm and fractionated on an ISCO Model 640 gradient fractionator coupled to an UA 5 absorbance monitor. The UV-absorbing fractions were collected and either dialysed against phosphate buffer overnight at 4^{0}C or diluted with phosphate buffer, and concentrated by high speed centrifugation.

B.4 Isopycnic density gradient centrifugation

Caesium chloride (CsCl) density gradients were used for preparative and analytical ultracentrifugation. The starting CsCl density at 22^0C was 1.377 g/ml in 0.1 M phosphate buffer, pH 7.0. Virus samples (200 μl, approx. 0.5 mg) were overlaid onto the CsCl solution and the gradient generated by centrifugation at 46 000 rpm for 18 h at 20^0C in a Beckman SW 50.1 rotor. Virus bands were visualised by light scattering and bands were harvested as described (B.3).

For the purpose of estimating virus density, 100 μl fractions were collected by bottom puncture and A_{260} read on a spectrophotometer (Beckman Model 25). The refractive indices of the fractions were determined in a refractometer (Bellingham and Stanley) at 22^0C. For correlation of density and refractive index, standard CsCl solutions ranging from 1.298 g/ml to 1.420 g/ml were made in 0.1 M phosphate buffer, pH 7.0 using a 10 ml volumetric flask and a 4-place digital balance (Mettler AE160). The refractive indices of these samples were determined and a linear regression equation correlating density to refractive index was computed using a Compucorp Statistician preprogrammed calculator ($y = bx + a$, where y represents the density and x the refractive index). The densities of experimental samples were calculated by substitution of their determined refractive index into the equation as recommended by Scotti (1985). This method eliminates the influence of buffer on density estimations.

B.5 Analytical ultracentrifugation

Sedimentation analyses were performed using an AN-D rotor in a Beckman Spinco Model E Analytical Ultracentrifuge, equipped with Schlieren optics. Virus samples (approximately 0.5 mg/ml) were centrifuged at 26 000 rpm at 20 - 22^{0}C and photographs were taken at 2 min intervals using Kodak Graphic Arts film sheets. Schlieren photographs were projected onto pre-calibrated graph paper and peak positions were read directly off the paper. Sedimentation coefficients ($S_{20.w}$) were then calculated according to Chervenka (1969).

B.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-PAGE on 12.5 to 15% polyacrylamide gels by the method of Laemmli (1970).

Analytical gels were run using the Hoefer SE-600 vertical slab gel apparatus (16 cm X 14 cm in size and 1.5 mm thick, Hoefer Scientific Instruments, San Francisco). Gels were run at ·35 mA/gel until the bromophenol blue front reached the bottom of the gel (12.5% gels) or ran off the end of the gel (15% gels) (approx. 4 h).

Samples were screened using a Hoefer "Mighty-Small" gel apparatus (8 cm X 7 cm in size and 1.5 mm thick). Gels were run at 20 mA for approximately 60 min.

B.6.1 Stock solutions

Resolving gel buffer

1 M Tris-HCl pH 8.8, kept at 4^0C.

Stacking gel buffer

1 M Tris-HCl pH 6.8, kept at 4^0C.

Acrylamide stock solution (40%)

200 g acrylamide monomer (BDH)

5.3 g N,N'-methylene bisacrylamide

Made up to a final volume of 500 ml with water. Insoluble material was removed by filtration and the solution was stored at 4^0C.

Electrophoresis buffer (10X)

0.25 M Tris

1.92 M Glycine

1% (w/v) SDS pH 8.3

Disruption mixture

10% (w/v) SDS

10% (v/v) 2-mercaptoethanol

15% (v/v) glycerol

0.01% (w/v) bromophenol blue

in 0.125 M Tris-HCl pH 6.8.

B.6.2 **Gel solutions**

Resolving gel

	Volume required (ml)	
	12.5%	*15%*
40% acrylamide stock	25.0	30.0
water	20.2	15.2
1 M Tris-HCl pH 8.8	30.0	30.0
1.5% (w/v) ammonium peroxydisulphate	4.0	4.0
10% (w/v) SDS	0.8	0.8

TEMED (N,N,N',N'-tetramethylethylenediamine) (60 µl) was added immediately before pouring and the gel was overlayed with isopropanol.

Stacking gel (5%)

	Volume required (ml)
40% acrylamide stock	3.8
water	18.8
1 M Tris-Cl pH 6.8	3.8
1.5% (w/v) ammonium peroxydisulphate	1.4
80% (v/v) glycerol	2.0
10% (w/v) SDS	0.3

TEMED (80 µl) was added immediately before pouring.

B.6.3 **Sample preparation**

Samples were mixed 1:1 (w/w) with disruption mix (B.6.1.) and heated at 95^0C for 5 min.

B.6.4 **Page blue stain**

Stain solution

Gels were stained in 0.1% (w/v) PAGE blue 83 (BDH, UK) dissolved in a solution of 45% (v/v) methanol and 10% (v/v) acetic acid. The PAGE blue 83 was dissolved in methanol before the addition of acetic acid and water. The solution was filtered before use and gels were stained for 2-16 h with gentle agitation.

Destain solution

Gels were destained in several changes of destain solution (25% v/v methanol, 10% v/v acetic acid).

Gels were either photographed immediately and stored in sealed plastic bags or dried on a Hoefer gel dryer (SE540). Where necessary, gels were scanned at 550 nm on a DU-8 spectrophotometer equipped with a transmission densitometer (Beckman Instruments).

B.7 **Autoradiography**

Radioactive gels or blots were exposed to Kodak XAR 5 X-ray film and stored at -70^{0}C for the appropriate time. Intensifying screens were used when increased sensitivity was needed for ^{32}P and ^{125}I isotopes. For gels and blots with a high number of radioactive counts as assessed on a hand held Geiger counter, less sensitive film was used (Kodak X-Omat). Autoradiographs were processed using Kodak GBX developer and fixed according to the manufacturers'

instructions. When necessary, autoradiographs were scanned at 700 nm on a Beckman DU-8 spectrophotometer.

B.8 Single-stranded RNA isolation and enzyme digestion

Virion RNA was isolated by the phenol-chloroform extraction method essentially as recommended by Brisco *et al.* (1985). The following precautions were taken for all manipulations involving RNA :

- Diethylpyrocarbonate (DEPC) treated water was used to make up buffers.
- All buffers were made with new chemicals, and were sterilised by autoclaving and stored in DEPC-treated bottles.
- New, sterile micropipette tips and Eppendorf vials were used for manipulations involving RNA.
- Gloves were worn throughout.

Virus pellets were resuspended in a solution of 1% (w/v) SDS, 1 mM EDTA and 0.1 M Tris-HCl pH 8.0 and dissociated by heating at 60^0C for 5 min. To 0.5 ml of dissociated virus, 0.5 ml of a buffer-saturated-phenol:chloroform:isoamylalcohol mixture (24:24:1) was added (Maniatis *et al.*, 1982). The mixture was vortexed for 1 min and the phases separated by centrifugation for 1 min in an Eppendorf microfuge. Phenol/chloroform extraction was repeated until a clean aqueous/organic phase interface was obtained (usually 3 times). Traces of phenol were removed from the aqueous phase by two extractions with cold, water-saturated ether. The ether was removed by evaporation, and the RNA precipitated by adding 1/25 volumes of 3 M sodium

acetate, pH 5.5 and 2.5 volumes of cold 96% (v/v) ethanol. RNA pellets were vacuum dried, resuspended in water and stored in aliquots at -70^{0}C.

The type and form of the nucleic acid was established by digestion with 0.1 mg/ml proteinase K-treated DNase (Sigma) (Tullis and Rubin, 1980) in 20 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$; 20 mM $MgCl_2$ for 60 min at 37^{0}C and with 10 μg/ml RNase A in 0.3 M NaCl for 60 min at 37^{0}C.

B.9 Plasmid DNA isolation

Plasmid DNA was isolated according to Ish-Horowicz and Burke (1981).

B.9.1 Small-scale plasmid DNA isolation

Cells from 2.2 ml of an overnight Luria-Bertani (LB) broth culture (5 ml) (Appendix A.3.1) containing 15 μg/ml tetracycline were harvested by centrifugation for 1 min in an Eppendorf microfuge. All subsequent centrifugation steps were performed in an Eppendorf microfuge. The pellet was drained and the cells resuspended in 100 μl Solution I (0.05 M glucose, 0.025 M Tris.HCl pH 8.0 and 0.01 M EDTA). After 5 min incubation at room temperature, 200 μl of fresh Solution II was added (0.2 M NaOH, 1% SDS). The solution was vortexed and left on ice for 5 min before adding 150 μl of Solution III (3 M potassium acetate pH 5.0). After at least 5 min on ice, the sample was centrifuged for 10 min, the supernatant transferred to a clean tube and the DNA precipitated by addition of an equal volume of isopropanol

and centrifugation for 10 min. The pellet was dried, resuspended in 200 μl of TE buffer (Appendix A.2.8), and 1/20 volumes of 3 M sodium acetate pH 5.5 and 2 volumes of 95% ethanol were added. The sample was incubated on ice for at least 20 min before centrifugation for 10 min. The DNA pellet was dried and resuspended in 10 μl TE buffer.

B.9.2 **Large-scale plasmid DNA isolation**

Cells were harvested from an overnight culture (400 ml LB broth containing 12.5 μg/ml Tetracycline) by centrifugation at 5 000 g for 5 min. The cells were resuspended in 30 ml of Solution I (Solutions I, II and III are the same as the small-scale plasmid isolation), incubated at room temperature for 5 min before adding 60 ml of Solution II. The sample was incubated on ice for 5 min before the addition of 45 ml of Solution III. After at least 10 min on ice the sample was centrifuged (10 000 x g for 10 min) and the supernatant removed to a clean centrifuge tube. The DNA was precipitated by the addition of an equal volume of isopropanol and the DNA collected by centrifugation. The DNA pellet was dried and resuspended in 4 ml of TE buffer. CsCl (final concentration of 1g/ml) and ethidium bromide (final conc 250 μg/ml) were added and the refractive index adjusted to 1.396. The samples were sealed in Beckman Quickseal ultracentrifuge tubes and centrifuged at 55 000 rpm in a VTi 60 rotor (Beckman) for at least 12 h. The DNA bands were visualised by fluorescence under UV illumination (320 nm) and DNA bands collected by drop fractionation of the gradient. The ethidium bromide was

removed from the sample by addition of 2 volumes of water and 3 volumes of isopropanol. The DNA was collected by centrifugation for 10-15 min in an Eppendorf microfuge. The DNA pellet was dried and resuspended in TE buffer.

B.10 Gel electrophoresis of nucleic acids

Agarose gel electrophoresis was performed using horizontal slab gels. Analytical gels (20cm length x 15 cm width) were run in custom made apparatus. Electrophoresis was performed at 40 V overnight or 100 V for 4-6h. Hoefer "minnie-gel" apparatus (10cm L x 7cm W) or custom made "slide-gel" apparatus (5cm L x 7cm W) were used for quick results and were run at 100V for approximately 60 min and 15 min respectively.

Vertical gel apparatus (Hoefer SE600, Appendix B.6) were used for acrylamide gel electrophoresis. These gels were run at 100 V for approximately 20 h at 4^0C.

B.10.1 Non-denaturing gel electrophoresis

Tris-borate-EDTA electrophoresis buffer (TBE, Appendix A.2.7) was used for all non-denaturing gels. Ethidium bromide (0.5 μg/ml) was either added to the gel and the electrophoresis buffer before pouring and running the gel or gels were stained after electrophoresis.

Agarose gel electrophoresis

Agarose (0.8% to 1.5% w/v) was dissolved in 1 X TBE by heating, and cooled to approximately 50^0C before pouring.

Acrylamide gel electrophoresis (6%)

	Volume required (ml)
Acrylamide (40%) (Appendix B.6)	24.0
water	35.9
TBE (5X)	16.0
1.5% (w/v) ammonium peroxydisulphate	4.0
TEMED	0.1

Loading buffer (agarose and acrylamide gels).

50% (v/v) glycerol

0.4% (w/v) bromophenol blue

0.4% (w/v) xylene cyanol

in TBE.

B.10.2 **Denaturing gel electrophoresis**

RNA sizes were determined by fractionation by agarose gel electrophoresis under denaturing conditions. Two different types of denaturing gels were run, formaldehyde and glyoxal gels.

Loading buffer (formaldehyde and glyoxal gels)

50% (v/v) glycerol

0.4% (w/v) bromophenol blue

0.4% (w/v) xylene cyanol

1 mM EDTA

B.10.2.1 Denaturation by formaldehyde (Maniatis *et al.*, 1982)

Electrophoresis buffer.

20 mM N-(3-morpholino)propanesulphonic acid (MOPS)

5 mM sodium acetate

1 mM EDTA, pH 7.0

The RNA was prepared in 1 X electrophoresis buffer, 50% (v/v) formamide (deionized with a mixed-bed resin), 6% (v/v) formaldehyde and incubated at 60^0C for 10 to 15 min. The RNA was electrophoresed in 0.8 - 1% (w/v) agarose containing 1 X electrophoresis buffer and 6% (v/v) formaldehyde. The formaldehyde was added to a dissolved agarose solution at 60^0C. Preparation and pouring of agarose gels was done in the fume hood. Gels were stained in 10 μg/ml ethidium bromide for 15 min and destained in several changes of water for 1 to 3 h.

B.10.2.2 Denaturation by glyoxal (McMaster and Carmichael, 1977).

Glyoxal (40% v/v) was deionized by passing through columns of mixed-bed resin until the pH was neutral. Deionized glyoxal was stored in aliquots at -20^0C. RNA samples were heated for 1 h at 50^0C in 1 M glyoxal, 50% (v/v) dimethyl sulphoxide (DMSO), 10 mM sodium phosphate pH 7.0. Glyoxylated RNA was electrophoresed in 0.8 - 1% agarose gels in 10 mM sodium phosphate electrophoresis buffer. The electrophoresis buffer was recirculated during running. The gels were stained with 33 μg/ml of acridine

orange for 30 min and destained in several changes of 1 mM EDTA.

B.11 Antiserum production

Antisera were raised in rabbits as described by Rybicki and von Wechmar (1981). Rabbits were given weekly intramuscular injections of a 1:1 (v/v) emulsion of Freund's incomplete adjuvant and virus (approximately 0.2 - 0.4 mg of virus). Rabbits were boosted after a 6 week interval and thereafter at 9 -12 week intervals for the life time of the rabbit.

Antisera to ALPV were prepared from virus purified by one or two cycles of zone electrophoresis (Appendix B.2). Antisera were checked before use by indirect ELISA (Appendix B.14) for antiserum titre and to ensure they were free of RhPV antibodies.

Antisera to RhPV was prepared from virus propagated in aphids infected with RhPV only. The RhPV inoculum was purified by sucrose gradient centrifugation (Appendix B.3).

To obtain antisera to SDS-PAGE fractionated RhPV capsid proteins, the protein bands were excised from a gel and transferred by electroblotting to nitrocellulose (Appendix B.15). The nitrocellulose was dissolved in the minimum volume of DMSO required to solubilize the paper (approx. 0.5 ml), the mixture added to an equal volume of Freund's incomplete adjuvant and injected subcutaneously in several locations in a rabbit. This procedure was repeated at weekly intervals for 3 weeks except subsequent injections were intramuscular.

B.12 Immunoglobulin preparation

Gamma-globulin (IgG) fraction of serum was prepared by ammonium sulphate salt precipitation and DEAE-cellulose filtration (Whatman DE-52) as described by Clark and Adams (1977). The IgG was stored at -20^{0}C in aliquots in half-strength phosphate-buffered saline pH 7.0 (PBS, Appendix A.2.4) at a concentration of 1 mg/ml.

B.13 Conjugation of alkaline phosphatase to IgG

Alkaline phosphatase was conjugated to IgG using glutaraldehyde (EM grade) as described by Clark and Adams (1977). Goat anti-rabbit alkaline phosphatase conjugate (GAR-AP) was purchased (Bio-Yeda, Israel).

B.14 Enzyme-linked immunosorbent assay (ELISA)

Double antibody sandwich (DAS-)ELISA and indirect ELISA were performed essentially as described by Rybicki and von Wechmar (1981), with PBS-based pH 7.0 buffers used throughout. DAS-ELISA was used to screen samples for the presence of virus and in serological relationship tests. Indirect ELISA was used to estimate antiserum titre, to screen antisera for contaminating virus antibodies and in serological relationships studies.

B.14.1 ELISA buffers

Buffers and solutions used in ELISAs are presented in Appendix A.

B.14.2 **Reaction conditions**

Reaction volumes

200 μl volumes were used throughout except for the substrate reaction when 300 μl were used.

Incubation conditions

To prevent evaporation, microtitre plates (Nunc) were incubated in a sealed container at the required temperature. Unless otherwise stated, plates were incubated at 37^0C for 90 min or overnight at 4^0C.

Washes

After each incubation step plates were emptied and washed (3 x) by flooding with PBS-Tween (PBS-T, Appendix A.2.5).

Substrate reaction

A stock solution of 10% (w/v) diethanolamine pH 9.8 was made and stored in the dark. The enzyme substrate was a 1 mg/ml solution of p-nitrophenyl phosphate (Merck, Darmstadt) in diethanolamine stock and was made up immediately before use. The substrate reaction step was incubated at room temperature until the desired colour intensity developed (1-3 h) and the A_{405} read on a eight-channel plate reader (Titertek Multiskan, Flow Laboratories).

Sample preparation

Liquid samples were diluted at least 1:1 (v/v) with PBS-Tween-milk powder (PBS-T-MP, Appendix A.2.6). Solid material was crushed directly in PBS-T-MP.

Controls

The following controls were included for all tests:-

A positive virus dilution series

A negative control e.g. virus-free aphids

An antigen-conjugate only reaction (negative control)

An antibody conjugate only reactions (negative control).

To avoid temperature effects causing apparent non-specific reactions, the outside wells of the microtitre tray were never used for experimental samples (Clark and Adams, 1977).

B.14.3 **Double antibody sandwich (DAS-) ELISA**

The antibody coating and conjugate concentrations were calibrated such that sensitive reactions were obtained with minimum background. Optimum IgG dilutions were usually between 1/400 to 1/600. For coating, IgG was diluted in PBS, and incubated for the required time. After washing, free binding sites were blocked by flooding the tray with PBS-T-MP, and trays were incubated on the bench for at least 20 min. The antigen was prepared (Appendix B.14.2) and trays incubated and washed (Appendix B.14.2). Conjugate (Appendix B.13) was diluted with PBS-T-MP to 1/400 - 1/600

and the trays incubated and washed as above. The substrate solution was added and results were recorded visually and by measuring absorbances (B.14.2)

B.14.5 **Indirect ELISA**

Plates were coated with 5 - 10 μg/ml of virus diluted in 0.1 M phosphate buffer, pH 7.0. The plates were washed and blocked as described above. A dilution series (5- or 10-fold) of antiserum to be tested was made in PBS-T-MP, with a starting dilution of 1/500. Plates were incubated, washed and goat anti-rabbit alkaline phosphatase (GAR-AP) was added at a dilution of 1/5000 in PBS-T-MP. Plates were washed and reacted with substrate as described (Appendix B.14.2).

B.15 **Immunoelectroblotting (Western blotting)**

Western blotting was performed essentially according to Rybicki and von Wechmar (1982b). Proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose paper (0.45 μm pore size, Schleicher and Schuell BA 85, NH, USA) in a Hoefer electroblot apparatus in 5 l of transfer buffer (25 mM Tris-HCl, 192 mM glycine and 20% v/v methanol, pH 8.3). Electrophoresis was for approximately 2 h at 1 A.

Electroblots were blocked overnight in a 1% (w/v) suspension of milk powder (MP) in a solution of 10 mM Tris-HCl and 0.15 M NaCl (Tris-saline-MP buffer) and reacted with antisera (diluted 1/100 to 1/200 in Tris-saline-MP) for 90 min with shaking. Blots were washed (3x) for 10 min in

saline containing 0.05% (v/v) Tween-20, and incubated in GAR-AP (1/5000 dilution in Tris-saline-MP) at room temperature for 90 min with agitation. Blots were washed as before and incubated in enzyme substrate solution (0.1 M NaCl, 0.1M Tris.HCl pH 9.5 and 5mM $MgCl_2$ solution containing 0.33 mg/ml nitro blue tetrazolium, 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.33% v/v N,N-dimethylformamide). The reaction was terminated by washing filters with a solution of 10 mM Tris.HCl pH7.5 and 1 mM EDTA.

APPENDIX C

APHID PROPAGATION, MAINTENANCE AND HANDLING

Conditions of aphid maintenance have been described in detail by von Wechmar (1987). Maintenance conditions described below were for *R. padi* aphids. Other aphids species were maintained similarly, temperature permitting.

C.1 APHID CLONES

Aphid colonies were reared from a single aphid; the origin of these are listed in Table C.1.

Table C.1. The origin of aphid clones

Colony name	Origin
Rhopalosiphum padi (Brits)	Brits, Transvaal
R. padi (Port.)	Porterville, Cape Province
R. padi (Stell.)	Stellenbosch, " "
R. maidis	Elsenberg, " "
Diuraphis noxia (UCT)	Bethlehem, Orange Free State
D. noxia (Brits)	Brits, Transvaal
D. noxia (Beth)	Bethlehem, Orange Free State
Metapolophium dirhodum	Elsenberg, Cape Province
Sitobion avenae	Elsenberg, " "
Myzus persicae	Cape Town, " "

C.2 APHID MAINTENANCE

C.2.1 Maintenance Conditions

Containers used for aphid propagation are described in Appendix C.2.3.

Aphids for virus propagation were maintained in cages at 24-26^{0}C. Under these conditions aphids were transferred weekly.

To safeguard aphid clones, stock colonies were kept in growth cabinets at 7-10^{0}C. Under these conditions aphids were transferred every 6 to 8 weeks.

Aphids for virus storage were kept at 4^{0}C in jars or cages. Under these conditions aphids were transferred every 12 and 8 weeks respectively.

C.2.2 Plants

Plants were grown in steam sterilized soil in growth rooms at controlled conditions of approximately 70% humidity, and a cycle of 14 h light/ 10 h dark with temperatures of 24^{0}C and 21^{0}C respectively. *R. padi* aphids were reared on barley cv. Clipper (*Hordeum vulgare* L.). Five to seven day old plants (approxmimately 30 seedlings/pot) were used to feed aphids.

C.2.3 Aphid containers

Cages

Wood frame, gauze-lined cages (50 x 50 x 50cm) could hold nine pots of plants. Cages were placed on custom made trolleys (maximum of eight cages/trolley) fitted with VHO

Grolux fluorescent lighting and kept at 24-26^{0}C on a 12h day/night cycle. Alternatively cages were maintained in a cold room at 4^{0}C on a 12h day/night cycle.

Growth cabinets

Growth cabinets were fitted with VHO Grolux fluorescent lighting and were temperature controlled. Cabinets could hold 36 pots of plants and were usually set on a 12 h day/night cycle with temperatures of 10^{0}C and 7^{0}C respectively.

Jars

Large preservative jars (23 cm x 11 cm) were fitted with a double gauze lid. Seeds were surface sterilised with formaldehyde, (Appendix C.2.5) and germinated seedlings were placed in approximately 6 cm of moist vermiculite in jars. The vermiculite was kept moist with Knops nutrient solution (Appendix A.2.14) and jars were kept in a cold room at 4^{0}C on a 12h day/night cycle.

Petri dishes

Petri dishes were used to maintain single or small numbers of aphids for short periods of time. Fresh leaves were placed on moist filter paper in the Petri dish. Leaves were changed every few days and the filter paper moistened with water daily.

C.2.4 Harvesting of Plants and Aphids Transfer

Plants were cut off at the base of their stems and excess aphids were shaken off the plants onto newspaper. Talcum powder was used to prevent aphids sticking together and sticking to the paper. Excess aphids were either transferred to new plants or stored at -20^0C. Harvested plants bearing aphids were stored in sealed plastic bags at 4^0C and were purified within 3 weeks of harvesting. Unwanted plant and aphid material was sprayed with Dazzel (containing Diazinon), and left for at least 12 h in a non-ventilated room before discarding for incineration.

C.2.5 Surface Sterilization of Seeds

Seeds were surface sterilized by submersion in 2% formaldehyde for 1 min and rinsing for 20 min under running water. Surface sterilized seeds were germinated in vermiculite under a fluorescent light.

C.3 APHID CONTAINMENT

The following precautions were taken to quarantine the different aphid clones.

1. A room was restricted for propagation of virus-infected aphids only. All manipulations were performed in this area in protective clothing, which remained in the room.
2. Virus-free aphids were kept in a sealed growth chambers in a separate, locked room. Entry was

restricted for maintenance purposes and to collect aphids for experimental purposes.

3. Fresh plants were placed around aphid cages and growth chambers to "catch" stray aphids. As aphids cannot easily traverse talcum powder, a line was placed at the exit of rooms and around growth chamber doors.
4. Aphid cages were dipped in biocide solution between use and specific cages were restricted to specific virus colonies.
5. When necessary, growth chambers and cages were sterilised with 2% (v/v) formaldehyde.
6. Jars were heat-sterilised at 100^0C for 120 min.
7. Unwanted plants and aphids were sprayed with insecticide, and kept an in unventilated room for 12 h before incineration
8. Stocks of "virus-free" aphids were screened monthly for virus by DAS-ELISA.

REFERENCES CITED

AHLQUIST, P. AND KAESBERG, P. (1979). Determination of the length distribution of poly(A) at the 3' terminus of the virion RNAs of EMC virus, poliovirus, rhinovirus, RAV-61 and CPMV and of mouse globin mRNA. *Nucleic Acids Research* **7:5**, 1195-1204.

AKAI, H., GATEFF, E., DAVIS, L.E. AND SCHNEIDERMAN H.A. (1967). Virus-like particles in normal and tumorous tissues of *Drosophila*. *Science* **157**, 810-813.

ALLEN, M.A., AND BALL, B.V. (1986). Properties of a virus pathogenic in the aphid *Sitobion avenae*. In Proceedings of "Applied Biology ... Pathways Forward". Residential meeting, Association of Applied Biologists, University of Warwick, 29 September - 1 October 1986.

AMBROS, V. AND BALTIMORE, D. (1978). Protein is linked to the 5' end of poliovirus RNA by a phosphodiester linkage to tyrosine. *Journal of Biological Chemistry* **253** (15), 5263-5266.

ANDERSON, D.L. AND GIBBS, A.J. (1988). Inapparent virus infections and their interactions in pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. *Journal of General Virology* **69**, 1617-1625.

ARGOS, P., KAMER, G., NICKLIN, M.J. AND WIMMER, E. (1984). Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families. *Nucleic Acids Research* **12** (18), 7251-7267.

ARNOLD, E., LUO, M., VRIEND, G., ROSSMANN, M.G., PALMENBERG, A.C., PARKS, G.D., NICKLIN, M.J.H. AND WIMMER, E. (1987). Implications of the picornavirus capsid structure for polyprotein processing. *Proceeding of the National Academy of Science (USA)* **84**, 21-25.

BAICHWAL, V.V. AND SUGDEN B. (1988). Latency comes of age for herpesvirus. *Cell* **52**, 787-789.

BAILEY, L. (1976). Viruses attacking the honey bee. *Advances in Virus Research* **20**, 271-302.

BAILEY, M.J. AND FUJITA, F.R.H. (1987). Specific immunological response against the granulosis virus of the codling moth (*Cydia pomonella*) in woodmice (*Apodemus sylvaticus*): field observations. *Annals of Applied Biology* **111**, 649-660.

BAILEY, L. AND MILNE, R.G. (1969). The multiplication regions and interaction of acute and chronic bee-paralysis viruses in adult honey bees. *Journal of General Virology* **4**, 9-14.

BAILEY, L. AND MILNE, R.G. (1978). Filamentous viruslike particles in honey bees in Britain. *Journal of Invertebrate Pathology* **32**, 15-20.

BAILEY, L. AND WOODS, R.D. (1977). Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses. *Journal of General Virology* **37**, 175-182.

BAILEY, L., BALL, B.V. AND PERRY, J.N. (1981). The prevalence of viruses of honey bees in Britain. *Annals of Applied Biology* **97**, 109-118.

BAILEY, L., BALL, B.V. AND PERRY, J.M. (1983). Association of viruses with two protozoal pathogens of the honey bee. *Annals of Applied Biology* **103** (1), 13-20.

BAILEY, L., GIBBS, A.J. AND WOODS, R.D. (1963). Two viruses from adult honey bees (*Apis mellifera* Linnaeus). *Virology* **21**, 390-395.

BAILEY, L., CARPENTER, J.M. AND WOODS, R.D. (1979). Egypt bee virus and Australian isolates of Kashmir bee virus. *Journal of General Virology* **43**, 641-647.

BAILEY, L., NEWMAN, J.F.E. AND PORTERFIELD, J.S. (1975). The multiplication of Nodamura virus in insect and mammalian cell cultures. *Journal of General Virology* **26**, 15-20.

BALL, B.V., OVERTON, H.A., BUCK, K.W., BAILEY, L. AND PERRY, J.N. (1985). Relationships between the multiplication of chronic bee paralysis virus and its associate particle. *Journal of General Virology* **66**, 1423-1429.

BLACKHURST, D.M. (1987). *Cleavage of the precursor coat protein of black beetle virus strain W17 in rabbit reticulocyte lysate*, pp. 64-68. MSc thesis, Rhodes University.

BRIESE, D.T. AND PODGWAITE, J.D. (1985). Development of viral resistance in insect populations. In *Viral Insecticides for Biological Control*, pp. 361-398. Edited by K. Maramorosch and K.E. Sherman. Academic Press, Florida.

BRISCO, M.J., HULL, R., and WILSON, T.M.A. (1985). The effect of extraction protocol on the yield, purity, and translation products of RNA from an isometric plant virus. *Journal of Virological Methods* **10**, 195-202.

BROWN, F. (1986). The classification and nomenclature of viruses: summary of results of meeting of the International Committee on Taxonomy of viruses in Sendai, September 1984. *Intervirology* **25**, 141-143.

CHAMBERS, R.J., AND SUNDERLAND, K.D., STACEY, D.L. AND WYATT, I.J. (1986). Control of cereal aphids in winter wheat by natural enemies: aphid-specific predators, parasitoids and pathogenic fungi. *Annals of Applied Biology* **108**, 219-231.

CHAO, Y.C. AND YOUNG, S.Y. (1986). Characterization of a picornavirus isolated from *Pseudoplusia includens* (Lepidoptera:Noctuidae). *Journal of Invertebrate Pathology* **47**, 247-257.

CHERVENKA, C.H. (1969). *A manual of methods for the analytical ultracentrifuge*, pp. 23-37. Palo Alto: Beckman Instruments, California.

CLARK, M.F. AND ADAMS, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475-483.

CLARK, M.F. AND BARBARA, D.J. (1987). A method for the quantitative analysis of ELISA data. *Journal of Virological Methods* **15**, 213-222.

CRUMP, W.A.L. AND MOORE, N.F. (1981). The polypeptides induced in *Drosophila* cells by a virus of *Heteronychus arator*. *Journal of General Virology* **52**, 173-176.

D'ARCY, C.J., BURNETT, P.A., HEWINGS, A.D. AND GOODMAN, R.M. (1981a). Purification and characterization of a virus from the aphid *Rhopalosiphum padi*. *Virology* **112**, 345-349.

D'ARCY, C.J., BURNETT, P.A. AND HEWINGS, A.D. (1981b). Detection, biological effects, and transmission of a virus of the aphid *Rhopalosiphum padi*. *Virology* **114**, 268-272.

DALL, D.J. (1985). Inapparent infection of honey bee pupae by Kashmir and sacbrood bee viruses in Australia. *Annals of Applied Biology* **106**, 461-468.

DALL, D.J. (1987). Multiplication of Kashmir bee virus in pupae of the honeybee, *Apis mellifera*. *Journal of Invertebrate Pathology* **49**, 279-290.

DAVID, J. AND PLUS, N. (1971). Le virus P de la Drosophile: comparaison de la longevite et de la fecondite des mouches infectees par injection ou par contamination naturelle. *Annals de l'Institute Pasteur* **120**, 107-119.

DAWSON, W.O. (1983). Tobacco mosaic virus protein synthesis is correlated with double-stranded RNA synthesis and not single-stranded RNA synthesis. *Virology* **125**, 314-323.

DIXON, A.F.G. (1985). *Aphid Ecology*, pp. 1-134. Blackie and Son Ltd., London.

DIXON, A.F.G. (1987a). The way of life of aphids: Host specificity, speciation and distribution. In *World Crop Pests; Aphids: their biology, natural enemies and control*, pp. 197-206. Edited by A.K. Minks and P. Harrewijn. Elsevier Science Publishers, Amsterdam.

DIXON, A.F.G. (1987b). Parthenogenetic reproduction and the rate of increase of aphids. In *World Crop Pests, aphids their biology, natural enemies and control*, pp. 269-285. Edited by A.K. Minks and P. Harrewijn. Elsevier Science Publishers, Amsterdam.

DORNER, A.J., SEMLER, B.L., JACKSON, R.J., HANECAK, R., DUPREY, E. AND WIMMER, E. (1984). *In vitro* translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. *Journal of Virology* **50** (2), 507-514.

DURR, H.J.R. (1983). A list of additional host plants of aphids (Hemiptera: Aphidoidea) in South Africa. *Phytophylactica* **15**, 67-69.

EASTOP, V.F. (1977). "Worldwide importance of aphids as virus vectors". *In Aphids as virus vectors*, pp. 3-62. Edited by K.F. Harris and K. Maramorosch. Academic Press, New York.

EATON, B.T. AND STEACIE, A. (1980). Cricket paralysis virus RNA has a 3' terminal poly(A). *Journal of General Virology* **50**, 167-171.

EMINI, E.A., SCHLEIF, W.A., COLONNO, R.J. AND WIMMER, E. (1985). Antigenic conservation and divergence between the viral-specific proteins of poliovirus type 1 and various picornaviruses. *Virology* **140**, 13-20.

ENTWISTLE, P.F. (1983). Viruses of insect pest control. *Span* **26** (**2**).

EWEIDA, M AND OXELFELT, P. (1985). Purification and properties of two Swedish isolates of barley yellow dwarf virus and their serological relationships with American isolates. *Annals of Applied Biology* **106**, 475-482.

FALCON, L.A. (1985). Development and use of microbial insecticides. In *Biological control in agricultural integrated pest management systems*, pp. 229-242. Edited by M.A. Hoy and D.C. Herzog. Academic Press Inc. (London).

FAULKNER, P AND BOUCIAS, D.G. (1985). Genetic improvements of insect pathogens: emphasis on the use of baculoviruses. In *Biological control in integrated pest management systems*, pp. 263-280. Edited by M.A. Hoy. Academic Press, Inc. USA.

FENNER, F. (1976). Classification and nomenclature of viruses. Second report of the International Committee on Taxonomy of Viruses. *Intervirology* **7**, 39-40.

FLANEGAN, J.B., YOUNG, D.C., TOBIN, G.J., STOKES, M.M., MURPHY, C.D. AND OBERSTE, S.M. (1987). Mechanism of RNA replication by the poliovirus RNA polymerase, HeLa cell host factor, and VPg. *In Positive Strand RNA Viruses*, pp. 273-284. Edited by M.A. Brinton and R.R. Rueckert. Alan R. Liss Inc., New York.

FLEXNER, J.L., LIGHTHART, B. AND CROFT, B.A. (1986). The effect of microbial pesticides on target, beneficial arthropods. *Agriculture, Ecosystems and Environments* **16**, 203-254.

FORD, D.J., RADIN, R. AND PESCE, A.J. (1978). Characterisation of glutaradehyde-coupled alkaline phosphatase-antibody and lactoperoxidase-antibody conjugated. *Immunochemistry* **15**:237.

FORSS, S., STREBEL, K., BECK, E. AND SCHALLER, H. (1984). Nucleotide sequence and genome organization of foot-and-mouth disease virus. *Nucleic Acids Research*, **12** (16), 6587-6601.

FRANSSEN, H., LEUNISSEN, J., GOLDBACH, ROB., LOMONOSSOFF, G. AND ZIMMERN, D. (1984). Homologous sequences in non-structural proteins from cowpea mosaic virus and picornaviruses. *EMBO Journal*, **3** (4), 855-861.

FRAVAL, A. AND LAPIERRE, H. (1970). Isolement a partir de graminees et de pucerons (Homoptera: Aphididae) d'un virus a RNA. *CR Acad. Sci. Paris*, **270**, 890-893.

GIBBS, A., AND HARRISON, B.D. (1976). Physical and chemical methods of assay and analysis. In *Plant Virology: the Principles*, pp. 110-128. Edward Arnold Ltd., London.

GILDOW, F.E. AND D'ARCY, C.J. (1988). Barley and oats as reservoirs for an aphid virus and the influence on barley yellow dwarf virus transmission. *Phytopathology*, in press.

GILDOW, F.E. AND ROCHOW, W.F. (1980). Role of accessory salivary glands in aphid transmission of BYDV. *Virology* **104**, 97-108.

GILDOW, F.E., BALLINGER, M.E. AND ROCHOW, W.F. (1983). Double-stranded RNA's associated with barley yellow dwarf virus infection of oats. *Phytopathology* **73**, 1570-1572.

GOLDBACH, R. (1987). Genome similarities between plant and animal RNA viruses. *Microbiological Sciences* **4:7,** 197-202.

GRIFFITHS, E., WRATTEN, S.D. AND VICKERMAN, G.P. (1985). Foraging by the carabid *Agonum dorsale* in the field. *Ecological Entomology* **10,** 181-189.

GRUBMAN, M.J. (1984). *In vitro* morphogenesis of foot-and-mouth disease virus. *Journal of Virology* **43** (3), 760-765.

GRUBMAN, M.J., ZELLNER, M. AND WAGNER, J. (1987). Antigenic comparison of the polypeptides of foot-and-mouth disease virus serotypes and other picornaviruses. *Virology* **158,** 133-140.

GUPTA, S., SHIH, D.S. AND SHIH, C.Y.T. (1985). A comparative study on the translation of cardiovirus RNAs in rabbit reticulocyte lysates. *Virology* **144,** 523-528.

HALL, R.A. (1976). A bioassay of the pathogenicity of *Verticillium lecanii* conidiospores on the aphid, *Macrosiphoniella sanborni*. *Journal of Invertebrate Pathology* **27,** 41-48.

HARPAZ, I. (1982). Nonpesticidal control of vector-borne viruses. *Pathogens, vectors and plant disease,* pp 1-21. Academic Press, Inc.

HARRAP, K.A. AND PAYNE, C.C. (1979). The structural properties and identification of insect viruses. *Advances in Virus Research* **25,** 273-355.

HARRAP, K.A., LONGWORTH, J.F., TINSLEY, T.W. AND BROWN, K.W. (1966). A noninclusion virus of *Gonometa podocarpi* (Lepidoptera: Lasiocampidae). *Journal of Invertebrate Pathology* **8,** 270-272.

HASHIMOTO, Y. and KAWASE, S. (1983). Characteristics of structural proteins of infectious flacherie virus from the silkworm, *Bombyx mori*. *Journal of Invertebrate Pathology* **41,** 68-76.

HASHIMOTO, Y., WATANABE, A. AND KAWASE, S. (1984). *In vitro* translation of infectious flacherie virus RNA in a wheat germ and a rabbit reticulocyte system. *Biochmica et Biophysica Acta* **781,** 76-80.

HASHIMOTO, Y., WATANABE, A. AND KAWASE, S. (1986) Evidence for the presence of a genome-linked protein in infectious flacherie virus. *Archives of Virology* **90,** 301-312

HATFILL, S.J. (1988). *The pathogenesis of aphid lethal paralysis virus.* MSc thesis, University of Cape Town.

HENDRY, D., HODGSON, V., CLARK, R. AND NEWMAN, J. (1985). Small RNA viruses co-infecting the pine emperor moth (*Nudaurelia cytherea capensis*). *Journal of General Virology* **66**. 627-632.

HEY, T.D., RICHARD, O.C. AND EHRENFELD, E. (1986). Synthesis of plus- and minus-strand RNA from poliovirion RNA template *in vitro*. *Journal of Virology* **58:3**, 790-796.

HOGLE, J.M., CHOW, M. AND FILMAN, D.J. 1985. Three-dimensional structure of poliovirus at 2.9 Å resolution. *Science* **229**, 1358-1365.

HUISMANS, H. AND CLOETE, M. (1987). A comparison of different cloned bluetongue virus genome segments as probes for the detection of virus-specified RNA. *Virology* **158**, 373-380.

ICENOGLE, J., GILBERT, S.F., GRIEVES, J., ANDEREGG, J. AND RUECKERT, R. (1981). A neutralizing monoclonal antibody against poliovirus and its reaction with related antigens. *Virology* **115**, 211-215.

ISH-HOROWICZ, D. AND BURKE, J.F. (1981). Rapid and efficient cosmid vector cloning. *Nucleic Acids Research* **9**, 2989-2998.

JACKSON, R.J. (1986). A detailed kinetic analysis of the *in vitro* synthesis and processing of EMC virus products. *Virology* **149**, 114-127.

JAEGLE, M. AND VAN REGENMORTEL, M.H.V. (1985). Use of ELISA for measuring the extent of serological cross-reactivity between plant viruses. *Journal of Virological Methods* **11**, 189-198.

JORE, J., DE GEUS, B., JACKSON, R.J., POUWELS, P.H. AND ENGER-VALK, B.E. (1988). Poliovirus protein 3CD is the active protease for processing of the precursor protein P1 *in vitro*. *Journal of General Virology* **69**, 1627-1636.

JOUSSET, F.X., BERGOIN, M. AND REVET, B. (1977). Characterisation of the *Drosophila* C virus. *Journal of General Virology* **34**, 269-285.

JUCKES, I.R.M. (1979). Comparison of some biophysical properties of the Nudaurelia β and viruses. *Journal of General Virology* **42**, 89-94.

KING, L.A., MASSALSKI, P.R., COOPER, J.I. AND MOORE, N.F. (1984). Comparison of the genome RNA sequence homology between cricket paralysis virus and strains of *Drosophila* C virus by complementary DNA hybridization analysis. *Journal of General Virology* **65**, 1193-1196.

KING, L.A., PULLIN, J.S.K. AND MOORE, N.F. (1986). Comparison of cricket paralysis virus with mammalian picornaviruses. *Fundamental and applied aspects of invertebrate pathology* pp73. Edited by R.A. Samson, J.M. Vlak and D. Peters. Wageningen: ICIP Foundation.

KING, L.A., PULLIN, J.S.K., STANWAY, G., ALMOND, J.W. AND MOORE, N.F. (1987). Cloning of the genome of cricket paralysis virus: sequence of the 3' end. *Virus Research* **6**, 331-344.

KITAJIMA, E.W. (1976). Isometric, viruslike particles in the green peach aphid, *Myzus persicae*. *Journal of Invertebrate Pathology* **28**, 1-10.

KLINGHAUF, F.A. (1987). Feeding, Adaptation and Excretion. In *World Crop Pests, Aphids: their biology, natural enemies and control*, pp. 244-246. Edited by A.K. Minks and P. Harrewijn. Elsevier Science Publishers, Amsterdam.

KOENIG, R. (1978). ELISA in the study of homologous and heterologous reactions of plant viruses. *Journal of General Virology* **40**, 309-318.

KORANT, B.D. (1972). Cleavage of viral precursor proteins *in vivo* and *in vitro*. *Journal of Virology* **10** (4), 751-759.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

LARSEN, G.R., ANDERSON, C.W., DORNER, A.J., SEMLER, B.L. AND WIMMER, E. (1982). Cleavage sites within the poliovirus capsid protein precursors. *Journal of Virology* **41** (1), 340-344.

LOMMEL, S.A., MORRIS, T.J. AND PINNOCK, D.E. (1985). Characterization of nucleic acids associated with Arkansas bee virus. *Intervirology* **23**, 207-216.

LONBERG-HOLM, K. AND BUTTERWORTH, B.E. 1976. Investigation of the structure of polio- and human rhinovirions through the use of selective chemical reactivity. *Virology* **71**, 207-216.

LONGWORTH, J.F. (1978). Small isometric viruses of invertebrates. *Advances in Virus Research* **23**, 103-157.

LONGWORTH, J.F. AND CAREY, G.P. (1976). A small RNA virus with a divided genome from *Heteronychus arator* (F.) (Coleoptera:scarabaeidae). *Journal of General Virology* **33**, 31-40.

LONGWORTH, J.P., PAYNE, C.C AND MACLEOD, R. (1973). Studies on a virus isolated form *Gonometa podocarpi* (Lepidoptera: Lasiocampidae). *Journal of General Virology* **18**, 119-12

LUO, M., VRIEND G., KAMER, G., MINOR, I., ARNOLD, E., ROSSMANN, M.G., BOEGE, U., SCRABA, D.G., DUKE, G.M. AND PALMENBERG, A.C. (1987). The structure of Mengo virus at atomic resolution. *Science* **235**, 182-191.

MAK, T.W., O'CALLAGHAN, D.J. AND COLTER, J.S. (1970). Studies of the pH inactivation of three variants of Mengo encephalomyelitis virus. *Virology* **40**, 565-571.

MANDEL, M. AND HIGA A. (1970). Calcium dependent bacteriophage DNA infection. *Journal of Molecular Biology* **53**, 154.

MANIATIS, T., FITCH, E.F. AND SAMBROOK, J. (1982). *Molecular cloning; a laboratory manual*. New York, Cold Spring Harbor Laboratory.

MANOUSIS T. AND MOORE, N.F. (1987). Cricket paralysis virus a potential control agent for the olive fruit fly, *Dacus-Oleae Gmel*. *Applied and Environmental Microbiology* **53:1**, 142-148.

MATTHEWS, R.E.F. (1982). Classification and nomenclature of viruses. Fourth Report of the International Committee on Taxonomy of Viruses. *Intervirology* **17**, 1-199.

M^{C}GREGOR, S. AND MAYOR, H.D. 1971. Internal components released from rhinovirus and poliovirus by heat. *Journal of General Virology* **10**, 203-207.

M^{C}MASTER, G.K. AND CARMICHAEL, G.G. (1977). Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proceeding of the National Academy of Science (USA)* **74**, 4835-4837.

MILLER, G.L. AND GOLDER, R.H. (1950). Buffers of pH 2 to 12 for use in electophoresis. *Archives of Biochemistry* **29**, 420-423.

MILNE, R.G. and LESEMANN D.E. (1984). Immunosorbent electron microscopy in plant virus studies. In *Methods in Virology 8*, pp. 85-101. Edited by K. Maromorosch and H. Koprowski. Academic Press Inc. Florida.

MIYAZAKI, M. (1987). Morphology of aphids. In *World Crop Pests, aphids their biology, natural enemies and control*, pp. 1-23. Edited by A.K. Minks and P. Harrewijn. Elsevier Science Publishers, Amsterdam.

MOORE, N.F. AND PULLIN, J.S.K. (1982). Plaque purification of cricket paralysis virus using an agar overlay on *Drosophila* cells. *Journal of Invertebrate Pathology* **39**, 10-14.

MOORE, N.F AND PULLIN, J.S.K. (1983). Heat shock used in combination with amino acid analogues and protease inhibitors to demonstrate the processing of proteins of an insect picornavirus (*Drosophila* C virus) in *Drosophila melanogaster* cells. *Annals of Virology (Pasteur Institute)* **134E** (3), 285-292.

MOORE, N.F. AND TINSLEY, T.W. (1982). The small RNA-viruses of Insects. Brief review. *Archives of Virology* **72**, 229-245.

MOORE, N.F., KEARNS, A. AND PULLIN, J.S.K. (1980). Characterization of cricket paralysis virus-induced polypeptides in *Drosophila* cells. *Journal of Virology* **33**, 1-9.

MOORE, N.F., KING, L.A. AND PULLIN, J.S.K. (1987). Insect Picornaviruses. *The molecular biology of the positive strand RNA viruses* pp. 67-74. Edited by D.J. Rowlands, B.W.J. Mahy and M. Mayo. Academic Press Inc. Ltd. (London).

MOORE, N.F., REAVY B. and KING L. (1985). General characteristics, gene organization and expression of small RNA viruses of insects. *Journal of General Virology* **66**, 647-659.

MOORE, N.F, McKNIGHT, L. and TINSLEY, T.W. (1981a). Occurrence of antibodies against insect virus proteins in mammals: simple model to differentiate between passive exposure and active virus growth. *Infections and Immunity* **31**, 825-827.

MOORE, N.F., REAVY, B. AND PULLIN, J.S.K. (1981b). The polypeptides induced in *Drosophila* cells by *Drosophila* C virus (strain Ouarzazate). *Virology* **112**, 411-416.

MOORE, N.F., REAVY, B. AND PULLIN, J.S.K. (1981c). Processing of CrPV induced polypeptides in *Drosophila* cells; production of high molecular weight polypeptides by treatment with iodoacetamide. *Archives of Virology* **68**, 1-8.

MOORE, N.F., PULLIN, J.S.K. AND REAVY, B. (1981d). Inhibition of the induction of heat shock proteins in *Drosophila melanogaster* cells infected with insect picornaviruses. *FEBS Letters* **128**, 93-96.

MOORE, N.F., PULLIN, J.S.K. AND REAVY, B. (1981e). The intracellular proteins induces by CrPV in *Drosophila* cells; the effect of protease inhibitors and amino acid analogues. *Archives of Virology* **70**, 1-10.

MOORE, N.F., PULLIN, J.S.K., CRUMP, W.A.L. AND PLUS, N. (1982). The proteins expressed by different isolates of *Drosophila* C virus. *Archives of Virology* **74**, 21-30.

MORRIS, T.J., HESS, R.T. AND PINNOCK, D.E. (1979). Physicochemical characterization of a small RNA virus associated with baculovirus infection in *Trichoplusia ni*. *Intervirology* **11**, 238-247.

MORROW, C.D., GIBBONS, G.F. AND DASGUPTA, A. (1985). The host proteins required for *in vitro* replication of poliovirus is a protein kinase that phosphorylates eukaryotic initiation Factor-2. *Cell* **40**, 913-921.

MOUSSA, A.Y. (1981). Studies of the housefly virus structure and disruption during purification procedures. *Micron* **12**, 131-138.

MURANT, A.F., ROBERTS, I.M. AND ELNAGER, S. (1976). Association of virus-like particles with the foregut of the aphid *Cavariella aegopodii* transmitting the semi-persistent viruses anthricus yellows and parsnip yellow fleck. *Journal of General Virology* **31**, 47-57.

MURANT, A.F., HEMIDA, S.K. AND MAYO, S.K. (1987). Plant viruses that resemble picornaviruses (Abstr.). In Proceedings, *VII International Congress of Virology*, pp. 183, Edmonton, Canada.

MUSSEN, E.C. AND FURGALA, B. (1977). Replication of sacbrood virus in larval and adult honeybees, *Apis mellifera*. *Journal of Invertebrate Pathology* **30**, 20-34.

NEWMAN, J.F.E. ROWLANDS, D.J. AND BROWN, F. (1973). A physico-chemical sub-grouping of the mammalian picornaviruses. *Journal of General Virology* **18**, 171-180.

ODINDO, M.O., PAYNE, C.C., CROOK, N.E. AND JARRETT P. (1986). Properties of a novel DNA virus from the Tsetse Fly, *Glossina pallidipes*. *Journal of General Virology* **67**, 527-536.

OFORI, F.A. AND FRANCKI, R.I.B. 1985. Transmission of leafhopper A virus, vertically through eggs and horizontally through maize in which it does not multiply. *Virology* **144**, 152-157.

PALMENBERG, A.C. (1987). Comparative organization and genome structure in picornaviruses. *In Positive Strand RNA Viruses*, pp. 25-34. Edited by M.A. Brinton and R.R. Rueckert. Alan R. Liss Inc., New York.

PALMENBERG, A.C. AND RUECKERT, R.R. (1982). Evidence for intramolecular self-cleavage of picornaviral replicase precursors. *Journal of Virology* **41** (1), 244-249.

PARRISH, W.B. and BRIGGS,J.D. (1966). Morphological identification of virus-like particles in the corn leaf aphid, *Rhopalosiphum maidis* (Fitch). *Journal of Invertebrate Pathology* **18**, 122-123

PELHAM, H.R.B. (1979). Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates. *Virology* **96**, 463-477.

PEROTTI, M.E. AND BAIRATI, A. (1968). Ultrastructure of the melanotic masses in two tumorous strains of *Drosophila melanogaster* (*tuB3* and *Freckled*). *Journal of Invertebrate Pathology* **10**, 122-138.

PETERS, D. (1967). Potato leafroll virus, its purification from its vector *Myzus persicae*. *Virology* **26**, 122-123.

PHILPOTT, D.E., WEIBEL, J., ATLAN, H., AND MIQUEL, J. (1969). Viruslike particles in the fat body, oenocytes, and central nervous tissue of *Drosophila melanogaster* imagoes. *Journal of Invertebrate Pathology* **14**, 31-38.

PLUS, N, AND DAVID, J. (1971). Le virus P de la *Drosophile*: comparaison de la longevite et de la fecondite des mouches infectees par injection ou par contamination naturelle. *Annals de l'Institute Pasteur* **120**, 107-119.

PLUS, N. AND SCOTTI, P.D. (1984). The biological properties of eight different isolates of cricket paralysis virus. *Annals de Virologie (Pasteur Institute)* **135**, 257-268.

PLUS, N., CROIZIER, G., REINGANUM, C. AND SCOTTI, P.D. (1978). Cricket paralysis virus and *Drosophila* C virus: serological analysis and comparison of capsid polypeptides and host range. *Journal of Invertebrate Pathology* **31**, 296-302.

PLUS, N., CROIZIER, G., VEYRUNES, J.C. AND DAVID, J. (1976). A comparison of buoyant density and polypeptides of *Drosophila* P, C and A viruses. *Intervirology* **7**, 346-350.

PLUS, N., CROZIER, G. REINGANUM, C. AND SCOTTI, P.D. (1978). Cricket paralysis virus and *Drosophila* C virus: Serological analysis and comparison of capsid polypeptides and host range. *Journal of Invertebrate Pathology* **31**, 296-302.

PODGWAITE, J.D. AND MAZZONE H.M. (1986). Latency of insect viruses. *Advances in Virus Research*, **31**, 293-319. Edited by K. Maramorosch, F.A. Murphy and A.J. Shalkin. Academic Press.

PUDNEY, M., NEWMAN, J.F.E. AND BROWN, F. (1978). Characterization of Kawino virus, an entero-like virus isolated from the mosquito *Mansonia uniformis* (Diptera: culicidae). *Journal of General Virology* **40**, 433-441.

PULLIN, J.K., BLACK, F., KING, L.A., ENTWISTLE, P,F. AND MOORE, N.F. (1984). Characterisation of a small RNA-containing virus in field-collected larvae of the Tussock Moth, *Lymantria ninayi*, from Papua New Guinea. *Applied and Environmental Microbiology* **48** (3), 504-507.

PULLIN, S.K., MOORE, N.F., CLEWLEY, J.P. AND AVERY R.J. (1982). Comparison of the genomes of two insect picornaviruses, cricket paralysis virus and *Drosophila* C virus, by ribonuclease T_1 oligonucleotide fingerprinting. *FEMS Microbiology Letters* **15**, 215-218.

RAE, P.M.M. AND GREEN, M.M. (1967). Virus-like particles in adult *Drosophila melanogaster*. *Virology* **34**, 187-212.

REAVY, B. AND MOORE, N.F. (1981a). *In vitro* translation of cricket paralysis virus RNA. *Archives of Virology* **67**, 175-180.

REAVY, B. AND MOORE, N.F. (1981b). Cell-free translation of cricket paralysis virus RNA: analysis of the synthesis and processing of virus-specified proteins. *Journal of General Virology* **55**, 429-438.

REAVY, B. AND MOORE, N.F. (1983a). Cell-free translation of *Drosophila* C virus RNA: Identification of a virus protease activity involved in capsid protein synthesis and further studies on *in vitro* processing of cricket paralysis virus specific proteins. *Archives of Virology* **76**, 101-115.

REAVY, B. AND MOORE, N.F. (1983b). An inhibitor-resistant protease specified by an insect picornavirus, and the role of cellular proteases in the rapid processing of capsid protein precursors. *Journal of General Virology* **64**, 1831-1833.

REAVY, B. AND MOORE, N.F. (1983c). The gene organisation of a small RNA-containing insect virus: comparison with that of mammalian picornaviruses. *Virology* **131**, 551-554.

REAVY, B., CRUMP, W.A.L. AND MOORE, N.F. (1983a). Characterisation of cricket paralysis virus and *Drosophila* C virus induced RNA species synthesised in infected *Drosophila melanogaster* cells. *Journal of Invertebrate Pathology* **41**, 397-400.

REAVY, B., PULLIN, J.S.K. AND MOORE, N.F. (1983b). Translational inhibition of heat-shock induced gene expression in picornavirus-infected *Drosophila melanogaster* cells. *Microbios* **38**, 91-98.

REINGANUM, C. (1975). The isolation of cricket paralysis virus from the emperor gum moth, *Antherae eucalypti* Scott, and its infectivity towards a range of insect species. *Intervirology* **5**, 97-102.

REINGANUM, C. AND SCOTTI, P.D. (1976). Serological relations between twelve small RNA viruses of insects. *Journal of General Virology* **31**, 131-134.

REINGANUM, C., O'LOUGHLIN, G.T. AND HOGAN, T.W. (1970). A nonoccluded virus of field crickets *Teleogryllus oceanicus* and *T. commodus* (Orthoptera: Gryllidae). *Journal of Invertebrate Pathology* **16**, 214-220.

REINGANUM, C., ROBERTSON, J.S., AND TINSLEY, T.W. (1978). A new group of RNA viruses from insects. *Journal of General Virology* **40**, 195-202.

REINGANUM, C., GAGEN, S.J., SEXTON, S.B. AND VELLACOTT, H.P. (1981). A survey for pathogens of the back field cricket, *Teleogryllus commodus*, in the Western District of Victoria, Australia. *Journal of Invertebrate Pathology* **38**, 153-160.

RICHARD-MOLARD, C., BLONDEL, D., WYERS, R. AND DEZELEE, S. (1984). Sigma virus: growth in *Drosophila melanogaster* cell culture; purification; protein composition and localization. *Journal of General Virology* **65**, 91-99.

RIGBY, P.W.J., DIECKMANN, M., RHODES, C. AND BERG, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *Journal of Molecular Biology* **113**, 237.

ROBERTS, T., KAUPP, W. AND FAULKNER, P. (1987) Isolation and characterization of small RNA viruses from field collections of Eastern spruce budworm. In proceeding to *VII International Congress of Virology*, Edmonton, Canada. pp. 209.

ROSSMANN, M.G. AND RUECKERT, R.R. (1987). What does the molecular structure of viruses tell us about viral functions? *Microbiological Sciences*, **4:7**, 206-214.

ROSSMAN, M.G., ARNOLD, E., ERIKSON, J.W. FRANKENBERGER, E.E., GRIFFITH, J.P. HECHT, H.J., JOHNSON, J.R., KAMER, J.R., LUO, M., MOSSER, A.G., RUECKERT, R.R. SHERRY, B. AND VRIEND, G. (1985). Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature* **317**, 145-153.

ROWLANDS, D.J., SHIRLEY, M.W.,SANGAR, D.V. AND BROWN.F. (1975). A high density component in several vertebrate enteroviruses. *Journal of General Virology* **29**, 223-234.

RUECKERT, R.R. (1976). On the structure and morphogenesis of picornaviruses. In *Comprehensive Virology* **6**, 131-213. Edited by H. Fraenkel-Conrat and R.R. Wagner. Plenum, New York.

RUECKERT, R.R. AND WIMMER, E. (1984). Systematic nomenclature of picornavirus proteins. *Journal of Virology* **50:3**, 957-959.

RYBICKI, E.P. (1984). *Investigations of viruses affecting South African small grains.* Ph.D. thesis, University of Cape Town.

RYBICKI, E.P. AND COYNE, V.C. 1983. Serological differentiation of brome mosaic virus morphomers. *FEMS Microbiolology Letters* **20**, 103-106.

RYBICKI, E.P. AND VON WECHMAR, M.B. (1981). The serology of the bromoviruses. 1. Serological inter-relationships of the bromoviruses. *Virology* **109**, 391-402.

RYBICKI , E.P. AND VON WECHMAR, M.B. (1982a). Characterization of an aphid-transmitted virus disease of small grains: Isolation and partial characterization of three viruses. *Phytopathologische Zeitschrift* **103**, 306-322.

RYBICKI, E.P. AND VON WECHMAR, M.B. (1982b). Enzyme-assisted immune detection of plant virus proteins electroblotted onto nitrocellulose paper. *Journal of Virological Methods* **5**, 267-278.

RYBICKI, E.P. AND VON WECHMAR, M.B. (1984). Serological, biophysical and biochemical investigations of aphid-transmitted viruses of small grains. *In (*Diuraphis noxia *Mordw.) Research in the Republic of South Africa*, pp. 42-43. Edited by M.C. Walters. Pretoria: Government Printer.

SCOTTI, P.D. (1976). Cricket paralysis virus replicates in cultured *Drosophila* cells. *Intervirology* 6, 333-342.

SCOTTI, P.D. (1977). End-point dilution and plaque assay methods for titration of cricket paralysis virus in cultured *Drosophila* cells. *Journal of General Virology* **25**, 393-396.

SCOTTI, P.D. (1985). The estimation of virus density in isopycnic cesium chloride gradients. *Journal of Virological Methods* **12**, 149-160.

SCOTTI, P.D. AND LONGWORTH, J.F. (1980). Naturally occurring IgM antibodies to a small RNA insect virus in some mammalian sera in New Zealand. *Intervirology* **13**, 186-191.

SCOTTI, P.D. AND WIGLEY, P.J. (1982). Factors affecting the use of immunoosmophoresis for the detection of two insect riboviruses. *Journal of Virological Methods* **4**: 129-137.

SCOTTI, P.D., LONGWORTH, J.F., PLUS, N., CROIZIER, G. AND REINGANUM, C. (1981). The biology and ecology of strains of an insect small RNA virus complex. In *Advances in Virus Research*, pp. 117-143. Academic Press Inc. (London).

SEMLER, B.L., JOHNSON, V.H., DEWALT, P.G. AND YPMA-WONG, M.F. (1987). Site-specific mutagenesis of cDNA clones expressing a poliovirus proteinase. *Journal of Cellular Biochemistry* **33**, 39-51.

SHIH, D.S., SHIH, C.T., KEW, O., PALLANSCH, M., RUECKERT, R. AND KAESBERG, P. (1978). Cell-free synthesis and processing of the proteins of poliovirus. *Proceeding of the National Academy of Science (USA)* **75** (12), 5807-5811.

SHIH, D.S., SHIH, C.T., ZIMMERN, D., RUECKERT, R.R. AND KAESBERG, P. (1979). Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. *Journal of Virology* **30** (2), 472-480.

SPIER, R.W. (1962). pH and thermal stability of Mengo virus. *Virology* **17**, 588-592.

STOLZ, D.B., KRELL, P., COOK, D., MACKINNON, E.A. AND LUCAROTTIS, C.J. (1988). An unusual virus from the parasitic wasp *Cotesia melanoscela*. *Virology* **162**, 311-320.

STRUTHERS, J.K. AND HENDRY, D.A. (1974). Studies on the protein and nucleic acid components of *Nudaurelia capensis β* virus. *Journal of General Virology* **22**, 355-362.

SUMMERS, M., ENGLER, R., FALCON, L.A. AND VAIL, P. (1975). Baculoviruses for insect pest control: safety considerations. American Society for microbiology, Washington.

TENINGES, D. AND PLUS, N. (1972). P virus of *Drosophila melanogaster*, as a new picornavirus. *Journal of General Virology* **16**, 103-109.

TINSLEY, T.W., MACCALLUM, F.O., ROBERTSON, J.S. AND BROWN F. (1984). Relationship of encephalomyocarditis virus to cricket paralysis virus of insects. *Intervirology* **21**, 181-186.

TOWBIN, H., STAEHELIN, T. AND GORDON, J. (1979). Electrophoresis transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceeding of the National Academy of Science (USA)* **76** (76), 4350-4354.

TOYODA H, NICKLIN M.J.H., MURRAY, M.V., ANDERSON, C.W., DUNN, J.J., STUDIER, F.W. AND WIMMER, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of polio virus polyprotein. *Cell* **45**, 761-770.

TULLIS, R.H. AND RUBIN,H. (1980). Calcium protects DNase 1 from Proteinase K: A new method for the removal of contaminating RNase from DNase 1. *Analytical Biochemistry* **107**, 260-264.

VALVERDE, R.A., DODDS, J.A. AND HEICK J.A. (1986). Double-stranded ribonucleic acid from plants infected with viruses having elongated particles and undivided genomes. *Phytopathology* **76** (4), 459-465.

VAN DER WERF, S., BREGEGERE, F., KOPECKA, H., KITAMURA, N., ROTHBERG, P., KOURILSKY, P., WIMMER, E. AND GIRARD, M. (1981). Molecular cloning of the genome of poliovirus type I. *Proceeding of the National Academy of Sciences (USA)* **78**, 5983-5987.

VAN REGENMORTEL, M.H.V. (1964). Purification of plant viruses by zone electrophoresis. *Virology* **23** (4), 495-503.

VAN REGENMORTEL, M.H.V. (1972). Electrophoresis, pp.390-412 *In Principles and techniques in plant virology*. Edited by C.I. Kado and H.O. Agrowal. Van Nostrand Reinhold Company, New York.

VAN REGENMORTEL, M.H.V. AND VON WECHMAR, M.B. (1970). A reexamination of the serological relationship between tobacco mosaic virus and cucumber virus 4. *Virology* **41**, 330-338.

VON WECHMAR, M.B. (1987). Short and long term maintenance of aphid cultures. In Proceedings of the Barley Yellow Dwarf Workshop, Udine, Italy July 1987; CIMMYT, Mexico.

VON WECHMAR, M.B. and RYBICKI, E.P. (1981). Aphid transmission of three viruses causes Free State streak disease. *South African Journal of Science* **77**, 488-492.

WEHMAN, H.J. AND BRAGER, M. (1971). Viruslike particles in *Drosophila* : constant appearance in imaginal discs imaginal discs *in vitro*. *Journal of Invertebrate Pathology* **18**, 127-130.

WIGLEY, P.J. AND SCOTTI, P.D. (1983). The seasonal incidence of cricket paralysis virus in a population of the New Zealand field cricket, *Pteronemobius nigrovus* (Orthoptera: Gryllidae). *Journal of Invertebrate Pathology* **41**, 378-380.

WILLIAMS, C.A. AND CHASE, M.W. (1967). *Methods in Immunology and Immunochemistry, Vol II*. Academic Press, New York and London.

WILLIAMSON, C. (1983). *A study of* Rhopalosiphum padi *virus*. Honours thesis. University of Cape Town.

WILLIAMSON, C., VON WECHMAR, M.B. AND RYBICKI, E.P. (1985). Barley plants - a reservoir for an insect virus. (Abstr.) *Phytophylactica*, **17**(1), 56.

WILLIAMSON, C., RYBICKI, E.P., KASDORF, G.G.F. AND VON WECHMAR, M.B. (1988). Characterization of a new picorna-like virus isolated from aphids. *Journal of General Virology* **69**, 787-795.

WIMMER, E. (1982). Genome-linked proteins of viruses. *Cell* **28**, 199-201.

YAMAGUCHI-KOLL, U, WIEGERS, K.J. AND DRZENIEK, R. (1975). Isolation and characterization of 'Dense Particles' from poliovirus-infected Hela cells. *Journal of General Virology* **26**, 307-319.