CHARACTERISTICS OF TRANSMISSION OF VELVET TOBACCO MOTTLE VIRUS BY THE MIRID, CYRTOPELTIS NICOTIANAE (KONINGS)

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References
Some characteristics of the transmission of velvet tobacco mottle virus (VTMoV) by the mirid, Cyrtopeltis nicotianae (Konings) have been determined using feeding experiments. Assay of virus concentration and distribution within the vector have been determined by the enzyme linked immunosorbent assay (ELISA) and the horse-radish peroxidase (HRP) dot-immunobinding assay.

C. nicotianae was not specific for VTMoV, as it also transmitted solanum nodiflorum mottle, sowbane mosaic and southern bean mosaic viruses, but not subterranean clover mottle, lucerne transient streak, tobacco ringspot, galinsoga mosaic, nor nicotiana velutina mosaic viruses. Tomato bushy stunt virus was transmitted to 1/58 test plants.

The acquisition threshold of VTMoV by C. nicotianae was shorter than 1 min, with an increase in the rate of transmission for acquisition periods of up to 1000 min. The inoculation threshold was between 1 and 2 h following an acquisition access period of 2 days, and the rate of transmission increased with increasing inoculation time. When the acquisition access period was 1 h, or if mirids were fasted after the 2 day acquisition, the inoculation threshold increased to between 4 and 8 h and between 2 and 4 h respectively. The minimum time from commencement of access to inoculation was 10 h, and any latent period would have been shorter than this.

Following access to VTMoV for 24 h, mirids which were transferred sequentially each day to a separate healthy test plant, transmitted intermittently for up to 10 days. Up to 50% of mirids transmitted after a moult, and this transstadial transmission was not
due to the mirids probing the shed cuticles or exudates of "infective" insects.

Ten percent of non-infective mirids given access to virus through a parafilm membrane transmitted VTMoV. Twenty-three percent of non-infective mirids injected with purified virus transmitted VTMoV. Transmission through a moult or after injection with virus was taken as evidence that VTMoV circulates in the mirid vector.

In experiments to test how long mirids can transmit VTMoV, mirids stopped transmitting the virus between 5 and 9 days after acquisition was completed. VTMoV was detected in nymphs by ELISA up to 8 days after acquisition was completed, however, no antigen was detected in nymphs 9 and 10 days after acquisition. These results indicate that VTMoV does not propagate in C. nicotianae.

Following acquisition by feeding, virus was detected in the gut, haemolymph and faeces of mirids but not in the salivary glands. Infective virus was detected in the faeces of nymphs for 6 days, but was not detected after 7 days. Patterns of defecation, monitored in nymphs fed the insoluble dye nigrosin, showed that it was eliminated intermittently from the gut up to 6 days after ingestion, thus matching the loss of virus infectivity.

Non-infective nymphs inoculated plants on which had been placed 1 ul deposits of infectious plant sap to simulate infective faecal deposits. Thus, nymphs may be able to transmit VTMoV by probing through faeces or other secretions containing VTMoV which have been deposited on the leaf surface.

The acquisition threshold of VTMoV by C. nicotianae is characteristic of a nonpersistent association, and the inoculation threshold and retention of infectivity for up to 10 days are
characteristic of either a semipersistent or circulative association. Transstadial transmission and inoculation after injection with virus are characteristic of a circulative association.

In general, circulative viruses are detected in the haemolymph and salivary glands of insect vectors (Sylvester, 1980). Although virus was detected in the haemolymph, it was not detected in the mirids's salivary glands. Thus, the ingestion-salivation mechanism proposed for circulative associations (Harris, 1981b), does not adequately explain transmission of VTMoV by mirids.

Some of the intermittent transmission observed over long inoculation periods may thus be explained by contamination of stylets from faecal deposits (an ingestion-defecation mechanism). It is not known whether mirids do this by ingesting infectious faeces and then regurgitating them during inoculation, or by contaminating the stylets while probing. Moreover, contamination of leaf surfaces by regurgitation is another possible route of infection, and this has yet to be investigated.

The VTMoV-mirid association has characteristics of both a noncirculative and circulative mechanism of transmission and is best explained by the ingestion-egestion (Harris, 1977) and ingestion-defecation models of transmission.
This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, does not contain material previously published or written by another person, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Signed,

Karen Gibb
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Abbreviations of commonly used terms

Viruses:

VTMoV  Velvet tobacco mottle virus
NVMV  Nicotiana velutina mosaic virus
SNMV  Solanum nodiflorum mottle virus
LTSV  Lucerne transient streak virus
SCMoV  Subterranean clover mottle virus
SoMV  Sowbane mosaic virus
SBMV  Southern bean mosaic virus
TBSV  Tomato bushy stunt virus
GMV  Galinsoga mosaic virus
TRSV  Tobacco ringspot virus
AYV  Anthriscus yellows virus
CaMV  Cauliflower mosaic virus
PYFV  Parsnip yellow fleck virus
PYY  Potato virus Y
MCDV  Maize chlorotic dwarf virus
PLRV  Potato leafroll virus
PEMV  Pea enation mosaic virus
BYDV  Barley yellow dwarf virus
BWYV  Beet western yellows virus
WTV  Wound tumour virus
WSMV  Wheat streak mosaic virus
MRDV  Maize rough dwarf virus
TMV  Tobacco mosaic virus
Units:

v  volume
w  weight
mM millimolar
M  molar
ppm parts per million
cpm counts per minute
mA milliamperes
nm nanometres
um micrometres
mm millimetres
cm centimetres
ng nanograms
ug micrograms
mg milligrams
nl nanolitres
ul microlitres
ml millilitres
sec seconds
min minutes
h  hours
Chapter 1
General Introduction

I Introduction

Velvet tobacco mottle virus (VTMoV) was isolated from velvet tobacco, Nicotiana velutina (Wheeler) growing wild at Cobbler's Sandhill and near Innamincka in northeastern South Australia (Randles et al., 1981). The leaves of diseased plants had a yellow mosaic with prominent blistering from which virus was readily transmitted by mechanical inoculation to N. clevelandii (Randles et al., 1981). N. velutina has previously been reported as a host of nicotiana velutina mosaic virus (NVMV) which causes a bright yellow mosaic (Randles et al., 1976). Adults and nymphs of Cyrtopeltis nicotianae (Konings) (Hemiptera: Miridae), collected from N. velutina at Cobbler's Sandhill transmitted VTMoV (Randles et al., 1981). The mechanisms of transmission of VTMoV by C. nicotianae are reported in this thesis.

II VTMoV and its relationship to other viruses

VTMoV is one of four viruses which have been isolated in Australia with unusual circular RNA. The other three viruses are solanum nodiflorum mottle virus (SNMV), lucerne transient streak virus (LTSV) and subterranean clover mottle virus (SCMoV). VTMoV and SNMV are serologically closely related (Randles et al., 1981) but LTSV and SCMoV are serologically only remotely related. No serological relationship has been detected between LTSV and VTMoV or SNMV, or
between SCMoV and VTMoV or SNMV (Francki et al., 1983b).

VTMoV has small polyhedral particles, about 30 nm in diameter and in addition to a linear single-stranded RNA (RNA 1) of molecular weight (M. Wt) $1.5 \times 10^6$, the virus also encapsidates a low M. Wt satellite RNA which occurs as either covalently linked circular (RNA 2) or linear (RNA 3) molecules of M. Wt $0.16 \times 10^6$ (Gould, 1981; Randles et al., 1981; Randles and Francki, 1986). Polypeptides of M. Wt 31500, 33,000 and 37,000 (Randles et al., 1981; Chu and Francki, 1983), have been isolated from virus preparations.

The RNA 2 molecule of VTMoV has extensive sequence homology (92-95%) with that of SNMV RNA 2, and is 11-12 nucleotides smaller (Gould and Hatta, 1981; Haseloff and Symons, 1982), whereas the RNAs 2 of the remaining viruses, all differ from each other in size and sequence (Francki et al., 1983b). Jones and Mayo (1984) showed that for selected isolates of SNMV, RNA-2 is not required either for the infectivity and replication of SNMV RNA-1 or for the production of SNMV particles. Jones and Mayo (1983) showed that although the RNA 2 molecules of VTMoV and SNMV differ in size and sequence from those of LTSV and SCMoV (Keese et al., 1983; Francki et al., 1985), they are replicated in association with LTSV. RNA 2 from LTSV can replicate with sowbane mosaic virus (SoMV), a virus which has never been isolated from the field with any low molecular weight RNA components (Francki et al., 1983a). LTSV RNA 2 can also replicate with southern bean mosaic virus (SBMV), which supports the conclusion that RNA 2 from LTSV behaves as a satellite RNA (Francki et al., 1985). Francki et al. (1986a) reported the isolation of a VTMoV devoid of RNA 2 which can support the replication of and can encapsidate RNA 2, and they concluded that this RNA species is also a
satellite RNA.

VTMVo, SNMV, LTSV and SCMoV are all members of the sobemovirus group (Randles and Francki, 1986; Greber and Randles, 1986; Forster and Jones, 1980; Francki et al., 1987).

III Cyrtopeltis nicotianae and Miridae as vectors of plant diseases

Cyrtopeltis nicotianae was first described as Enqytatus nicotianae by Koningsberger in 1903. China (1938) concluded that nicotianae Koningsberger and three other species were all synonyms of tenuis Reuter. Usinger (1946) found the left clasper and the last abdominal segment of nicotianae were strikingly different to that of tenuis so the species was reinstated. Usinger (1946) showed that C. nicotianae was the same as Kirkaldy's Cyrtopeltis nicotianae from Fiji (Kirkaldy, 1908) and C. nicotianae has been reported from Java, New Zealand, New Caledonia, Fiji, western Micronesia (Carvalho, 1956) and Australia (Randles et al., 1981).

C. nicotianae transmits both VTMVo (Randles et al., 1981) and SNMV (Greber and Randles, 1986) but mirids have not been recorded on the natural host of SNMV, Solanum nodiflorum, so it is unlikely that transmission of this virus by mirids is important in nature (Greber, 1981). SNMV is efficiently transmitted by three coccinellid beetles (Greber, 1981).

Mirid transmission has been reported for the uncharacterised spinach blight disease by the tarnished plant bug Lygus pratensis (McLintock and Smith, 1918) and transmission was suspected of being via a mechanical mechanism (Harris, 1981a). Transmission of fire
blight bacteria *Erwinia amylovora* to pear fruit by *Lygus* spp., was also considered to be by mechanical inoculation (Stahl and Luepschen, 1977). Miridae are not regarded as important vectors of disease, and no case of transmission of a known virus by mirids has been previously described.

IV Mechanisms of plant virus transmission by arthropods

There are about 383 known species of animal vectors of plant viruses (Harris, 1981a). About 94% of these vectors are arthropods, and the remainder are nematodes. Of the 358 known arthropod vectors, 356 are insects and 2 are mites. About 76% or 273 of the insect vectors belong to the order Homoptera: 214 species in the suborder Sternorrhyncha (aphids, mealybugs, whiteflies and psyllids) and 59 in the suborder Auchenorrhyncha (leafhoppers, spittle-bugs, membracids and planthoppers) (Harris, 1980). Most studies have been done on the three major homopterous vectors, aphids, leafhoppers and planthoppers and criteria used to categorise virus-vector associations have been developed from these studies.

Virus-vector associations can be classified as nonpersistent (Watson and Roberts, 1939), semipersistent (Sylvester, 1956) or persistent (Watson and Roberts, 1939). Transmission categories used in this thesis follow those proposed by Harris (1977) in which virus-vector associations are classified as either noncirculative (including both nonpersistent and semipersistent associations) or circulative (synonymous with persistent). Circulative viruses may be subcategorised as either propagative or nonpropagative.
The noncirculative, nonpersistent mode occurs only with aphids and is the most transient of the virus-vector relationships (Sylvester, 1980). The main features of nonpersistent transmission are that the virus can be picked up by the aphid after a very short time on the infected plant and can transmit it immediately to one or only a few healthy plants since the ability to transmit is soon lost (Matthews, 1981). Kennedy et al. (1962) proposed that the term "stylet-borne" replace "nonpersistent" to accommodate work by Bradley and Ganong (1955) in which styles of viruliferous aphids were treated with formalin or UV radiation to render the insects noninfective. This was interpreted as evidence that transmissible virus was carried on the styles, more specifically near the tips of the maxillae (Bradley, 1966). The failure of aphids to transmit following such treatments may well have been the result of an effect on the styles, rather than on the virus (Garrett, 1973). Damage to the chemoreceptors at the tips of the mandibular styles affects the probing behaviour of aphids, and this could be responsible for the lack of transmission (Harris, 1977).

Electron microscopy has been used to obtain evidence that stylet-borne virus is involved in transmission. Jellison (1986) detected the lentil strain of pea seed-borne mosaic virus (PSbMV-L) on the styles of Acyrthosiphon pisum using an enzyme-linked antigen distribution assay. Lim et al. (1977) used indirect immunolatex labelling to localise the Madison pea strain of PSbMV on styles of the potato aphid, Macrosiphum euphorbiae Thomas. These studies demonstrated only that virus or viral antigens can be found in vector's styles, and the possible relevance of this virus to the transmission process could not be established (Berger and Pirone, 1986).
Harris (1977) proposed an ingestion-egestion mechanism of transmission for nonpersistent viruses in which aphids sample sap of a virus-infected plant which contaminates the foregut with virus-laden material. Some or all of this material would be then egested during subsequent sap-sampling probes of healthy plants to effect transmission.

An assumption that active uptake would be rare during the brief probes in which noncirculative, nonpropagative viruses are ideally acquired, and the lack of evidence for a mechanism of virus egestion by aphids has prevented full acceptance of this hypothesis (Harris and Bath, 1973; Pirone, 1969). However Harris and Bath (1973), found that aphids ingested and subsequently egested, carbon black particles when feeding through artificial membranes. Pirone and Harris (1977) discuss the evidence for and against this hypothesis in detail.

Semipersistent viruses have some characteristics in common with both nonpersistent and persistent transmission (Harris, 1977). This type of transmission seems compatible with an ingestion-egestion mechanism of transmission (Harris, 1977; Pirone and Harris, 1977). Semipersistent viruses are thought to accumulate in the vector's alimentary canal and resist quick dissociation from the vector either by egestion or flushing through with virus-free sap from healthy plants (Harris et al., 1981). One way in which virus might persist is by selective "adsorption" or "attachment" of virus to the vector directly by reciprocity between binding sites on viral coat protein with sites on the cell-membrane linings of the anterior alimentary canal (Harris, 1977). Viruses may also "adsorb" or "attach" to the vector indirectly via an intermediate helper component (HC). Because the HC must be present at the time virus is acquired by the aphid, it
has been suggested that it might act by (1) regulating virus uptake, (2) binding virus to sites in the food canal from which it can subsequently be eluted or (3) protecting virus from adverse conditions in the alimentary tract (Raccah and Pirone, 1984).

Anthriscus yellows virus (AYV) is transmitted by the aphid Cavariella aegopodii in a semipersistent manner and, like the potyviruses and cauliflower mosaic virus (CaMV), AYV acts as a helper for aphid transmission of parsnip yellow fleck virus (PYFV) (Harrison and Murant, 1984). Murant et al. (1976) found virus-like particles thought to be AYV, at a specific site in the foregut of C. aegopodii carrying AYV, or AYV and PYFV. Myzus persicae were given access to $^{125}$I-labelled tobacco etch virus or potato virus Y mixed with HC or with inactivated HC. In the presence of HC, label was associated with the maxillary stylets and with portions of the alimentary canal anterior to the gut, as well as with the gut region. Label accumulated only in the gut in control aphids. This selective localisation of virus acquired in the presence of HC supports a binding mechanism for the mode of action of HC (Berger and Pirone, 1986).

Rice tungro disease is caused by a complex of two viruses, rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV), which are transmitted in a semipersistent manner by leafhoppers (Hibino et al., 1978, 1979). RTBV is transmitted by the leafhopper Nephotettix virescens, but only when RSTV is acquired previously or at the same time (Hibino et al., 1978, 1979). Thus the RSTV/RTBV complex resembles the aphid-transmitted AYV/PYFV complex (Harrison and Murant, 1984). Maize chlorotic dwarf virus (MCDV) has characteristics of semipersistent transmission by its
leafhopper vector (Harris, 1979) and its particles, like those of AYV, are reported to occur at a specific site of retention in the foregut of insect vectors (Harris, 1981c).

In circulative transmission, virus is acquired via the maxillary food canal, absorbed, translocated and following a latent or incubation period in the vector, inoculated to plants in virus-laden saliva ejected from the maxillary saliva canal during probing and feeding: an ingestion-salivation mechanism of transmission (Harris 1981b).

Most of the viruses that are considered to be circulative in aphids are nonpropagative, but three rhabdoviruses are known to be propagative; lettuce necrotic yellows, sowthistle yellow vein and strawberry crinkle viruses (Sylvester, 1980). Luteoviruses are transmitted by aphids in a circulative and nonpropagative manner (Harrison and Murant, 1984). Early evidence that a luteovirus, potato leafroll virus (PLRV) multiplies in the vector aphid Myzus persicae (Stegwee and Ponsen, 1958) was not substantiated by a study in which M. persicae injected with PLRV remained infective for approximately 5 days and in a series of serial passage trials, infectivity of aphids was not maintained beyond the first transfer (Eskandari et al., 1979). A study using ELISA showed that the virus content of aphids fell after the end of the acquisition access period (Tamada and Harrison, 1981).

Pea enation mosaic virus (PEMV) is transmitted by the aphid vector Acyrthosiphon pisum in a circulative manner and as with the luteoviruses, no evidence was obtained by ELISA that the virus multiplies in the vector (Fargette et al., 1982). PEMV was the first circulative virus to be localised by electron microscopy in
situ in the salivary system of \textit{A. pisum} and thus provided the link between virus circulation and inoculation to plants through the saliva.

Particles of the luteovirus, barley yellow dwarf virus (BYDV) were also found in the basal lamina of the accessory salivary glands of \textit{Sitobion (Macrosiphum) avenae}. Particles of both transmissible (MAV) and of non-transmissible (RPV) forms of BYDV were found in the basal lamina and in plasmalemma invaginations of the accessory salivary gland. However, virus-like particles were observed in cytoplasmic vesicles in cells of the accessory salivary gland only in aphids carrying MAV (Gildow and Rochow, 1980). Virus movement from the plasmalemma to the salivary ducts may involve coated vesicles, which have been observed to contain virus-like particles in accessory salivary gland cells of aphids carrying BYDV, PLRV or beet western yellows virus (BWYV) (Gildow, 1982).

Most leafhopper-borne viruses are circulative and propagative, with the exception of the circulative, nonpropagative beet curly top virus (Harris, 1979), and the noncirculative semipersistent rice tungro disease and MCDV, already mentioned. Viruses known to multiply in their leafhopper vectors include wound tumour virus (Shikata et al., 1964; Reddy and Black, 1972), rice dwarf virus (Fukushi et al., 1962; Fukushi and Shikata, 1963a; 1963b), wheat striate mosaic virus (Sinha and Chiykowski, 1969; Vela and Lee, 1974), rice transitory yellowing virus (Chen and Shikata, 1972), barley yellow striate mosaic virus (Conti and Plumb, 1977), maize rayado fino virus (Gamez, 1973; Kitajima and Gamez, 1983; Rivera and Gamez, 1986) and oat blue dwarf virus (Banttari and Zeyen, 1976).
Planthoppers (Delphacidae), transmit 10 viruses in a persistent manner and these viruses appear to multiply in their insect vectors (Harris, 1979). Examples of these viruses are maize mosaic virus (Falk and Tsai, 1985), maize rough dwarf virus (Caciagli and Casetta, 1986) and Fiji disease virus (Francki and Grivell, 1972). Sugarbeet leaf curl virus is transmitted by a piesmid and also appears to multiply in its vector (Harris, 1980).

Transmission characteristics of noncirculative and circulative virus-vector associations are listed in Table 1.

Terms used to categorise virus-vector associations in this thesis are defined below:

Acquisition access period: duration of vector access to a virus source.
Acquisition feeding period: duration of the acquisition feed.
Inoculation access period: duration of access of an "infective" vector to a healthy test plant.
Inoculation feeding period: duration of the inoculation feed.
Latent period: the interval between the start of the acquisition access period and the start of that inoculation access period in which the first successful transmission is made (Toros et al., 1978).
Persistence: the time for which a vector remains infective after leaving a virus source (Gibbs and Harrison, 1976).
Table 1: Criteria used to categorise virus-vector associations (derived from Gibbs and Harrison, 1976)

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<th>Parameter</th>
<th>Non-circulative</th>
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<td></td>
<td>Nonpersistent</td>
<td>Semipersistent</td>
</tr>
<tr>
<td>Acquisition &amp; inoculation thresholds</td>
<td>Seconds</td>
<td>Minutes</td>
</tr>
<tr>
<td>Acquisition period increases</td>
<td>Transmission decreases</td>
<td>Transmission increases</td>
</tr>
<tr>
<td>Preacquisition fast</td>
<td>Transmission increases</td>
<td>No effect</td>
</tr>
<tr>
<td>Latent period</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Persistence</td>
<td>Seconds-minutes</td>
<td>Hours-days</td>
</tr>
<tr>
<td>Transstadiol transmission</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Virus in haemocoel</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Infective after injecting with virus</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Virus propagates</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Transovarial transmission</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>


The categories used to define virus transmission by vectors are based on stylet-bearing insects and this may not be appropriate when applied to chewing insects, such as beetles. Even with aphid transmitted viruses there are a number of viruses that do not fit into these categories or that display retention properties that fit more than one category (Pirone and Harris, 1977). Some noncirculative viruses are transmitted both nonpersistently and semipersistently i.e. bimodally by the same aphid species e.g. CaMV (Chalfant and Chapman, 1962) and pea seed borne mosaic virus (PSbMV) (Lim and Hagedorn, 1974).

The characteristics of bimodal transmission of CaMV include acquisition of virus during both short probes of seconds to minutes and also during longer probes of hours to days. Two peaks of transmission were observed when rate of transmission was plotted against acquisition access time. The first peak represented the noncirculative nonpersistent phase of aphid transmission and the second peak represented the noncirculative semipersistent phase of transmission. Bimodal transmission of viruses is reviewed by Lim and Hagedorn (1977).

Mechanisms of plant virus transmission by mealybugs, beetles and mites do not easily fit the criteria described here and alternative mechanisms have been described. Mealybug transmission of cacao swollen shoot virus (CSSV) closely resembles a noncirculative, semipersistent association (Harris, 1981a), but transstadial passage, characteristic of a circulative association, has been reported (Roivainen, 1971). It is not known whether virus is recoverable from the haemolymph of infective mealybugs, or if injection of virus into the haemocoel renders insects infective. Whether mealybugs transmit
virus via ingestion-egestion or ingestion-salivation mechanisms, or both, remains to be determined (Harris, 1981a).

Beetle transmission has been studied intensively but little is known about how virus is transferred from the beetle to the plant during transmission. The bean leaf beetle, *Cerotoma trifurcata* transmitted the cowpea strain of southern bean mosaic virus (CP-SBMV), intermittently for up to 19 days (Walters and Henry, 1970), characteristic of a circulative association. Generally viruses can be retained by beetles for several days or longer and the longer the acquisition feed, the longer is the retention period (Walters and Henry, 1970). One exception is SNMV transmission by three coccinellid beetles which remain infective for only 10 h if transferred to new plants at 2 h intervals. Moreover, with this system infectivity is not retained through pupation to adults (Greber, 1981).

Adult beetles were infective after injection with CP-SBMV (Slack and Scott, 1971; Sanderlin, 1973) and SBMV was detected in the regurgitant and CP-SBMV in the faeces of adult beetles (Slack and Scott, 1971; Scott and Fulton, 1978). SBMV, CP-SBMV and bean pod mottle virus (BPMV) were detected in the haemolymph of the adult bean leaf beetle *Cerotoma trifurcata* (Slack and Fulton, 1971; Slack and Scott, 1971; Sanderlin, 1973). Maize chlorotic mottle virus (MCMV) was recovered from the gut and haemolymph of three species of adult and larval corn rootworms (Jensen, 1985), and urdbean leaf crinkle virus was detected in the faeces of the Epilachna beetle vector from both adults and larvae, (Bharathan and Beniwal, 1984).

BPMV transmitted by the bean leaf beetle was recovered from regurgitated fluid and faeces of the beetle but tobacco ringspot virus (TRSV) which was not transmitted by the beetle was also detected in
regurgitated fluid and faeces (Slack and Fulton, 1971). The role of faeces in virus transmission is unknown as this phenomenon is not correlated with any mechanism(s) of transmission (Fulton et al., 1975). Inoculation by faecal contamination of plant areas damaged by feeding may be possible (Harris, 1981a).

The role of regurgitant in virus inoculation has been studied recently. Scott and Fulton (1978) reported that beetles which had been acquisition-fed on virus-infected tissue deposited both a beetle-transmissible virus (SBMV) and a non-beetle transmissible virus, the cowpea strain of tobacco mosaic virus (CP-TMV), on the leaf surface during feeding. Gergerich et al., (1983) found that regurgitant from leaf-feeding beetles contained a factor(s) that prevented infection by CP-TMV and TRSV, but had no effect on the beetle-transmissible viruses SBMV and BPMV. Ribonuclease (RNase) activity was detected in the regurgitant of leaf-feeding beetles and results of experiments with various virus-host combinations indicated that purified pancreatic RNase reproduced the selective effect that regurgitant had on virus transmission. It was concluded that RNase is the factor in beetle regurgitant which is responsible for the specificity of plant virus transmission by leaf-feeding beetles (Gergerich et al., 1986).

Wheat streak mosaic virus (WSMV) is transmitted in a circulative manner by mites (Paliwal, 1980), and while this appears to be the main mechanism of transmission, other concurrent mechanisms similar to those postulated for beetles are possible. Virus particles persisted in the posterior midgut for up to 5 days after removal of the mites from the virus source and it was postulated that this virus may be defecated, smeared on the leaf surface and introduced into the
leaf cells through feeding punctures or other injury. Alternatively, a backflow of virus through the mid- and foregut to the mouthparts, and introduction into the leaf cells during feeding was suggested by (Paliwal and Slykhuis, 1967).

Mechanisms of plant virus transmission by mealybugs, beetles and mites are different to those proposed for aphid- and leafhopper-borne viruses. Transmission by new vectors like mirids may also be difficult to categorise using the accepted criteria. Combinations of transmission mechanisms may be needed to explain the association, as has been necessary for mealybugs, beetles and mites.

V Objective of this thesis and an outline of the study

This thesis describes the first detailed study of virus transmission by a mirid. Feeding trials were done to determine acquisition, inoculation and retention periods, and transmission through a moult was also studied. From these basic parameters more refined experiments were done. Infectivity was tested after mirids were fed virus through a membrane or were injected with virus. ELISA was used to monitor antigen levels in mirids with time after acquisition and a dot-immunobinding assay was used to detect antigen in haemolymph, gut and salivary glands. Retention of infective virus in the insect gut was studied using an infectivity assay.

VTMoV transmission by C. nicotianae has characteristics similar to those found in some beetle and mite associations. It was difficult to explain the mechanisms of transmission using the accepted criteria therefore it was necessary to invoke and test unusual mechanisms such as inoculation of virus by faecal contamination of the leaf surface,
Chapter 2
General Materials and Methods

I Plants

1. Plants for assays

Plants for infectivity assays and insect culture were grown in the glasshouse at 18-25°C. \textit{Nicotiana clevelandii} A. Gray at the 8-10 leaf stage were used both for culturing \textit{C. nicotianae} and as source plants for VTMoV acquisition by mirids. Plants at the 4 leaf stage were used in insect feeding trials with VTMoV and plants at the 6 leaf stage were used as indicator plants for virus. \textit{Lycopersicon esculentum} cv. Rutgers does not support the replication of VTMoV (Francki \textit{et al.}, 1986\textit{c}), therefore these plants were used at the 5 leaf stage, to feed mirids in virus clearance studies. Plants used for the transmission by mirids of other viruses were at the 4 leaf stage and those used for mechanical inoculation were at the 6 leaf stage. The test plants used for each virus are listed as "Experimental hosts" in Table 2.

2. Staining the leaf surface of \textit{N. clevelandii} for probing punctures made by \textit{C. nicotianae}

Probing punctures made by the stylets of \textit{C. nicotianae} during feeding on excised leaves, were stained with erythrosine (Naito, 1965). The leaf was placed in the dark for 15-30 min to let the stomata close, dipped in 70% alcohol, then transferred to a 1%
solution of erythrosine, which then selectively stained the probing puncture. After 1-5 min, the stained leaf was transferred into water and washed for several min, then the leaf was dried and pressed between two glass microscope slides and observed at 6x magnification.

II Viruses

1. Virus isolates

VTMoV was originally isolated from native velvet tobacco, *Nicotiana velutina*, in South Australia (Randles et al., 1981). The isolate was maintained in the glasshouse by regular mechanical and mirid inoculation of *N. clevelandii*. Plants infected by mirid transmission were included in the culture to ensure that the isolate remained transmissible by the insect. The satellite-free VTMoV-K1 isolate of VTMoV, which was originally obtained from a mirid transmission trial (Francki et al., 1986a), was maintained in the glasshouse in mechanically inoculated *N. clevelandii*. The sources of other viruses used are listed in Table 2 and they were all maintained in the glasshouse in mechanically inoculated test plants, as listed in Table 2.
Table 2: Virus isolates and their experimental hosts

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Source of isolate</th>
<th>Experimental host</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNMV</td>
<td>Solanum nodiflorum (Greber, 1973)</td>
<td>N. clevelandii A. Gray</td>
</tr>
<tr>
<td>SBMV</td>
<td>P. vulgaris (Randles et al., 1981)</td>
<td>Phaseolus vulgaris L. cv. Hawkesbury Wonder</td>
</tr>
<tr>
<td>SoMV</td>
<td>C. trigonon (Teakle, 1968)</td>
<td>Chenopodium quinoa Willd.</td>
</tr>
<tr>
<td>TBSV</td>
<td>Type isolate (Martelli et al., 1971)</td>
<td>N. clevelandii</td>
</tr>
<tr>
<td>GMV</td>
<td>G. parviflora (Behncken, 1970)</td>
<td>Galinsoga parviflora Cav.</td>
</tr>
<tr>
<td>NVMV</td>
<td>N. velutina (Randles et al., 1976)</td>
<td>N. glutinosa L.</td>
</tr>
<tr>
<td>TRSV</td>
<td>Gladiolus sp. (Randles and Francki, 1965)</td>
<td>N. clevelandii</td>
</tr>
<tr>
<td>LTSV</td>
<td>Medicago sativa provided by D. J. Dall, (unpublished)</td>
<td>C. quinoa</td>
</tr>
<tr>
<td>SCM0V</td>
<td>T. subterraneum (Francki et al., 1983)</td>
<td>Trifolium subterraneum L. cv. Dinninup</td>
</tr>
</tbody>
</table>

* See Abbreviations for virus names
2. Purification of VTMoV and VTMoV-K1

The purification procedure (Gould et al., 1981a) was used for both VTMoV and VTMoV-K1. Systemically infected N. clevelandii leaves were harvested and extracted with two volumes (w/v) of 0.1 M PB (PB is phosphate buffer, pH 7.4) containing 0.1% thioglycollic acid and an equal volume of a mixture of chloroform and butanol (v/v). The slurry was stirred at 4°C for 20 min. After clarification by centrifugation in a Sorvall SS-34 rotor at 27,000 g for 10 min, the supernatant fluid was centrifuged in a Beckman 30 rotor at 78,000 g for 90 min and the pellets were resuspended in 20 mM PB. After clarification by centrifugation at 27,000 g for 10 min, the supernatant fluid was centrifuged in a Beckman 65 rotor at 270,000 g for 30 min, and the pellets were resuspended in 20 mM PB. The preparation was again clarified at 27,000 g and then centrifuged at 270,000 g as described above, and the final virus pellets were resuspended in 20 mM PB.

Preparations were examined in a Beckman DU-8B UV-Visible spectrophotometer (Beckman Instruments, U.S.A.) to estimate virus concentration. The $E_{260nm}^{0.1cm}$ for VTMoV was assumed to be 5 on the basis of its approximate RNA content (Randles et al., 1981).

Partial purifications were done when total plant nucleic acids were required. Leaf material was extracted and clarified as described above. To the aqueous phase was added 6% polyethylene glycol (PEG) 6000 and 0.2 M sodium chloride. The preparation was stirred for 2 h at 4°C followed by centrifugation in a Sorvall SS-34 rotor at 10,000 g for 25 min. The precipitate was suspended in 20 mM PB and clarified at 27,000 g for 10 min. The supernatant was retained for nucleic acid extraction.
3. Nucleic acid extraction

Nucleic acids were extracted using heat sterilised glassware and autoclaved solutions to remove RNases. Preparations of VTMoV and VTMoV-K1 suspended in 20 mM PB were shaken with one volume of 2% sodium dodecyl sulphate (SDS) in 0.2 M sodium acetate and two volumes of 90% phenol containing 0.1% 8-hydroxyquinoline, at room temperature for 1 h. The aqueous phase was recovered after centrifugation at 10,000 g for 10 min in a Sorvall SS-34 rotor and re-extracted twice with a half volume of phenol. Nucleic acids were precipitated with 3 vol of redistilled ethanol and left at -20°C for at least 3 h, recovered by centrifugation and washed again with cold ethanol (Gould, 1981). Nucleic acids were dried under vacuum and resuspended in TBE buffer (90 mM Tris-HCL, 90mM boric acid, and 3 mM EDTA, pH 8.3). Concentrations were determined spectrophotometrically assuming 
\[ \text{Abs}_{260\text{nm},10\text{mm}} = 25. \]

4. Polyacrylamide gel electrophoresis

RNA species were separated by polyacrylamide gel electrophoresis under denaturing conditions (3.3% acrylamide, 0.17% methylene bisacrylamide, 7 M urea in TBE buffer) (Gould, 1981). An equal volume of 0.02% xylene cyanol marker dye in 50% glycerol was added to each RNA sample before loading (Schumacher et al., 1983). Electrophoresis using a vertical gel electrophoresis system (Bethesda Res. Labs, U.S.A.), was for 4 h at 30 mA in a 140 x 120 x 1.5 mm slab gel.

Three alternative staining methods were used to locate RNA bands:

(I) Gels were stained for 30 min in a solution of 0.05% toluidine blue
in 5% acetic acid and destained in sterile distilled water overnight at room temperature.

(II) Gels were fixed in 0.5% acetic acid-10% ethanol for at least 1 h, washed in sterile water, stained in 10 mM silver nitrate for 10-15 min, washed again and developed in 0.4 M sodium hydroxide, 2mM sodium borohydride and 0.4% v/v formaldehyde for 10-20 min. The reaction was stopped in 5% acetic acid (Sammons et al, 1981).

(III) The bands were located by UV fluorescence after staining the gel for 1 h in a solution of 0.5 ppm ethidium bromide in the electrophoresis buffer (Schumacher et al., 1983).

5. VTMoV detection assay using VTMoV-K1

To detect low levels of VTMoV, an assay was developed in which the detection of the satellite RNA of VTMoV was achieved by co-inoculation of test plants with the satellite free VTMoV-K1 isolate. The increased sensitivity over a direct infectivity assay was demonstrated in chapter 4.

All co-inoculated plants were tested by agar gel double-immunodiffusion (ch. 2.III.2) for the presence of VTMoV. Plants which had been inoculated with VTMoV plus VTMoV-K1, and control plants inoculated with PB or VTMoV-K1, were harvested and weighed individually. Total nucleic acids extracted from partially purified samples were separated by polyacrylamide gel electrophoresis. RNAs from purified VTMoV and VTMoV-K1 (10 ug) were used as molecular weight markers. Satellite RNA (sat-RNA) bands were identified after staining in toluidine blue or silver.
III Serology

1. Antisera

Antisera to a range of viruses, obtained from the sources listed in Table 3, were used in transmission tests with C. nicotianae. All antisera were checked by agar gel double-immunodiffusion tests for the presence of antibodies to normal host plant antigens. None of the antisera reacted with sap from healthy plants. All antisera were stored in 50% glycerol (w/v) at -20°C.

Table 3: Sources of antisera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Titre in agar gel double-diffusion tests</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTMoV</td>
<td>1/256</td>
<td>J. W. Randles</td>
</tr>
<tr>
<td>SNMV</td>
<td>1/128</td>
<td>R. I. B. Francki</td>
</tr>
<tr>
<td>SBMV</td>
<td>1/258</td>
<td>&quot;</td>
</tr>
<tr>
<td>SoMV</td>
<td>1/128</td>
<td>&quot;</td>
</tr>
<tr>
<td>TBSV(type)</td>
<td>1/1024</td>
<td>&quot;</td>
</tr>
<tr>
<td>GMV</td>
<td>1/256</td>
<td>&quot;</td>
</tr>
<tr>
<td>TRSV</td>
<td>1/252</td>
<td>&quot;</td>
</tr>
<tr>
<td>LTSV</td>
<td>1/512</td>
<td>&quot;</td>
</tr>
<tr>
<td>SCM oV</td>
<td>1/256</td>
<td>D. J. Graddon</td>
</tr>
</tbody>
</table>

a W.A.R.I. = Waite Agricultural Research Institute
2. Agar gel double-immunodiffusion tests

Radial double-immunodiffusion tests were done in 0.75% Bacto-agar (Difco Labs, U.S.A.) in 10 mM PB, with 0.02% sodium azide as preservative (Francki and Habili, 1972). Plant sap was ground in 20 mM PB and purified virus was diluted in 20 mM PB and samples were pipetted into the outer wells (12 ul/well). Serum diluted in PB was pipetted into the central well(s). The plates were observed for precipitin lines after 24-36 h incubation at 25°C.

IV Manipulation of C. nicotianae

1. Rearing C. nicotianae

C. nicotianae were collected from N. velutina Wheeler in north-eastern South Australia by J. W. Randles in 1979. Insects were reared on a group of N. clevelandii plants of various ages in an insectary maintained at 25 ± 4°C.

2. Stages of C. nicotianae used for experiments

In preliminary experiments, the transmission efficiency of adult C. nicotianae (Fig. 1a) was less than for the nymphs. In addition, adults took longer to anaesthetise before injecting or before blood sampling and the actual manipulation took longer than with nymphs, because the wings covered the preferred injection site between the last thoracic and first abdominal segments. It was also
found that salivary glands of adults were more easily disrupted after dissection than were those from nymphs. For these reasons the adult C. nicotianae were not used in further experiments.

Nymphal stages (Fig. 1b) were identified according to colour and wing-bud length relative to body size. The first two stages were yellow and therefore easily distinguished but they were not used in experiments as they were small and the lower food capacity compared to other stages may have significantly affected transmission efficiency. Third and fourth-stages were of similar length and since size differences were to be minimised, large third-stage, and fourth-stage mirids were used. These stages were 2-2.5 mm long and wing buds were obvious (Fig. 1b).

Fifth-stage nymphs were easily identified by the well developed wing-buds which occupied 1/4 of the insect's body-length and by the shape of the abdomen which was more pointed than other nymphal stages (Fig. 1b). The larger fifth-stage nymphs were used in experiments involving micromanipulation and for transstadial transmission trials because moulting to the winged adult was an easily identifiable event. In this thesis, the term "nymphs" is used to describe the nymphal stages only, and the term "mirids" is used more generally to describe both nymphs and adults.

3. Collection of C. nicotianae and fasting before experiments

Mirids were collected by aspiration into 4 x 6 cm cylindrical plastic cages with screw-on lids. A 2 cm diameter disc had been removed from the base of each cage and the hole covered with gauze. Each cage was placed gauze side down in a plastic container 24 x 34
Fig. 1: Adult and nymphal stages of *C. nicotianae*

a  Adult *C. nicotianae*; green body with light brown antennae.
   Antennal segment 1 black in the medial 1/2, segment 2 black in the
   proximal 1/4 and distal 1/2-1/4. Legs are light brown, mid and
   hind tibia with a small black patch dorsally at the proximal end.
   Wings are transparent with black patches at the costal fracture,
   the distal corner of the cuneus and at the distal corner of the
   clavus; also a black line is apparent along the anterior border of
   the clavus.

b  The five nymphal stages of *C. nicotianae*; from left to right
   the 1st, 2nd, 3rd, 4th and 5th stages. Nymphs are classified
   according to stage by wing-bud development and colour. The 3rd,
   4th and 5th nymphal stages are green. The first two stages are
   yellow.

Bar represents 1 mm
cm, which had been lined with damp paper towel, and covered. Mirids were fasted in this way for 1000 min before each experiment to minimise behavioural variations which might affect feeding efficiency. Mirids were also fasted in this way for the controlled feeding and moulting experiments. All experiments were done under continuous lighting of ca. 5000 lux at 25°C.

4. Control experiments

To ensure that the mirid culture was non-infective, all trials included the use of control mirids. Mirids were fed on healthy plants and transferred to test plants for a specific "inoculation" period.

5. Acquisition feeding by *C. nicotianae*

In this thesis, the acquisition period is defined as the time during which mirids were given access to an infected or healthy plant or excised leaf. This is equivalent to the acquisition access period of Gibbs and Harrison (1976). To determine the minimum time required for mirids to acquire VTMOV, acquisition times between 1 and 1000 min were tested. Nymphs fasted singly in cages were given access to an excised leaf from a source plant, i.e. either systemically infected *N. clevelandii* or healthy plants. The acquisition period was measured from the time when the nymph was observed to settle on the leaf.

When acquisition periods of 1000 min or greater were used, mirids were given access to virus-infected or healthy *N. clevelandii* plants rather than to excised leaves which tended to
wilt after about 1000 mir. Nymphs were collected and stored in groups of 5 before being placed at 20 per source plant. Plastic cylinders 12 x 20 cm were placed over the plants and covered with gauze. Cages were removed at the end of the acquisition period and mirids recovered.

Experiments to test the transmission of other viruses by C. nicotianae were done using acquisition periods ranging from 1 to 3 days. Nymphs were collected in groups of 5, deposited onto infected plants and caged for the specified acquisition period.

6. Inoculation feeding by C. nicotianae

The inoculation period is defined as the duration of access to healthy test plants, i.e. inoculation access period of Gibbs and Harrison (1976). The ages and species of test plants used for mirid inoculation are given in Table 2. Single mirids in 4 x 6 cm cages were placed on the test plants and the inoculation period commenced when the mirid settled onto the plant. Single mirid inoculations of plants were used in all experiments except where otherwise stated.

In experiments to determine the inoculation threshold, infective and control nymphs were given access to test plants for periods of between 1 and 28 h. Each mirid was placed onto the healthy test plant and the cage placed on the plant. Mirids were given access to plants for the specified inoculation period before being removed by aspiration. After inoculation, all test plants were transferred from continuous light at 25°C to the glasshouse at 18-24°C. Plants were observed for up to 1 month for symptom expression. During this period, some test plants developed "damping off" symptoms and died, which is the reason why sample sizes varied in some of the feeding trials (chapter 4), unless otherwise stated.
7. Injection of \textit{C. nicotianae}

The method used to inject mirids was based on that used for the injection of the beet leafhopper \textit{Circulifer tenellus} with curly top virus (Maramorosch, 1955), except that anaesthesia with carbon dioxide was replaced by chilling of insects before injection. The injection method has been described again here because the equipment used was different to that used by Maramorosch (1955).

Fifth-stage nymphs collected singly into plastic cages were fasted (Ch.2.IV.3), before being taken to the laboratory at \(22 \pm 4^\circ\text{C}\) for injection. The inoculum was injected into fasted mirids because as reported by Harris \textit{et al.}, (1975), loss of inoculum by bleeding was substantially reduced by subjecting the insects to a pre-injection starvation period to reduce pressure within the haemocoel. During a successful injection, nymphs could be seen swelling as the inoculum entered the haemocoel.

Each mirid to be injected was chilled by placing its cage on ice for 2 min to reduce mobility. The mirid was transferred to a cold concave glass block where it was held by its abdomen to a 50 ul micro-pipette attached to a Dymax MK II air pump (Charles Austen Pumps Ltd., Eng.) by flexible plastic tubing (Dow Corning Corp.n, U.S.A.). The nymph was held above a frigistor block (Mectron Frigistor Ltd., Eng.) mounted on the stage of a dissecting microscope (Fig. 2) so as to maintain low activity.

Borosilicate tubes 100 um in diameter, (Clark Electromedical Instruments, England) were drawn on a microforge (Sylvester \textit{et al.}, 1970) and mounted on Terumo 27 G x 1/2 (0.4 x 13.0 mm) disposable needles (Neolus Terumo Corp.n, Japan) and sealed with
Araldite™ adhesive. The needle was attached to an Agla micrometer syringe (Wellcome Res. Labs, England) containing the inoculum and mounted on the microapplicator (Instrumentation Specialties Co. (ISCO), U.S.A.) (Fig. 2).

The needle tip was positioned so it could be seen through the microscope and injections were done while looking through the microscope (12x magnification). The nymph was manipulated until the needle had been inserted just beneath the cuticle between the last thoracic and first abdominal segments on the dorsal surface. Mueller and Rochow (1961) found less haemolymph was lost when they injected aphids dorsally between the abdomen and thorax. When the needle was in position, the microapplicator foot-pedal was depressed for 0.5 sec to deliver the inoculum. The nymph was removed from the needle and placed onto a healthy test plant. Nymph survival and feeding was observed daily during the inoculation access period.

8. Membrane feeding by C. nicotianae

The method used was based on that described by Mittler and Dadd (1964) for feeding aphids on artificial diets. Nymphs were collected in groups of 5 and fasted before being given access to the test solution through a membrane. A glass cylinder 35 mm x 10 mm (length x width), was placed on damp filter paper in a plastic petri-dish and a layer of Parafilm® (American Can Co., U.S.A.) was stretched over the top of the cylinder and gently indented. The test solution was pipetted into the indentation and another layer of parafilm stretched over the top. The two edges of parafilm were rolled downwards (Fig. 3).
Fig. 2: Apparatus used to inject nymphs

A 50 ul micropipette (a) attached to an air pump (b) was used to hold nymphs for injection. A frigistor block (c) was taped over the microscope stage to reduce mobility of the insect. The syringe and needle were mounted on the microapplicator (d). When the needle was inserted into the nymph, the microapplicator foot-pedal (e) was depressed for 0.5 sec to deliver the inoculum.
Fig. 3 : Membrane feeding chamber

A diagrammatic representation of the system used to feed nymphs through a membrane. Diets of either $^{32}$P-labelled or unlabelled solutions of 5% sucrose containing VTMoV, and a diet of 0.4% nigrosin in 5% sucrose were used in membrane feeding trials.
The fasted nymphs were cooled on ice and when partially immobilised, were transferred to the feeding chamber(s) kept at 20 ± 4°C. Some nymphs remained on the filter paper initially but after a few min, most were observed on the parafilm. Since test solutions either contained an aqueous solution of 32P-labelled ortho-phosphate (specific radioactivity 1.86 x 10^5 cpm/ul) (BRESA, South Aust.) (Chapter 5) or Nigrosin dye (Chapter 8), it was possible to determine which nymphs had acquired the test solution.

9. Detection of VTMoV in C. nicotianae using ELISA

The double antibody sandwich enzyme-linked immunosorbent assay (ELISA), (Clark and Adams, 1977) was used for both qualitative and quantitative determination of the virus. VTMoV antiserum, titre > 1/64 (prepared by D.J. Dall) was used to prepare both the coating antibody (1/750 dilution in 50 mM carbonate coating buffer, pH 9.6), and for conjugation with bovine alkaline phosphatase (BAP, Sigma Type VII-S E.C. No. 3.1.3.1., Sigma Chemical Co., U.S.A.).

Polystyrene microtest plates (Medical Disposable Products Pty. Ltd., Aust.) were coated with antibody (100 ul/well), incubated for 16 h at 5°C, and washed three times with PB-Tween (20 mM phosphate buffer, pH 7.4 containing 0.05% (v/v) Tween 20). Fifty ul of conjugated antibody (1/750 dilution in PB-Tween containing 2% (w/v) polyvinylpyrrolidone (PVP)) was added to each well. Samples diluted in PB-Tween + PVP (50 ul/well) were added last. Infected and healthy plant samples were ground without buffer and 30 ul of this extract was diluted in 120 ul PB-Tween + PVP. Individual nymphs to be tested were ground in 50 ul PB-Tween + PVP and the extract was serially diluted to
1/5, 1/10, 1/50, 1/100, 1/500 and 1/1000. Insect controls were nymphs given access to healthy plants and plant controls were healthy. Non-infective nymphs were assayed after a single dilution to 1/5. These controls were compared with wells containing PB-Tween + PVP only and in all assays the control and buffer readings were not different.

Conjugate and samples were each incubated for 3 h at 37°C. After extensive washing, 100 ul of substrate buffer (9.7% (w/v) diethanolamine, adjusted to pH 9.8 with HCl) containing 0.075% (w/v) p-nitrophenyl phosphate disodium salt was added to each well and incubated at room temperature. After 30-40 min, absorbance at 405 nm was measured with a Model 2550 EIA Reader (Bio-Rad, U.S.A.).

Estimates of the virus concentration in each sample were made using a calibration curve of absorbance vs standard virus concentrations of 9.5, 4.8, 0.5 and 0.2 ug/ml. The dilution of mirid samples for multiple testing gave a range of absorbance values, at least one of which fell on the standard curve. Absorbance readings from control nymphs were averaged and values more than twice the control mean were considered to be positive. Mean absorbance readings from control nymphs were subtracted from positive sample readings. For each positive sample, the virus concentration in ug/ml was calculated from the standard curve and multiplied by the dilution factor. This value was multiplied by the ratio of buffer volume to mirid weight to determine the amount of virus per mirid.
10. Location of VTMoV in *C. nicotianae* using the HRP
dot-immunobinding assay

A horse-radish peroxidase (HRP) dot-immunobinding assay
(Hawkes *et al.*, 1982) was useful for qualitative determination of
virus in dissected bodyparts of nymphs, particularly where only small
volumes were available for assay. A nitrocellulose sheet marked with 5
mm grids (0.45 μm, Schleicher and Schuell D-3354) was cut to size,
washed for 5 min by gentle agitation in distilled water and left at
room temperature to dry. Purified virus was diluted in TBS (50 mM
Tris-HCl, 200 mM NaCl, pH 7.4) to 0.95, 0.50 and 0.10 mg/ml and 1 ul
of each dotted onto the filter as a standard and left to dry.

Each "infective" and non-infective (control) nymph was
subjected to the manipulations described below:

The nymph was killed in ethyl acetate vapour and haemolymph
was immediately sampled by injection using a borosilicate needle
(Ch.2.IV.7) attached to a vacuum pump. Haemolymph taken up in the
needle was dotted onto the filter. Faeces were then collected from the
same nymph by gently pressing the abdomen of the dead nymph and taking
up expressed faeces in a borosilicate needle. The sample was dotted
onto the filter. The same nymph was then dissected, the gut removed
with forceps and ground in 2 ul TBS. One ul of this sample was
pipetted onto the filter. The rest of the same nymph was ground in 4
ul TBS and 1 ul pipetted onto the filter.

In other experiments, salivary glands were extracted from
"infective" and non-infective (control) nymphs as now described. Each
killed nymph was placed in TBS and the abdomen was gently pressed as
the head was removed with fine forceps. The salivary glands were
released from the thoracic cavity but still attached by the salivary ducts. The glands were removed and applied directly to the filter using the heat glazed end of a fine glass rod. Samples of the gut from these same nymphs were ground in 2 ul TBS and 1 ul of the test solution was dotted onto the filter. TBS samples were included in each test to establish background levels.

When the filter was dry, it was washed for 5 min in TBS, followed by 15 min in blocking buffer (20 ml of 3% w/v bovine serum albumin, Fraction V 96-99% albumin, Sigma Chemical Co., U.S.A.) to block non-specific antibody binding sites. The primary antibody (20 ul in 10 ml blocking buffer) was that used for the ELISA coating antibody (Ch.2.IV.9). The filter was gently shaken with the primary antibody at room temperature for 16 h. The filter was washed for 30 min in several changes of TBS. After further blocking in blocking buffer, the filter was incubated for 2 h in horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Miles Lab. Inc., U.S.A.), (20 ul in 10 ml blocking buffer) and then washed for 30 min in several changes of TBS.

The HRP colour development reagent containing 4-chloro-1-naphthol (Bio-Rad Labs, U.S.A.), was prepared as a 3 mg/ml stock solution in methanol. Just before use, it was diluted with five volumes of TBS to a final concentration of 0.01% (v/v) in hydrogen peroxide (34% w/w). Ten ml of the developer were added to the filter and positive samples gave a purple-black spot in 2-5 min after which the filters were washed with distilled water and dried.
V Statistical methods

For all statistical tests, the probability (P) for rejection of null hypotheses was \( P \leq 0.05 \) (i.e. 5\% or less). In some cases the exact P was reported and in others, P was reported as being between two probabilities.

The Student's t test was used to compare two population means (Zar, 1984) and is written as \( t_{n-1} \), where: \( n \) = the sample size and \( n-1 \) = the degrees of freedom.

A one-way analysis of variance (ANOVA), was used to test multisample hypotheses (Zar, 1984) and is represented by the F statistic \( (F_{k-1,N-k}) \) where: F is the ratio of the variability among groups to the variability within groups. \( N \) = the total sample size and \( k \) = the number of groups being compared. If the variability among groups is large relative to the variability within the groups, the resulting large F value indicates differences among the group means (Zar, 1984).

Simple linear regression was used to test hypotheses of functional dependence of one variable (Y) on another variable (X) (Zar, 1984). The statistical significance of a regression equation is tested by an ANOVA in which F is a ratio of the variability due to a linear regression to the residual variability (defined as the variance of Y after taking into account the dependence of Y on X) (Zar, 1984). The amount of variability that is accounted for by the regression equation is called the coefficient of determination \( (r^2) \), which can be expressed as a percentage of the total variability and ranges between 0 and 100\%. This value is an indication of the strength of the straight-line relationship (Zar, 1984).
Contingency tables were used to test hypotheses concerning the frequencies of two variables measured simultaneously. The null hypothesis for contingency tables is that the frequencies in the various categories of one variable are independent of the other variable (column frequencies are independent of row frequencies) (Zar, 1984). This statistic was written as $\chi^2_{df}$, where the degrees of freedom (df) are calculated as $(r-1)(c-1)$ in which $r$ = the number of rows and $c$ = the number of columns. Chi-square ($\chi^2$) analysis was used to analyse contingency table data where possible, and Fisher's exact test was used to analyse 2x2 contingency tables in which there were insufficiently large frequencies for a legitimate $\chi^2$ analysis (Zar, 1984). This test calculates the exact probability ($P$) of the hypothesis being true.
Chapter 3

Viruses transmitted by C. nicotianae

I Introduction

C. nicotianae transmits VTMOV (Randles et al., 1981) and the serologically related SNMV (Randles et al., 1981; Greber and Randles, 1986). Eight viruses, besides VTMOV and SNMV, were used in tests of mirid transmissibility. Two of these viruses, LTSV and SCMoV were also recently isolated in Australia and found to encapsidate a viroid-like RNA component similar to that of VTMOV and SNMV (Francki et al., 1985). These viruses are serologically only remotely related to each other but are not related to either VTMOV or SNMV. VTMOV, SNMV, LTSV and SCMoV are members of the sobemovirus group (Randles and Francki, 1986; Greber and Randles, 1986; Forster and Jones, 1980; Francki et al., 1987). SBMV, the type member of the sobemovirus group (Matthews, 1979) and SoMV, another member of this group (Matthews, 1982; Zebzami et al., 1987) were also tested for transmissibility by C. nicotianae.

Like VTMOV, NVMV occurs in N. velutina in the wild (Randles et al., 1976), and while it is structurally distinct from the other viruses, the possibility that it was transmissible by C. nicotianae was tested. Three other icosahedral single-stranded RNA viruses which were tested fall outside this taxonomic group and do not encapsidate a satellite RNA.
II Experimental

1. Transmission tests with *C. nicotianae*

The acquisition and inoculation periods, and the number of test plants used in transmission experiments with each virus, are listed in Table 4.

Only test plants with mirid feeding damage i.e. silvering on the leaf surface, were included in the results. *P. vulgaris* and *N. glutinosa* were the least palatable host plants for *C. nicotianae*, judging by the barely discernible feeding damage on test plants, and fewer mirids survived on these hosts when compared to other hosts. Mirids found dead on *P. vulgaris* had been pierced by plant hairs but this was not observed on *N. glutinosa*.

In transmission experiments with SNMV, SBMV and SoMV, infected test plants and randomly chosen symptomless plants were tested by gel immunodiffusion. In all cases, only samples from plants with symptoms reacted against the homologous antiserum, and there was no reaction against host plant antigens (Fig.s 4-6). As reported previously (Randles et al., 1981), SNMV was serologically distinct from VTMoV as indicated by the spur (Fig. 4a) and no serological reaction was observed when sap from a VTMoV infected plant was tested against antiserum to either SBMV (Fig. 5a, [well 10] & Fig. 5b, [well 23]) or SoMV (Fig. 6c, [well 3]) (see figure legends for numbering pattern of wells). The single transmission of TBSV was verified serologically but this result could not be repeated in a later experiment. Of the remaining viruses, extracts of 10 symptomless plants from each experiment did not react against the appropriate antiserum.
Fig. 4: Serological identification of SNMV in plants inoculated by *C. nicotianae*

Double gel immunodiffusion test of antiserum to SNMV (1/16 dilution), (central wells of each pattern), against the samples listed below. Sample wells are numbered clockwise from number 1.

(a) 20mM PB (wells 1 and 4), purified SNMV (wells 2 & 5) and purified VTMoV (wells 3 & 6).

(b) Sap from SNMV infected *N. clevelandii* (well 4), sap from a plant subjected to feeding by a non-infective control nymph (well 2) and sap from plants showing symptoms after inoculation by infective nymphs (remaining wells).

(c) Sap from an SNMV infected plant (well 4), sap from a plant subjected to feeding by a non-infective control nymph (well 2), sap from a symptomless plant subjected to feeding by an "infective nymph" (well 5) and sap from plants showing symptoms after inoculation by infective nymphs (remaining wells).

(d) Sap from an SNMV infected plant (well 4), sap from a plant subjected to feeding by a non-infective control nymph (well 2), sap from a symptomless plant subjected to feeding by an "infective" nymph (well 1) and sap from plants showing symptoms after inoculation by infective nymphs (remaining wells).
Fig. 5: Serological identification of SBMV in plants inoculated by

*C. nicotianae*

Double gel immunodiffusion test of antiserum to SBMV (1/32 dilution),
(central wells of each pattern), against the samples listed below.
Sample wells are numbered clockwise from number 1.
(a) Sap from SBMV infected *P. vulgaris* (well 17), sap from VTMoV
infected *N. clevelandii* (well 10), sap from *P. vulgaris*
subjected to feeding by non-infective nymphs (wells 4, 7, 20 and 23)
and sap from *P. vulgaris* subjected to feeding by nymphs given
pre-inoculation access to SBMV infected plants (remaining wells with
positive samples in wells 6, 12, 14 and 16).
(b) Sap from SBMV infected *P. vulgaris* (wells 7 and 20), sap from
VTMoV infected *N. clevelandii* (well 23), sap from *P. vulgaris*
subjected to feeding by non-infective nymphs (wells 4, 10 and 17) and
sap from *P. vulgaris* subjected to feeding by nymphs given
pre-inoculation access to SBMV infected plants (remaining wells with
positive samples in wells 1, 2, 3, 5, 6, 8, 9, 11, 13, 14, 16, 22, 24
and 26).
Fig. 6: Serological identification of SoMV in plants inoculated by

*C. nicotianae*

Double gel immunodiffusion test of antiserum to SoMV (dilution 1/4), (central wells of each pattern), against the samples listed below.

Samples wells are numbered clockwise from number 1.

(a) Sap from SoMV infected *C. quinoa* (wells 4 and 7), sap from plants showing symptoms after inoculation by nymphs (wells 3 and 13) and sap from symptomless plants subjected to feeding by non-infective controls or by non-transmitting nymphs given pre-inoculation access to SoMV (remaining wells).

(b) Sap from an SoMV infected plant (well 3), sap from plants showing symptoms after inoculation by nymphs (wells 1 and 10) and sap from symptomless plants subjected to feeding by non-infective controls or by non-transmitting nymphs (remaining wells).

(c) Sap from SoMV infected plants (wells 4, 7, 14 and 17), sap from plants showing symptoms after inoculation by nymphs (wells 6 and 18), sap from VTMoV infected *N. clevelandii* (well 3) and sap from symptomless plants subjected to feeding by non-infective controls or by non-transmitting nymphs (remaining wells).
As shown in Table 4, nymphs of *C. nicotianae* transmitted SoMV and SBMV in addition to VTMoV and SNMV. The single transmission of TBSV indicates low efficiency, and requires repetition on a larger scale. SCMoV, LTSV, TRSV, GMV and NVMV were not transmitted in these tests although repetition is also required to test for low transmission efficiencies.

Table 4: Transmission of selected viruses by *C. nicotianae*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Test plant</th>
<th>Acquisition period (days)</th>
<th>Inoculation period (days)</th>
<th>Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>VTMoV</td>
<td>N. clevelandii</td>
<td>1</td>
<td>2</td>
<td>8/18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SNMV</td>
<td>N. clevelandii</td>
<td>1</td>
<td>2</td>
<td>21/30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCMoV</td>
<td>T. subterraneum</td>
<td>1</td>
<td>2</td>
<td>0/60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>4</td>
<td>0/60</td>
</tr>
<tr>
<td>LTSV</td>
<td>C. quinoa</td>
<td>2</td>
<td>4</td>
<td>0/30</td>
</tr>
<tr>
<td>SoMV</td>
<td>C. quinoa</td>
<td>2</td>
<td>4</td>
<td>2/30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>2/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>2/25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBMV</td>
<td>P. vulgaris</td>
<td>1</td>
<td>3</td>
<td>4/40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5</td>
<td>14/40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TBSV</td>
<td>N. clevelandii</td>
<td>1</td>
<td>2</td>
<td>1/28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&quot;</td>
<td>&quot;</td>
<td>0/30</td>
</tr>
<tr>
<td>TRSV</td>
<td>N. clevelandii</td>
<td>2</td>
<td>2</td>
<td>0/30</td>
</tr>
<tr>
<td>GMV</td>
<td>G. parviflora</td>
<td>2</td>
<td>2</td>
<td>0/25</td>
</tr>
<tr>
<td>NVMV</td>
<td>N. glutinosa</td>
<td>2</td>
<td>2</td>
<td>0/30</td>
</tr>
</tbody>
</table>

<sup>a</sup> the numerator represents the number of infected plants and the denominator represents the number of plants tested

<sup>b</sup> trials were repeated to confirm the result
III Conclusions

Tests with a range of viruses indicate that the C. *nicotianae*-virus association has some specificity. Of the four viruses containing a viroid-like RNA component (VTMoV, SNMV, LNTS and SCMoV), only VTMoV and SNMV were transmitted as previously recorded (Randles et al., 1981; Greber and Randles, 1986), and C. *nicotianae* transmitted SBMV and SoMV.

In one experiment a single nymph transmitted TBSV but this was not repeated in a later experiment so transmission by C. *nicotianae* has been established for only four of the viruses tested and these viruses all belong to the sobemovirus group.

Francki et al, (1985) reported that the number of virus particles is greater in cells infected with VTMoV, SNMV, SBMV and SoMV than those with LNTS or SCMoV and it therefore seems possible that the low concentrations of LNTS and SCMoV in infected plants may have contributed to lack of transmission by mirids.

Host plant palatability was unlikely to have interfered with transmission since only test plants with feeding damage were included in the experiment and C. quinoa was a host of both the non mirid-transmissible LNTS and the mirid-transmissible SoMV. Also, fewer nymphs survived on P. vulgaris and feeding damage was only just discernible on these plants. Despite this, nymphs still transmitted SBMV to these plants. Even when the preferred host of the mirid, N. clevelandii, was used in transmission experiments, mirids did not transmit TRSV.

Large scale transmission experiments using LNTS, SCMoV, TRSV, GMV and NVAV are required to determine whether transmission can occur
at low frequencies.

The transmission of SNMV, SBMV and SoMV by the mirid vector of
VTMoV, would support the view that these viruses have biological
affinities and thus support their inclusion in the sobemovirus group.

Since the colony of mirids used in these experiments were
selected from one area (Ch.2.IV.1), it is possible that they are a
specific biotype, able to transmit a unique range of viruses. It would
be of interest to test this by collecting C. nicotianae from
elsewhere, for example, Fiji, and testing the transmission of a range
of viruses using this colony.
Chapter 4

Parameters of the Transmission of VTMoV by C. nicotianae

I Introduction

Plant viruses are transmitted in a noncirculative (nonpersistent or semipersistent), or circulative (persistent) manner by their arthropod vectors (Harris, 1977). Characteristics of each mechanism are described in chapter 1. It is not known whether VTMoV is transmitted in a noncirculative or circulative manner by C. nicotianae, so experiments were done to test this.

Thresholds of acquisition and inoculation, and the period of retention of virus by the mirid were determined. Transstadial transmission was observed and tests were done to determine whether this could be explained by mirids probing the shed cuticles or exudates of "infective" insects. An assay was developed to detect low levels of virus on moulted cuticles.

II Experimental

1. Characteristics of acquisition of VTMoV by C. nicotianae

To test the relationship between acquisition period and rate of transmission, fasted nymphs were given access to excised leaves infected with VTMoV, for the times shown in Fig. 7. Twenty-four nymphs were used for each of the first three acquisition times and 18 for each of the remaining times. The individual nymphs were transferred immediately to a test plant for a 2 day inoculation feed. In control trials, 18 nymphs were allowed an acquisition feed of 1000 min.
Fig. 7: Relationship between acquisition time and rate of transmission

Fasted nymphs were given access to VTMoV infected leaves for the times shown, then transferred immediately to test plants for a two day inoculation feed. Each point is the percentage of test plants infected for each acquisition period.

The analysis was done on $\log_{10}$ acquisition periods, and the actual times in min are shown on the figure. The log values corresponding to the acquisition periods of 1, 3.2, 10, 31.6, 100 and 1000, are 0, 0.5, 1, 1.5, 2 and 3.
Percentage of infected plants vs. acquisition period (min).
After a 1 min acquisition feed, 20% of nymphs transmitted VTMoV, suggesting that the acquisition threshold was shorter than 1 min. The rate of transmission significantly increased \( (F_{1,4} = 24.2, \ p = 0.009, \ r^2 = 87\% ) \) with increasing acquisition time over the periods tested.

2. Characteristics of inoculation of VTMoV by \( C. \ nicotianae \)

An experiment was done to determine both the inoculation threshold and the effect of acquisition period and pre-inoculation fasting on the inoculation threshold and the rate of transmission.

In each treatment, nymphs were given access to a source of VTMoV for the times shown below. Where there was no pre-inoculation fast (treatments 1 and 3), nymphs which had access to VTMoV, were immediately transferred to test plants for the specified inoculation period. In treatment 2, nymphs were fasted for 16 h after acquisition was completed, before being transferred to test plants.

The 3 treatments used are listed below:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acquisition period</th>
<th>Pre-inoculation fast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 h</td>
<td>0 h</td>
</tr>
<tr>
<td>2</td>
<td>2 days</td>
<td>16 h</td>
</tr>
<tr>
<td>3</td>
<td>2 days</td>
<td>0 h</td>
</tr>
</tbody>
</table>

Inoculation periods were between 1 and 28 h and 15 nymphs, at 1 per plant were tested at each time. In a control treatment, 15 nymphs were allowed an inoculation feed of 28 h.
As the data were assumed to be binomially distributed, they were analysed using a logit function, and the data were fitted to a generalised linear model (Baker and Nelder, 1978), of the form:

\[ Y_i = \frac{n_i e^{\beta_0 + \beta_i t}}{1 + e^{\beta_0 + \beta_i t}} \]

where \( i \) = treatments 1, 2, 3

\( n_i \) = the number of plants at each inoculation period for each treatment

\( e \) = the base of the natural log

\( \beta_0 \) = the intercept of a linear function

\( \beta_i \) = the slope of a linear function for each treatment

\( t \) = the inoculation period (h)

In Fig. 8, the symbols indicate the actual points and the solid line indicates the fitted points. From the actual data, the inoculation threshold of nymphs which had access to VTMOV for 2 days with no post-acquisition fast (treatment 3), was between 1 and 2 h (Fig. 8). However when a fast was imposed after acquisition (treatment 2), the inoculation threshold was between 2 and 4 h and when acquisition access was 1 h before inoculation (treatment 1), the threshold was between 4 and 8 h (Fig. 8).

From the fitted lines (Fig. 8), the slopes of the lines for treatments 1 and 2 were not significantly different when compared by Student's t-test (\( t_{23} = 0.21, P = 0.83 \)) but those for treatments 1 and 3 were significantly different (\( t_{23} = 4.13, P = 0.0006 \)). There was a significant increase in the number of infected test plants with increasing inoculation time when nymphs acquired for 2 days with no pre-inoculation fast (treatment 3), (\( t_{23} = 2.53, P = 0.02 \)).
Fig. 8: The relationship between acquisition time, inoculation threshold and rate of transmission

In treatment 1 (bottom graph), fasted nymphs were allowed an acquisition time of 1 h with no post-acquisition fast. In treatment 2 (middle graph), fasted nymphs were given an acquisition time of 2 days with a 16 h post-acquisition fast, and in treatment 3 (top graph), the acquisition time was 2 days with no post-acquisition fast. The symbols (○), indicate the actual points and the solid line indicates fitted points (see page 42 of text). Inoculation of test plants was for the times shown and each point represents a test with 13 to 15 insects.
3. Calculation of the latent period

The latent period is defined as the interval between the start of the acquisition access period and the start of that inoculation access period in which the first successful transmission was made (Toros et al., 1978). The latent period is usually determined using experiments with short acquisition times followed by short, sequential inoculation periods on test plants.

The inoculation threshold depends on the duration of the acquisition access period so that after a 2 day acquisition, the inoculation threshold was between 1 and 2 h whereas after a 1 h acquisition, the inoculation threshold was between 4 and 8 h. Using the sequential transfer method, the latent period could only be estimated within 8 h periods and since short acquisition feeds resulted in low rates of transmission, only one or two nymphs would be expected to transmit at each transfer. This precluded the calculation of the latent period for half the vector individuals i.e. an LP 50 (Gibbs and Harrison, 1976). Given these constraints, the latent period was calculated from the inoculation threshold data.

After a 1 h acquisition, the first transmission occurred after an 8 h inoculation, the previous inoculation interval being for 4 h. Thus if it exists, the latent period would be estimated to lie between 5 h and 9 h,
4. Retention of infectivity by C. nicotianae

To determine the retention time of virus by mirids, 30 fasted nymphs were given an acquisition access of 24 h and then transferred individually at daily intervals to a series of healthy test plants. Mirids were given inoculation access for 8 h to preclude the possibility of reacquisition from infected plant cells, and transfers continued for 12 days or until the death of the mirid. Between inoculation access periods, mirids were removed and fasted for 16 h at 25°C. Five mirids used in the control treatment were given access to healthy plants before sequential transfers to test plants.

The virus was retained by mirids for up to 10 days but was transmitted intermittently and was only occasionally transmitted on successive days during its period of retention. Mirids were able to transmit following moulting (Fig. 9).

5. Transmission by moulted and unmoulted C. nicotianae

The frequency of retention of infectivity through a moult was tested in a further experiment. In treatment 1, 40 fasted fifth-stage nymphs were given access to a virus source for 16 h. They were maintained in individual cages at 25°C without food until they moulted to the adult and were then allowed a 2 day inoculation access on test plants. In treatment 2, 40 nymphs were given access to virus for 16 h and maintained without food in individual cages at 25°C as above, for 24 h. After this period, unmoulted nymphs were allowed a 2 day inoculation access on test plants. This experiment was repeated 3 times.
Fig. 9: Duration of retention of infectivity by *C. nicotianae*

Time course of transmission of VTMOV following a 24 h acquisition access, showing intermittent sequential transmission and frequent transstadial transmission by mirids. Transfers of mirids to test plants continued daily for 12 days or until the death of the mirid (d). The time of moulting was recorded (m) as was virus transmission (-----).
<table>
<thead>
<tr>
<th>Mirid No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
<td>1</td>
<td>d</td>
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<td></td>
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</tr>
<tr>
<td>2</td>
<td>d</td>
<td></td>
<td></td>
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</table>
In each trial, both moulted and unmoulted mirids transmitted VTMoV (Table 5). Transmission by unmoulted nymphs was expected, but in trials 1 and 3 the rates of transmission by unmoulted nymphs was lower than that by moulted mirids (Table 5). The reason for this is not known but it is clear from these results that moulting does not adversely affect the rate of transmission of VTMoV by C. nicotianae.

Table 5: Transmission by moulted and unmoulted C. nicotianae

<table>
<thead>
<tr>
<th>Trial</th>
<th>Moulted mirids</th>
<th>Unmoulted mirids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16/31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10/30</td>
</tr>
<tr>
<td>2</td>
<td>9/24</td>
<td>8/26</td>
</tr>
<tr>
<td>3</td>
<td>15/29</td>
<td>6/25</td>
</tr>
</tbody>
</table>

<sup>a</sup> numerator indicates number of plants infected; denominator, number of test plants used

6. Testing transmission through a moult when C. nicotianae are denied access to their shed cuticle

Tests were done to determine whether transmission after moulting was due to re-acquisition of virus from cuticles which had been shed at moulting. In treatment 1, 40 fasted fifth-stage nymphs were given access to a virus source for 16 h and were then maintained in individual cages without food until they had moulted.
The mirids which had moulted were given access to their shed cuticles for 16 h before being transferred to test plants for a two day inoculation feed. In treatment 2, another 40 fasted fifth-stage nymphs were given access to virus for 16 h and then maintained in individual cages without food at 25°C. The nymphs were observed at 15 min intervals and as each nymph moulted, its cuticle was immediately removed and the mirid transferred to a test plant.

The results in Table 6 show that transmission occurred when newly moulted adults were denied access to their shed cuticle.

Table 6: Transmission after moulted mirids were denied access to their shed cuticle

<table>
<thead>
<tr>
<th>Mirids infective before moult</th>
<th>Access to shed cuticle</th>
<th>Rate of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>4/20\textsuperscript{a}</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>12/32</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0/16</td>
</tr>
</tbody>
</table>

\textsuperscript{a} numerator indicates number of plants infected; denominator, number of test plants used

7. Tests to determine whether non-infective \textit{C. nicotianae} can acquire \textit{VTMoV} from shed cuticles or from plastic cages previously occupied by "infective" \textit{C. nicotianae}

The previous experiment showed that mirids could still transmit through a moult when denied access to their shed cuticle. An experiment was done to test whether non-infective nymphs could acquire \textit{VTMoV} from either cuticles shed from "infective" mirids or from
exudates left inside the plastic cages previously occupied by "infective" moulted mirids. If it could be shown that non-infective nymphs could acquire VTMoV from these sourcees, then this would suggest that post-moult contamination of mouthparts by probing cuticles or mirid exudates was responsible for transstadial transmission.

In treatment 1, 50 fasted fifth-stage nymphs were fed on a virus source for 16 h and were then maintained at one per cage, without food at 25°C. After 18 nymphs had moulted to adults they were transferred singly, in a new cage, to a test plant for a 2 day inoculation feed. In treatment 2, each of the cuticles shed from the 18 mirids used in treatment 1, were collected from the cages and transferred singly to a gelatin capsule which contained a non-infective nymph, and left for 24 h. In treatment 3, a non-infective nymph was kept in each of the 18 cages previously occupied by the moulted mirid, for 24 h. Nymphs from treatments 2 and 3, were transferred singly to a test plant for a 2 day inoculation feed.

Ten of the 18 moulted mirids transmitted VTMoV. Non-infective nymphs given 24 h access to either shed cuticles from "infective" mirids or cages previously occupied by "infective" mirids did not transmit VTMoV.
8. Transstadiial transmission by *C. nicotianae* after short acquisition periods

An experiment was done to determine whether mirids transmit through a moult after short acquisition periods. Three groups of 40 fasted fifth-stage nymphs were given access to a virus source for either 10, 100 or 1000 min. Nymphs were maintained without food until they moulted to adult and were then allowed a 2 day inoculation feed on test plants. As an unmoulted control treatment, 3 groups of 40 fasted fifth-stage nymphs were given the same acquisition access periods as described above, and were maintained without food for 24 h. Unmoulted nymphs were transferred to test plants for a 2 day inoculation feed.

Some of the mirids which had access to a virus source for only 10 or 100 min, were able to transmit through a moult (Table 7).

Table 7: Transmission through a moult when different acquisition times were tested

<table>
<thead>
<tr>
<th>Acquisition period (min)</th>
<th>Moulted mirids</th>
<th>Unmoulted mirids</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4/40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5/20</td>
</tr>
<tr>
<td>100</td>
<td>5/33</td>
<td>6/20</td>
</tr>
<tr>
<td>1000</td>
<td>6/19</td>
<td>5/16</td>
</tr>
</tbody>
</table>

<sup>a</sup> numerator indicates number of plants infected; denominator, number of test plants used
The duration of the acquisition period did not significantly affect the rate of transmission by moulting mirids 
\( \chi^2 = 4.45, 0.1 < P < 0.25 \) or the rate of transmission by unmoulted nymphs \( \chi^2 = 0.201, .9 < P < .95 \).

9. Detecting low levels of VTMoV on cuticles shed by \textit{C. nicotianae}

Although previous experiments have shown that shed cuticles are not a source of virus for non-infective nymphs, they may contain levels of VTMoV that are too low for transmission by \textit{C. nicotianae}. To detect low levels of VTMoV, an assay that makes use of the satellite free isolate of VTMoV, VTMoV-K1 (Francki et al., 1986a), was developed. This assay was then used to detect VTMoV on shed cuticles.

(a) VTMoV detection assay using VTMoV-K1

The initial method for detecting VTMoV on shed cuticles was to sonicate cuticles and inoculate them onto healthy test plants. It was assumed that levels of VTMoV on the cuticles would be low, so an assay was developed to increase the sensitivity of such an infectivity assay. As the satellite free VTMoV-K1 isolate supports the replication of satellite RNA (sat-RNA) (Francki et al., 1986a), an experiment was done to test whether levels of VTMoV RNA too low to detect, might be detected by the ability of VTMoV-K1 to "rescue" the satellite of VTMoV. If an infective amount of VTMoV-K1 was inoculated onto a plant with a non-infective amount of VTMoV, any sat-RNA detected would have been contributed by VTMoV, thus revealing its presence.
To test this hypothesis, groups of 10 *N. clevelandii* were each mechanically inoculated with one of four dilutions of purified VTMoV in 20 mM PB: $6.4 \times 10^{-3}$, $1.3 \times 10^{-3}$, $2.6 \times 10^{-4}$ and $5.1 \times 10^{-5}$ ug/ml. This treatment was replicated. In a second treatment, plants were inoculated as above, with the addition of 100 ug/ml of purified VTMoV-K1 in 20 mM PB. Two control groups each of 5 plants were inoculated with either 20 mM PB or 100 ug/ml VTMoV-K1.

All plants were tested by gel immunodiffusion using VTMoV antiserum and only those plants with symptoms were positive. In addition, total nucleic acids were extracted from plants which had been inoculated with VTMoV plus VTMoV-K1, and from control plants inoculated with PB or VTMoV-K1. These samples were separated by polyacrylamide gel electrophoresis and sat-RNA bands were identified after staining in toluidine blue or silver (Ch.2.II.5). It was assumed that infected plants which had been inoculated with VTMoV alone, contained the sat-RNA so total nucleic acids were not extracted from these plants.

No sat-RNA bands were observed from samples inoculated with PB or VTMoV-K1 alone (Fig. 10, Gel 1). Some plants inoculated with VTMoV plus VTMoV-K1 contained sat-RNA (Fig. 10, Gels 2-5). The verification diagram on the legend for Fig. 10 shows which numbered tracks contained sat-RNA for each gel.

The number of plants infected with VTMoV alone, and assumed to contain sat-RNA, at each of the four concentrations of inoculated VTMoV, and the number of plants containing sat-RNA after inoculation with VTMoV plus VTMoV-K1 are listed in Table 8.
Fig. 10: Detection of sat-RNA by electrophoresis of total nucleic acids (TNA) from samples inoculated with a range of concentrations of VTMoV, with or without the addition of a fixed concentration of VTMoV-K1

<table>
<thead>
<tr>
<th>Gel number</th>
<th>Sample</th>
<th>Track number</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>VTMoV standard</td>
<td>1, 2 &amp; 14</td>
</tr>
<tr>
<td></td>
<td>VTMoV-K1 standard</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>TNA from each of the 5 plants inoculated with VTMoV-K1 (100 ug/ml)</td>
<td>3-7</td>
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<tr>
<td></td>
<td>TNA from each of the 5 plants inoculated with phosphate buffer</td>
<td>8-12</td>
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<tr>
<td></td>
<td>Gel stained in toluidine blue</td>
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</tbody>
</table>

2-5 VTMoV standard
VTMoV-K1 standard
TNA from each of the 10 plants inoculated with:

<table>
<thead>
<tr>
<th>Track</th>
<th>VTMoV conc.n in inoculum (ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.4 x 10^{-3} ug/ml VTMoV + 100 ug/ml VTMoV-K1</td>
</tr>
<tr>
<td>2</td>
<td>1.3 x 10^{-3}</td>
</tr>
<tr>
<td>3</td>
<td>2.6 x 10^{-4}</td>
</tr>
<tr>
<td>4</td>
<td>5.1 x 10^{-5}</td>
</tr>
</tbody>
</table>

Gels were stained with silver
Arrows indicate positions for sat-RNA 2 (2) and sat-RNA 3 (3).

Verification diagram of gels 2-5 showing which tracks, i.e. test plants, contain detectable sat-RNA. For gel 1, no samples contained detectable sat-RNA.

<table>
<thead>
<tr>
<th>Gel number</th>
<th>VTMoV conc.n in inoculum (ug/ml)</th>
<th>Track number</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>6.4 x 10^{-3}</td>
<td>+ + - - - + + + +</td>
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<tr>
<td>3</td>
<td>1.3 x 10^{-3}</td>
<td>+ + + - - + + - +</td>
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<tr>
<td>4</td>
<td>2.6 x 10^{-4}</td>
<td>+ - + + + - + + +</td>
</tr>
<tr>
<td>5</td>
<td>5.1 x 10^{-5}</td>
<td>+ - + + + + + + +</td>
</tr>
</tbody>
</table>

+ = sat-RNA band observed
- = sat-RNA band not observed
Table 8: Number of plants containing the sat-RNA following inoculation with different concentrations of VTMoV in the absence or presence of a fixed concentration of VTMoV-K1 (100 ug/ml)

<table>
<thead>
<tr>
<th>Virus</th>
<th>VTMoV concentration (ug/ml) in inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inoculated</td>
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<tr>
<td></td>
<td>6.4x10^{-3} 1.3x10^{-3} 2.6x10^{-4} 5.1x10^{-5}</td>
</tr>
<tr>
<td>VTMoV</td>
<td>3^a 6 3 0</td>
</tr>
<tr>
<td>VTMoV</td>
<td>3 4 2 0</td>
</tr>
<tr>
<td>VTMoV + VTMoV-K1</td>
<td>7 6 8 9</td>
</tr>
</tbody>
</table>

^a each test was done with 10 plants

Infection levels were similar when VTMoV was inoculated to plants at $6.4 \times 10^{-3}$, $1.3 \times 10^{-3}$ and $2.6 \times 10^{-4}$ ug/ml. The addition of VTMoV-K1 had little effect at the two highest concentrations of virus but at $2.6 \times 10^{-4}$ ug/ml, more plants were infected. At a VTMoV concentration of $5.1 \times 10^{-5}$ ug/ml, no plants were infected unless VTMoV-K1 was included in the inoculum, in which case 9/10 plants were shown to contain sat-RNA, contributed by VTMoV. Thus, at VTMoV concentrations too low for detection by direct infectivity assay, sat-RNA from the inoculum can be detected, apparently as a result of the associated replication of VTMoV-K1 RNA1. Samples containing very low levels of VTMoV (for example, the insect cuticle) may therefore be identified by detecting the sat-RNA component when the samples are inoculated with VTMoV-K1.
(b) An assay to detect \( \text{VTMoV} \) on cuticles shed by \( \text{C. nicotianae} \)

An infectivity assay based on the detection of sat-RNA in plants co-inoculated with \( \text{VTMoV-K1} \) was used to determine whether virus was associated with shed cuticles.

Cuticles were obtained from 3 sources:

1. 32 cuticles from infective mirids collected immediately after moulting
2. 35 cuticles from infective mirids collected 16-18 h after moulting
3. 18 cuticles from non-infective mirids.

Cuticles from (1) and (2) were sonicated for 2 min with 250 ul 20 mM phosphate buffer (PB), pH 7.4, using a Branson Sonifier Cell Disrupter B 15 with Microtip (Branson Sonic Power Co., U.S.A.) and cuticles from (3) were sonicated with 125 ul PB. A second set of inocula were prepared by mixing 40 ul of each of the above samples with 40 ul of a 2 mg/ml solution of \( \text{VTMoV-K1} \). Four test plants were inoculated with each of the cuticle samples from (1), (2) and (3) and another 4 were inoculated with the samples containing \( \text{VTMoV-K1} \). Four plants were mock inoculated with buffer only and 4 plants were inoculated with a 2 mg/ml solution of \( \text{VTMoV-K1} \).

Plants were observed for symptom expression and groups of systemically infected leaves from each treatment were harvested separately and assayed for virus by double immunodiffusion tests. Total nucleic acids were isolated from leaf material and pooled samples for each treatment were separated by polyacrylamide gel electrophoresis. A \( \text{VTMoV} \) standard was used as a molecular weight marker for sat-RNA and 10-15 ug of the nucleic acid was loaded per well. The RNA bands were located by fluorescence after staining for 30 min in a 1 ug/ml solution of ethidium bromide, and the presence of sat-RNA (Fig. 11, tracks 6-8) indicated the presence of \( \text{VTMoV} \) in the
Fig. 11: Polyacrylamide gel electrophoretic assay of total nucleic acids extracted from plants inoculated with mirid cuticles with or without the addition of a fixed concentration of VTMoV-K1 (100 μg/ml)

Track

1 20mM phosphate buffer (PB), pH 7.4
2 18 cuticles from non-infective mirids
3 as above + VTMoV-K1
4 VTMoV-K1
5 32 cuticles collected from infective mirids immediately after moultng
6 as above + VTMoV-K1
7 35 cuticles collected from infective mirids 16-18 h after moultng
8 as above + VTMoV-K1
9 VTMoV standard

Gel stained in ethidium bromide.

Arrows indicate position for sat-RNA 2 (2) and sat-RNA 3 (3).
cuticles.

When cuticle extracts were inoculated alone, only the group collected 16-18 h after moulting contained infective virus (Table 9). No experiments were done to investigate this, but one could speculate that "infective" moulted adults may have probed their shed cuticles, and in so doing, contaminated their cuticles with enough virus to infect test plants without the involvement of VTMoV-K1.

The infectivity assay based on the detection of sat-RNA in plants inoculated with the satellite free VTMoV-K1 isolate was more sensitive than inoculation of cuticle extracts alone (Table 9). Using this assay, groups of cuticles collected from mirids both 16-18 h and immediately after moulting were shown to contain infective virus (Table 9).

Table 9: Infectivity of moulted cuticles inoculated onto plants which were then assayed by symptoms, immunodiffusion, and electrophoretic detection of sat-RNA

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>- VTMoV-K1</th>
<th>+ VTMoV-K1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. infected plants</td>
<td>No. infected plants</td>
</tr>
<tr>
<td>Buffer</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Cuticles (non-infective)</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Cuticles (infective) c</td>
<td>0/4</td>
<td>4/4</td>
</tr>
<tr>
<td>Cuticles (infective) d</td>
<td>2/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

a = satellite not detected in plant nucleic acid (samples pooled for each treatment)
b = satellite detected in plant nucleic acid (samples pooled for each treatment)
c = cuticles collected immediately after moulting
d = cuticles collected 16-18 h after moulting
III Conclusions

Experiments to investigate characteristics of VTMoV transmission by *C. nicotianae* showed that the acquisition threshold was shorter than one min which is characteristic of a noncirculative, nonpersistent association. Transmission efficiency increased with increasing acquisition time, characteristic of either a semipersistent or a circulative association.

The inoculation threshold was between 1 and 2 h, and the rate of transmission increased with increasing inoculation time, also characteristic of either a semipersistent or a circulative association. When a short acquisition period of 1 h or a pre-inoculation fast of 16 h were imposed, the inoculation thresholds increased to between 4 and 8 h and between 2 and 4 h respectively. These results could not exclude a latent period of between 5 and 9 h, but a longer period was not observed.

With acquisition periods of 24 h, virus infectivity persisted for up to 10 days, which is again characteristic of either a semipersistent or a circulative association.

Thus far, the parameters of VTMoV transmission by *C. nicotianae* are characteristic of both a noncirculative (nonpersistent and semipersistent) and circulative association.

*C. nicotianae* were able to transmit VTMoV transstadially and this was shown not to be due to post-moult contamination of mouthparts by probing either the shed cuticles or mirid exudates on plastic surfaces. Mirids transmitted through a moult after acquisition periods as low as 10 min which suggests that there is no requirement for accumulation of large amounts of virus in the insect before
transstadial transmission can occur. Although virus was detected on
the shed cuticles, mirids given access to cuticles did not transmit
VTMoV.

The evidence supporting transstadial transmission shows that
VTMoV is retained in the mirid at sites that are not shed during
moulting of the insect. One possible site of retention is the midgut
as suggested for beetle transmission (Harris, 1981a) since the midgut
is not shed during moulting, and the other possibility is that VTMoV
circulates in the mirid. Further studies are required to test
circulation of VTMoV in C. nicotianae and chapter 5 describes
experiments in which transmission was tested after introduction of
virus directly into the haemocoel by injection.

In summary, these results show that VTMoV is acquired in a
manner similar to that of a nonpersistent virus, but is retained for
periods characteristic of a semipersistent or circulative virus.
Infectivity is not lost when the mirids moult which is typical of a
circulative virus.

The parameters of transmission are characteristic of both a
noncirculative and circulative association, and these results suggest
that transmission of VTMoV by C. nicotianae may not be adequately
explained by one transmission mechanism.
Chapter 5
Acquisition of VTMoV by Membrane Feeding and Injection

1 Introduction

Circulatively transmitted viruses are often transmitted by their vectors following acquisition either by membrane feeding or by injection. Beet leafhoppers can acquire the circulative, nonpropagative geminivirus, curly top virus, by feeding through a membrane on partially purified virus (Bennett, 1971) as well as by injection of the virus into the body of nonviruliferous insects (Maramorosch, 1955). In membrane feeding tests with another circulative, nonpropagative geminivirus, maize streak virus (MSV), rates of virus transmission by the leafhopper vector were similar to those following acquisition from infected plants (Bock et al., 1974).

Some circulative viruses can be transmitted by their vectors following injection into the insect body cavity (Markham and Townsend, 1979), but there are no reports of noncirculative viruses being transmitted following injection of vectors with virus. Storey (1933) first used this technique to show that the leafhopper vector Cicadulina mbila, could transmit MSV, after injection with extracts of infected plants or infective leafhoppers. Needle inoculation was originally used to measure the amounts of inocula injected (Maramorosch, 1955) and to relate these to the amounts of virus required by vectors for transmission. Thus, transmission following injection with virus is a useful technique for determining whether the virus circulates in the vector. This technique also makes
it possible to introduce measured quantities of virus into the vector and to relate these values to transmissibility.

Nonpersistently transmitted viruses may be acquired through a membrane by their insect vectors, but one group of nonpersistent viruses require a helper component (HC) in the inoculum to allow them to be transmitted by their vectors. Govier and Kassanis (1974) showed that aphids (*Myzus persicae*) did not transmit PVY after probing into purified virus preparations through parafilm membranes, but did if these preparations were first mixed with extracts of infected plants from which PVY particles had been removed by centrifugation. They concluded that transmission of purified PVY by aphids depended on an additional sap component that was not found in healthy plants, and which they defined as HC. Loss of aphid transmissibility upon purification seems to be a general property of viruses in the potyvirus and caulimovirus groups and for some carlaviruses. Several of these nonpersistent viruses have been shown to be transmissible by aphids that were fed on a mixture of the purified virus preparation and PVY HC (Pirone, 1977).

Nonpersistent viruses which apparently do not require a helper include the viruses alfalfa mosaic (Pirone, 1964) and cucumber mosaic (Pirone and Megahed, 1966), but it is possible that in these cases a helper is present which is bound tightly to the virus and is not removed during the purification process (Pirone, 1977). Thus, transmission following acquisition of purified virus through a membrane may indicate that a free HC is not required for transmission.
In experiments described in this chapter, transmission was tested after mirids were either given access to VTMoV through a membrane or injected with virus. Transmission following acquisition of purified virus through a membrane may be taken as partial evidence that VTMoV does not require HC for transmission by *C. nicotianae*, and transmission following injection may be taken as evidence that VTMoV circulates in *C. nicotianae* and reaches a point from which it can be inoculated.

II Experimental

1. Transmission of VTMoV after acquisition of a solution containing virus, through a membrane

In this experiment, unlabelled, purified VTMoV, was mixed with a solution of $^{32}$P-labelled orthophosphate and offered to mirids through a parafilm membrane. Assuming that virus particles were distributed evenly throughout the mixture and that the amount of label measured in each mirid was proportional to the dose, then the amount of virus acquired by each mirid could be calculated from the volume acquired.

The relationship between cpm and dose was found to be linear ($r^2 = 98\%$) over the range used (1.86x10$^5$ to 7.4x10$^1$ cpm/ul).

For all samples, "Cerenkov" counting was used without the addition of scintillation fluid. Samples were counted in a Tri-Carb Liquid Scintillation Spectrophotometer Model 3320 (Packard, U.S.A.) with a gain of 50% and window setting of 20-1000.
One hundred mirids were given access to a mixture of purified VTMoV (1mg/ml) in 5% sucrose containing an aqueous solution of $^{32}$P-labelled ortho-phosphate (BRESA, South Aust), (specific radio-activity of the mixture was $1.86 \times 10^5$ cpm/ul), through a parafilm membrane (Ch.2.IV.8).

At 2, 4, 6, 8, 10, 12, 24, 26, 28, 30 and 32 h after access to virus commenced, mirids observed on the parafilm membrane, were removed and counted. These mirids were placed in a 500 ul plastic centrifuge tube, which was then placed in a glass scintillation vial and counted as described above. Those mirids registering more than twice the background level (mirids fed 5% sucrose only) were transferred to a test plant for 2 days, and those registering less than twice the background level were returned to the membrane feeding chamber.

For each mirid, cpm were used to determine virus dose directly from the specific radioactivity.

Forty-three mirids acquired between 1 and 700 ng of virus by feeding through a membrane but 31 of them acquired less than 15 ng of virus (Fig. 12). Five of the mirids which acquired more than 165 ng of virus transmitted VTMoV (Fig. 12). Mirids fed on unlabelled solutions without virus, did not transmit VTMoV.
2. Transmission of VTMoV after injection into the haemocoele of *C. nicotianae*

(a) Injection of *C. nicotianae* with a solution containing virus

To determine whether mirids could transmit after direct introduction of virus into the haemocoele, mirids were injected with a solution containing virus. The inoculum also contained $^{32}$P-labelled orthophosphate, so that the amount of virus introduced into the mirid could be measured and related to transmissibility, as described in section 1 above.

The relationship between cpm and dose was found to be linear ($r^2 = 96\%$) over the range used (7.95 x $10^3$ to 7.95 cpm/ul) and samples were counted as described in section 1.

Sixty fasted fifth-stage nymphs were each injected with a mixture of purified VTMoV (2 mg/ml) in sterile 2/3 strength Insect Ringer's (IR) solution (2/3 IR is 100 mM sodium chloride, 2 mM potassium chloride, 1mM calcium chloride), and an aqueous solution of $^{32}$P-labelled ortho-phosphate (specific radioactivity of the mixture was 7.95 x $10^4$ cpm/ul). Inoculum was filtered through a MILLEX-HA 0.45 um filter unit (Millipore corporation, Bedford, U.S.A.) before injection.

Injected nymphs were counted as described in section 1, and background radiation was determined from 10 nymphs injected with 2/3 IR only. For each nymph injected with virus, cpm were used to determine virus dose directly from the specific radioactivity.

Following injection, each nymph was placed on a plant for 2 days to test for survival, ability to feed, and the ability to
inoculate the test plants. Test plants with no obvious feeding damage at the end of the inoculation period were discarded.

Sixty nymphs received doses of between 1 and 160 ng of virus by injection (= 0.5 and 80 nl) and 32 of these received less than 15 ng of virus (Fig. 12). Two of the injected nymphs received 50 and 63 ng of virus respectively, and transmitted VTMoV (Fig. 12), but the remaining nymphs did not transmit VTMoV.

(b) Injection of C. nicotianae with an increased concentration of virus

To determine whether an increased dose of virus delivered by injection, increased the rate of transmission, 60 nymphs were injected with 30 mg/ml of virus in sterile 2/3 IR. The 10 control nymphs were injected with 2/3 IR only. Assuming that the range of volumes of labelled solution injected into nymphs was between 0.5 and 80 nl as described above, it was estimated that between 15 and 2,400 ng of virus was injected into the mirids. As above, nymphs were given access to test plants for 2 days after injection, and plants without feeding damage at the end of this period were discarded. Control nymphs did not transmit VTMoV, but 11/47 nymphs injected with virus transmitted VTMoV.
Fig. 12: Frequency distribution of virus taken up by mirids following either injection (solid bars) or membrane feeding (hatched bars).

One hundred nymphs were fed through a membrane containing a mixture of VTMoV (1 mg/ml) and $^{32}$P-labelled ortho-phosphate (specific radioactivity of the mixture was $1.86 \times 10^5$ cpm/ul). Sixty nymphs were injected with a mixture of VTMoV (2 mg/ml) and $^{32}$P-labelled ortho-phosphate (specific radioactivity of the mixture was $0.95 \times 10^4$ cpm/ul). Mirids were then given a 2 day inoculation feed on test plants and the numbers above the bars indicate the number of mirids which transmitted in each class.
III Conclusions

*C. nicotianae* can acquire purified virus through a membrane and can then transmit it to test plants. This indicates that WMoV does not require a free helper component for transmission by mirids.

The volume of virus suspension acquired by mirids through a membrane, as measured by uptake of $^{32}$P, varied from 1 to 700 ng (Fig. 12). Garrett (1971) also found a wide variation in acquisition of $^{32}$P-labelled ortho-phosphate by aphids through a membrane. Thirteen out of 43 mirids acquired more than 15 ng of virus, and it was from this group only that 5 mirids transmitted VTMoV. Thus, the ability to transmit appeared to be dose dependent.

Mirids were able to transmit VTMoV following injection with purified virus, which is characteristic of a circulative association. When mirids were injected with a solution containing a low concentration of virus, the range of virus levels in mirids was lower than for membrane-fed mirids (Fig. 12), and only 2/60 mirids transmitted VTMoV. When the concentration of injected virus was increased (and presumably the range of virus amounts in mirids increased), the rate of transmission increased to 11/47. As with membrane-fed mirids, the ability to transmit following injection with virus seems to be dose dependent.

An alternative explanation may be that $\beta$-emission from the $^{32}$P could have reduced the infectivity of injected virus in the first transmission experiment and this might account for the low rate of transmission. However, the specific radioactivity of
the solution was low, and the use of unlabelled virus may have reduced the risk of loss of infectivity. Takagi and Ikegami (1972) found that the infectivity of unlabelled TMV in a $^{32}$P-labelled solution was not reduced, whereas the infectivity of TMV in which $^{32}$P was incorporated, was 1/8th of the original infectivity over the same period (28 days). The radiation sensitivity of VTMOV is not known, and so a more appropriate experiment to compare dose dependence would have been to inject mirids with 30 mg/ml of virus in a labelled solution.

Thus, VTMOV can be acquired without the requirement of a helper component. The ability to transmit VTMOV, whether it is acquired through a membrane or by injection, seems to be dose dependent and when a dose of about 50 ng of virus is exceeded, mirids are able to transmit the virus in a circulative manner. The next chapter describes experiments to test the duration of retention of virus in the vector.
Chapter 6
Retention of VTMoV by C. nicotianae

I Introduction

Circulative viruses can be characterised by a number of criteria including transstadial transmission, a latent period and prolonged retention of inoculativity by infective insects (Eskandari et al, 1979), (Table 1, Ch.1). Propagative viruses also circulate in their vector. To test whether a virus is propagative, experiments have been done either by serial injection of vectors to determine whether infectivity is retained, or by serological tests to determine whether antigen is retained. For example, Reddy and Black (1966) monitored the multiplication of a reovirus, wound tumour virus (WTV), in Agallia constricta (Von Duzee) by injecting serially diluted insect extracts into non-infective hoppers and testing infectivity.

Gamez and Black (1968) used the particle counting method to estimate directly the number of particles of WTV in A. constricta. They established that the minimal infective dose by injection was about 400 virus particles and they detected increasing numbers of particles with time, indicating that virus particles replicated in the insect. Another technique used by Reddy and Black (1972) was to monitor WTV concentration in A. constricta by counting infection foci in monolayer cultures of vector cells.

In experiments with PEMV, and the luteoviruses, PLRV and BYDV (see abbreviations list for full names of viruses), infective aphid extracts were serially injected into non-infective aphids and infectivity tested. For all three viruses, infectivity was maintained
for only one passage (Clarke and Bath, 1973; Eskandari et al., 1979; Mueller and Rochow, 1961), and it was concluded that these viruses were circulative but probably not propagative in their aphid vectors.

The lack of evidence for propagation of PEMV and PLRV was further shown in ELISA studies, in which there was no increase in the quantity of antigen after acquisition of PEMV by aphids (Fargette et al., 1982), and the PLRV content of aphids decreased with increasing time after transfer to turnip (immune to PLRV) (Tamada and Harrison, 1981).

Caciagli and Casetta (1986) used ELISA to study the multiplication of a reovirus, maize rough dwarf virus (MRDV), in the vector Laodelphax striatellus Fallen. They showed that well after maximal infectivity was reached, the virus was still increasing in concentration exponentially in the insects. Kelly et al., (1978) used ELISA to show two phases of exponential growth in the number of polyhedra of Heliothis armigera polyhedrosis virus in H. armigera larvae. Falk and Tsai (1985) used ELISA as a quasi-quantitative assay to detect the rhabdovirus, maize mosaic virus (MMV) in individual Peregrinus maidis and found that MMV antigen concentrations increased with time in the vector.

In this chapter, the duration of virus retention by C. nicotianae has been tested by feeding trials and ELISA, to determine the duration of persistence of virus in the vector, and to test whether virus propagation occurs.
II Experimental

1. Retention of infectivity by C. nicotianae

To estimate the period during which mirids could transmit VTMoV, infective nymphs were maintained on immune plants, with test feeds at regular intervals on susceptible test plants. Thirty nymphs which had been given access to a virus source for 2 days were transferred individually to single test plants for a 24 h inoculation access period. These nymphs were then transferred individually to single immune tomato plants (Ch.2.I.1) for 3 days. This feeding cycle of susceptible test plant followed by immune plant, was repeated 5 times and 10 non-infective controls were similarly treated.

Transmission to test plants ceased between 5 and 9 days after acquisition following two cycles on tomato, and no further transmission occurred up to 17 days (Table 10). Loss of infectivity with time provided evidence that VTMoV cleared from and did not propagate in the vector.
Table 10: Loss of infectivity by C. nicotianae

<table>
<thead>
<tr>
<th>Days after acquisition completed</th>
<th>Rate of Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^a)</td>
<td>11/28(^b)</td>
</tr>
<tr>
<td>5</td>
<td>2/28</td>
</tr>
<tr>
<td>9</td>
<td>0/25</td>
</tr>
<tr>
<td>13</td>
<td>0/23</td>
</tr>
<tr>
<td>17</td>
<td>0/19</td>
</tr>
</tbody>
</table>

\(^a\) between each 24 h inoculation period on susceptible test plants, mirids were maintained singly on tomato plants for 3 days

\(^b\) numerator indicates number of plants infected; denominator, number of test plants used

2. Characteristics of clearance of antigen as estimated by ELISA

An experiment was done to measure the amount of antigen in mirids with increasing time after removal from the virus source plant, and thus compare the rate of loss of infectivity with the rate of loss of detectable antigen. VTMoV is referred to as "virus" in infectivity assays and as "antigen" in ELISA.

Two hundred and fifty nymphs were given access to virus source plants for 2 days and then transferred to tomato seedlings (5 per plant). Each day for 10 days, 20 mirids were removed from tomatoes, 10 were individually tested by ELISA and 10 were individually given inoculation access to single test plants for 2 days. In addition, 8 non-infective control nymphs were removed from tomatoes each day, and
4 were tested by ELISA and 4 were tested for infectivity.

The number of mirids found to be positive by ELISA decreased linearly between 1 and 8 days after acquisition ($F_{8,9} = 24.24$, $P = .0015$, $r^2=76$%), and no mirids contained detectable antigen 9 and 10 days after acquisition (Table 11).

In contrast, the number of mirids transmitting virus showed a sharp decrease 2 days after acquisition (Table 11), and thereafter levels remained low ($F_{5,6} = 2.71$, $P = .16$). The ability to transmit VTMoV was lost in 90% of mirids two days after leaving the virus source but at least 1 mirid transmitted in each group (except day 4), up to 7 days after acquisition (Table 11).

Table 11: ELISA and infectivity assays of mirids, with time after completion of acquisition.

<table>
<thead>
<tr>
<th>No. of days after acquisition completed</th>
<th>No. of mirids positive by ELISA</th>
<th>Mean virus content of mirids positive by ELISA (ug ± S.E.)</th>
<th>Rate of Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/10</td>
<td>1.76 ± .50</td>
<td>8/10(^a)</td>
</tr>
<tr>
<td>2</td>
<td>9/10</td>
<td>2.44 ± .90</td>
<td>1/10</td>
</tr>
<tr>
<td>3</td>
<td>3/10</td>
<td>0.63 ± .16</td>
<td>1/10</td>
</tr>
<tr>
<td>4</td>
<td>2/10</td>
<td>1.03 ± .03</td>
<td>0/10</td>
</tr>
<tr>
<td>5</td>
<td>4/10</td>
<td>0.72 ± .18</td>
<td>1/10</td>
</tr>
<tr>
<td>6</td>
<td>3/10</td>
<td>0.49 ± .15</td>
<td>1/10</td>
</tr>
<tr>
<td>7</td>
<td>2/10</td>
<td>0.47 ± .32</td>
<td>1/10</td>
</tr>
<tr>
<td>8</td>
<td>1/10</td>
<td>0.33</td>
<td>NT(^b)</td>
</tr>
<tr>
<td>9</td>
<td>0/10</td>
<td>0.00</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>0/10</td>
<td>0.00</td>
<td>NT</td>
</tr>
</tbody>
</table>

\(a\) numerator indicates number of plants infected; denominator, number of test plants used

\(b\) NT = not tested
ELISA and infectivity assays were done simultaneously, so it was appropriate to compare the means of the samples using the Student's t-test for paired samples (Ch.2.V). At a given time after acquisition, the number of mirids positive by ELISA was greater than the number able to transmit the virus ($t_{6} = 3.23, P = .018$), thus more mirids contained detectable antigen than could transmit WMoV.

When mean amounts of virus in mirids shown to be positive by ELISA were considered, there was no linear decrease with time ($F_{32,33} = 3.44, P = .07$). That is, even though the number of mirids with detectable antigen decreased linearly with time, the amount of virus in these mirids did not. This result suggests an "all or nothing" pattern of virus loss rather than a dilution of virus content and decrease with time. This conclusion should be treated with caution however, since differences in amounts of antigen between sample days were obscured by the high variability in levels of antigen detected in mirids within each day, as indicated by the high standard deviations.

3. Effect of acquisition access period and a post acquisition inoculation feed on virus accumulation and clearance

An experiment was done to test the effect of 10 and 100 min acquisition access periods on the number of mirids containing antigen and the mean amounts of virus antigen in these mirids. Following acquisition, some mirids were given an inoculation access period, to test its effect on clearance.

In treatment 1, 60 nymphs were aspirated into cages (5 per cage), and given access to a virus infected leaf in each cage, for
10 min. At the end of the acquisition period, 30 nymphs were collected and immediately tested by ELISA, and the other 30 nymphs were placed on test plants for a 24 h inoculation access period before being tested by ELISA. In treatment 2, 60 nymphs were given an acquisition access of 100 min and tested as above. In the control treatment, 16 non-viruliferous nymphs were given access to healthy leaves for 100 min, 8 were tested by ELISA and the remainder were given an inoculation feed on test plants for 24 h before being tested by ELISA.

Table 12 shows the number of nymphs positive by ELISA and their mean antigen content following acquisition periods of either 10 or 100 min, with and without a 24 h inoculation period. The results in Table 12 are compared with those obtained for the 3000 min acquisition access period in Table 11.

Table 12: The effect of acquisition and inoculation period on the number of nymphs positive by ELISA and their mean content of antigen.

<table>
<thead>
<tr>
<th>Acquisition period (min)</th>
<th>Number of nymphs positive by ELISA after inoculation of:</th>
<th>Mean amount of antigen in positive nymphs [(ug)+S.E.] after inoculation of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>10</td>
<td>12/30</td>
<td>0/30</td>
</tr>
<tr>
<td>100</td>
<td>24/30</td>
<td>6/30</td>
</tr>
<tr>
<td>3000^a</td>
<td>10/10</td>
<td>NT^b</td>
</tr>
</tbody>
</table>

a  data obtained from Table 11

b  NT = not tested
When no inoculation feed was given, the number of nymphs containing antigen increased with length of acquisition feed
\( (F_{11,13} = 8.643, P = .0005) \). Least significant difference (LSD) pair-wise comparisons showed that the number of nymphs positive by ELISA was greater for the 100 min acquisition than for the 10 min acquisition (0.40 > 0.27) and no further increase was observed for the 3000 min acquisition (0.20 < 0.38). There was a significant increase in the amount of antigen detected in nymphs,
\( (F_{11,13} = 26.10, P < 0.0001) \) at each of the acquisition times tested.

All nymphs which had an acquisition access period of 10 min, cleared after 24 h, but some nymphs which had an acquisition period of 100 min, still contained antigen after a 24 h inoculation period. Of the nymphs that had an acquisition period of 100 min, there was significantly less antigen in nymphs which had an inoculation period of 24 h compared to those that did not,
\( (F_{39,41} = 57.24, P < 0.0001) \).
II Conclusions

Mirids given an acquisition access period of 2 days stopped transmitting VTMoV between 5 and 9 days after completion of acquisition access, and this can be compared with a previous experiment (Ch. 4. II. 4), in which some mirids retained infectivity for 10 days. Virus antigen was not detectable in mirids 9 days after acquisition. Such a loss as detected in these assays, would be consistent with either a semipersistent or circulative mode, but not a propagative mode of VTMoV transmission by C. nicotianae.

When the number of mirids found to be positive by ELISA was compared to the number of mirids transmitting VTMoV after acquisition, it was found that more mirids contained detectable antigen as estimated by ELISA than could transmit VTMoV. This observation is not uncommon and Tamada and Harrison (1981), working with the nonpropagative luteovirus, PLRV, found that the virus content of each aphid vector was not related to infectivity. Similarly, Fargette et al. (1982), found that the transmission efficiency of each aphid vector was not highly correlated with the amount of PEMV ingested. Caciaglì et al. (1985) found that 15-20% of Laodelphax striatellus which were positive for the propagative MRDV by ELISA, were unable to transmit the virus to host plants, and they concluded that a proportion of hoppers were merely carriers of the virus but not vectors, perhaps because the virus had not invaded their salivary glands. Using immunoelectron microscopy to detect the propagative Fiji disease virus (FDV) in its planthopper vector, Francki et al. (1986b) found that of 82 infected insects, only 34 transmitted FDV.
A possible explanation of this phenomenon is that non-transmitting mirids contain detectable virus in the gut but virus particles do not reach sites from which transmission can occur. Further studies on the distribution of VTMoV in the mirid are described in Chapter 7.

The amount of virus antigen detected in mirids with increasing time after acquisition was highly variable within each sampling day (Table 11). Variability in mirids may have been due to variations in the amount of virus acquired by each mirid, and this may be affected by the time spent feeding, the site of feeding, and the effect of moulting on feeding behaviour. Defecation rates may also have affected the amount of virus antigen detected, if it is assumed that virus accumulates in the gut. Some mirids contained as much virus 7 days after acquisition as on day 1 whereas others showed no antigen after 7 days. This implies an "all or nothing" effect in which virus may be either lost by rapid movement through the gut or retained at some site. Further studies are described in Chapters 7 and 8 to determine whether virus is concentrated in the gut and whether excretion behaviour affects virus retention or loss.

Studies of factors affecting virus accumulation and clearance showed that nymphs contained more antigen if they had a longer acquisition access period, which probably accounts for the increase in rates of transmission with increasing acquisition time (Ch.4.II.1). An inoculation access period of 24 h resulted in significant loss of antigen. After an acquisition access of 10 min, inoculation for 24 h resulted in complete clearance of virus from nymphs (Table 12), however, after an acquisition access of 3000 min (i.e. 2 days), clearance did not occur until after 8 days (Table 11). This indicates
that more virus accumulates in the insect with increasing acquisition period and is lost more slowly when a large amount is acquired. These results further emphasize the possible importance of the gut in accumulating virus for up to 9 days, but the effect this has on infectivity is to be studied further (chapter 8).

To summarise, virus is cleared from mirids after 8 days when mirids acquire for long periods but is lost more quickly after short acquisitions. Virus accumulation in the gut may partly explain this retention, but further studies are required to localise the virus in the vector and to determine how virus is transmitted from the sites of retention. The clearance of VTMoV from the mirid vector is characteristic of either a semipersistent, or circulative, non-propagative association.
Chapter 7
Location of VTMoV in C. nicotianae

I Introduction

In general, circulatively transmitted viruses accumulate in the insect gut as sap is ingested. For example, an analysis of the PEMV content of the gut and haemolymph of the pea aphid vector A. pisum, by ELISA, Fargette et al. (1982) showed that most of the ingested virus accumulated in the intestinal tract.

Circulatively transmitted viruses are thought to move through the gut wall into the haemocoele and once there, virus can be transported into the salivary glands and saliva, which becomes the inoculum for plants (Harrison and Murant, 1984). Studies with PEMV (Harris et al., 1975) and BYDV (Gildow and Rochow, 1980), indicated that virions were associated with the accessory glands, which implicated the accessory gland basal lamina in virus transport and showed the involvement of the salivary gland in transmission.

Gildow (1982), used electron microscopy to observe BWYV in invaginations of basal lamina and plasmalemma, in accessory salivary glands of M. persicae. Virus particles were frequently observed in tubular vesicles and coated vesicles in cytoplasm near salivary canals. A cellular mechanism, involving coated-vesicle transport of virions from tubular vesicles to the salivary canal, was suggested as a model for transport of luteoviruses through accessory gland cytoplasm (Gildow, 1982).
In contrast to the information known about transmission of PEMV and some of the luteoviruses, transmission by beetles for example, is poorly understood (Ch.1.IV). Beetle transmitted viruses have been detected in the haemolymph, (Freitag, 1956; Slack and Scott, 1971; Slack and Fulton, 1971; Sanderlin, 1973; Fulton et al., 1980), the regurgitant (Fulton et al., 1980) and the faeces (Slack and Fulton, 1971). The pathway by which virus moves from the haemocoele to the regurgitant is not known, but it has been suggested that virus may move from the haemocoele through the midgut into the gut lumen and subsequently be passed to the exterior by regurgitation or defecation, or both (Harris, 1981a). Although more work is required to determine a mechanism of transmission for beetle vectored viruses, localisation of virus in these insects has provided a basis from which to start.

The aim of the experiments described in this chapter was to assay the gut, faeces, haemolymph and salivary glands of *C. nicotianae* for VTMoV. The detection of virus in the haemolymph and salivary gland would show that virus which circulates in the mirid (see Ch.5), may do so via these tissues, and that the mechanism of transmission is therefore likely to be similar to that suggested for the luteoviruses and PEMV.
II Experimental

1. Detection of VTMoV in *C. nicotianae* using an HRP dot-immunobinding assay

Previous experiments (Ch. 6), showed that ELISA allows quantitative assay of virus in mirids, and so it was used in studies to detect virus in the haemolymph and gut. Pooled haemolymph samples from 5 nymphs gave a positive result using ELISA, but antigen was not detected in samples from individual nymphs. An assay to detect virus in small volumes was required, so a dot-immunoassay was chosen. A single antibody dot immunoassay (SADI), based on alkaline phosphatase was considered, but the presence of endogenous phosphatase activity in mirids prevented its use (Graddon and Randles, 1986). A horseradish peroxidase (HRP) dot-immunobinding assay developed by Hawkes *et al.*, (1982), and used by Berger *et al.*, (1985) to detect potyviruses, was used. The small sample volumes required for this technique permitted the detection of virus in haemolymph and other body samples from individual nymphs.

(a) Detection of VTMoV in haemolymph, faeces, gut and remainder of the nymph

The aim of this experiment was to assay the haemolymph, gut, faeces and remainder of the nymph for VTMoV.

Twenty nymphs given access to a virus source plant for 16 h were assayed immediately after acquisition. Haemolymph, faeces, gut and remainder of the nymph were collected from each nymph and tested
for virus by the HRP dot-immunobinding assay (Ch.2.IV.10). Before the
gut was removed from the dissected nymph, it was examined to make sure
it had not been pierced during haemolymph sampling, and in all cases
the gut was intact.

Results showed that 6 of these nymphs contained VTMoV in the
haemolymph, 12 in the faeces, 19 in the gut and 17 in the remainder of
the nymph (Fig. 13). All 5 nymphs with virus in the haemolymph
contained virus in all of the components assayed, and all 12 nymphs
with virus in the faeces contained virus in the gut and remainder of
the nymph. Seven nymphs contained virus in the gut but not in the
faeces, which suggests that virus particles may be unevenly
distributed throughout the gut and that faecal samples are not a
reliable indicator of the presence of virus in the gut. Two nymphs
with virus in the gut (but not in the haemolymph) did not contain
virus in the remainder of the nymph, which suggests that it is
possible to remove the gut without contaminating the rest of the body.
One nymph given access to virus did not contain virus in any of the
components assayed. The diagram presented in association with Fig. 13,
shows the virus positives, and the distribution of virus throughout
the body of each of the 20 nymphs.

The number of nymphs containing virus in the gut was
significantly greater than the number of nymphs containing virus in
either the blood ($\chi^2_{0.05,1} = 15$, $P = 0.0001$) or the faeces
($\chi^2_{0.05,1} = 4.8$, $P < 0.028$). The number of nymphs containing virus
in the gut was not significantly different to the number of nymphs
containing virus in the remainder of the body
($\chi^2_{0.05,1} = 0.23$, $P = 0.63$). This was expected, because virus in
the gut probably contaminated the remainder of the body during
dissection.
Fig. 13: Detection of virus in samples of mirid components by an HRP dot-immunobinding assay immediately after a 1000 min acquisition

Rows 1-8: Nymphs given 1000 min access to virus infected plants
9-10: Nymphs given 1000 min access to healthy plants

Verification diagram of virus positives (+) and negatives (-):

<table>
<thead>
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<th>Column</th>
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<td>8</td>
</tr>
</tbody>
</table>

9 - - - - - - - - column 1-5 = haemolymph & 6-10 = gut
10 - - - - - - - - column 1-5 = remainder & 6-10 = faeces
11 + + + + virus stds: 0.05, 0.50, 0.95 & 9.50 mg/ml

For rows 1, 3, 5 & 7, each set of squares in the same column contains components from the same nymph and similarly for rows 2, 4, 6 & 8
For rows 9 & 10, components from the same nymph are in columns 1 & 6 and similarly for columns 2 & 7, 3 & 8, 4 & 9, 5 & 10
(b) Attempts to detect VTMoV in salivary glands

The salivary glands of 5 nymphs were examined and one was drawn in detail (Fig. 14), so that it would be easier to identify and extract glands for the experimental assay.

Since salivary glands are bathed in haemolymph that has been shown to contain virus, the glands were washed to remove virus from their outside surface. To test the effectiveness of washing virus particles from the salivary glands, 40 glands were isolated and treated as follows. In treatment 1, 10 glands from "non-infective" nymphs were washed twice in fresh IR solution and applied to the nitrocellulose filter. In treatment 2, 10 glands from "non-infective" nymphs were washed in IR and then dipped in a 1 mg/ml purified preparation of VTMoV before application to the filter. In treatment 3, 10 glands from "non-infective" nymphs were washed, dipped in the virus solution and washed twice in IR before being applied to the filter. In treatment 4, 10 glands from "infective" nymphs were washed in IR and applied to the filter. Samples were tested for VTMoV antigen using the HRP dot-immunobinding assay, and glands from "non-infective" nymphs washed in IR produced purple-black spots of the same intensity as all the other samples. The experiment was repeated but with the same result,
Fig. 14: Salivary gland of fifth-stage C. nicotianae (paired)

The principal glands each consist of a pair of lobes, the anterior lobe (0.5-0.8 mm) and the posterior lobe (0.25-0.30 mm) which are richly tracheated and are joined at the hilus where the salivary ducts (2.0-2.5 mm) emerge. The accessory gland lies just beneath the posterior lobe of the principal gland and is vesicular shaped (0.25-0.30 mm). The convoluted accessory duct (2-3 mm) proceeds from the hilus and makes a sharp turn forming a loop which is closely associated with the anterior lobe by tracheal attachments. It proceeds posteriorly and then loops back again thus crossing over the salivary duct twice. The second loop is closely associated with the posterior lobe by tracheal attachments and above this loop the duct becomes closely associated with the salivary duct and proceeds toward the head. From there it loops back to continue to the point at which the accessory and salivary ducts are separate near the hilus. Here the accessory duct becomes "beaded", the last 7-8 "beads" becoming larger until they join the large vesicular accessory gland.
To test that the "non-infective" nymphs were virus free, 3
groups of 20 such nymphs were given either an acquisition access of
100 min, an acquisition access of 3000 min or were fed on healthy
plants for 3000 min. The salivary glands and guts were tested for
VTMoV using the HRP dot-immunobinding assay. The intensity of staining
of gut samples from mirids which had fed for 3000 min was greater than
that of nymphs which had fed for 100 min and gut samples from
"non-infective" nymphs were negative (Fig. 15). In all three
treatments however, salivary gland samples were positive (Fig. 15).
The experiment was repeated but with the same result. It was concluded
that salivary glands contained endogenous peroxidases which were
reacting with the substrate.

An attempt was made to remove these endogenous peroxidases by
grinding isolated glands from "infective" and "non-infective" nymphs in
5 ul TBS and 5 ul chloroform, and centrifuging them at 10,000 g for 10
min. Guts from these nymphs were treated identically and the aqueous
layer of the supernatant from glands and guts were tested for virus,
using the HRP dot-immunobinding assay.

Gut samples from "infective" nymphs were positive and those
from "non-infective" nymphs were negative, whereas all salivary glands
were negative. It appears that the endogenous peroxidase activity was
removed during extraction with chloroform and that no virus was
released from the salivary glands into the aqueous supernatant.

Four groups of 10 salivary glands isolated from "infective"
nymphs were washed and sonicated in buffer (PB-Tween + PVP), for 15
min at 0°C and half of each group's sample was tested for VTMoV
antigen by ELISA. The other half was mixed with standard
concentrations of purified virus and tested by ELISA to make sure that
Fig. 15: HRP dot-immunobinding assay of salivary glands (glands) from "infective" and "non-infective" nymphs showing the activity of endogenous peroxidases in salivary glands (rows 1, 3 & 6)

Rows 1-2: 20 nymphs given 100 min access to virus infected plants
3-4: 20 nymphs given 3000 min access to virus infected plants
6-7: 20 nymphs given 3000 min access to healthy plants

<table>
<thead>
<tr>
<th>Column</th>
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<tbody>
<tr>
<td>Row 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20</td>
</tr>
<tr>
<td>1 ++++++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ ++++++ glands</td>
</tr>
<tr>
<td>2 +++++++ - +++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ - gut</td>
</tr>
<tr>
<td>3 +++++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ - glands</td>
</tr>
<tr>
<td>4 +++++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ +++++ + gut</td>
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<td>5 + - - - - - - - - - - - - - - - - - stds a</td>
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<td>6 + - - +++++ +++++ +++++ +++++ +++++ +++++ +++++ - gut</td>
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<tr>
<td>7 - - - - - - - - - - - - - - - - - - - - - - - - glands</td>
</tr>
</tbody>
</table>

For rows 1 & 2 each set of squares in the same column represents bodyparts from the same nymph and similarly for rows 3 & 4, and for rows 6 & 7

a stds = virus standards: 9.5, 0.95, 0.50, 0.05 mg/ml (1 ul)
<table>
<thead>
<tr>
<th>Row number</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Column number</td>
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<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
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</tbody>
</table>
the salivary glands did not contain a factor which would inhibit
detection of VTMoV. Guts from these nymphs were also tested by ELISA
and "non-infective" nymphs were treated as described for "infective"
nymphs.

"Non-infective" gut samples were negative and those from
"infective" nymphs were positive. All salivary gland samples were
negative and the detection of purified virus was not affected by the
addition of salivary gland samples, when compared with standard
concentrations of virus only. This indicates that under these
experimental conditions, salivary glands do not contain detectable
virus, and that the immunodetection of purified virus is not affected
by the presence of salivary gland samples.

(c) Rate of VTMoV clearance from the gut and haemolymph

An experiment was done to determine the rate and site of virus
loss from C. nicotianae over a period of 9 days. In treatment 1,
nymphs were given access to a virus source plant for 100 min before
being transferred to tomato plants at 25°C. Haemolymph and gut samples
from 20 nymphs were tested for virus immediately after acquisition
(day 1), and thereafter, 20 nymphs were tested for virus antigen after
3, 5, 7 and 9 days using the HRP dot-immunobinding assay. In treatment
2, nymphs fed on a healthy plant for 100 min were transferred to
tomato and 5 nymphs were tested for virus every 2 days as described
above. In treatment 3, nymphs were given access to a virus source for
3000 min and in treatment 4, a control group was fed on a healthy
plant for the same period. Haemolymph and gut samples from 20
"infective" (treatment 3), and 5 "non-infective" (treatment 4), nymphs
were taken every 2 days as described above and tested for VTMoV antigen using the HRP dot-immunobinding assay. Insufficient "infective" nymphs were available for sampling at day 9 and so samples of only 12 and 15 were assayed for the 100 min acquisition and 3000 min acquisition groups respectively.

Gut samples from nymphs which had acquired virus for 3000 min stained more intensely than gut samples from nymphs which had acquired virus for 100 min (Fig. 16). Control samples were negative (Fig. 16). The number of positive haemolymph and gut samples is listed in Table 13.

Table 13: Virus distribution and clearance from *C. nicotianae* placed on tomato for 1 to 9 days, after acquisition for either 100 or 3000 min.

<table>
<thead>
<tr>
<th>Days after acquisition</th>
<th>Acquisition period (min)</th>
<th>100</th>
<th>3000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>haemolymph</td>
<td>gut</td>
<td>haemolymph</td>
</tr>
<tr>
<td>1</td>
<td>6/20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13/20</td>
<td>9/20</td>
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<tr>
<td>3</td>
<td>3/20</td>
<td>5/20</td>
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<tr>
<td>9</td>
<td>0/12</td>
<td>0/12</td>
<td>3/15</td>
</tr>
</tbody>
</table>

<sup>a</sup> The numerator represents the number of samples positive for virus and the denominator represents the total number tested
Fig. 16: HRP dot-immunobinding assay for monitoring virus clearance from the haemolymph and gut over 9 days

A Nymphs given 100 min access to virus infected plants
B Nymphs given 3000 min access to virus infected plants
C Nymphs given 3000 min access to healthy plants

A and B:
Each square represents a sample from 1 nymph and for each column the samples in rows 1 & 6 are from the same nymph and similarly for rows 2 & 7, 3 & 8 etc.
Rows 1-5 represent haemolymph samples and rows 6-10 represent gut samples

<table>
<thead>
<tr>
<th>A</th>
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<tbody>
<tr>
<td>Row</td>
<td>1</td>
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<tr>
<td>1</td>
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<td>9</td>
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<tr>
<td>10</td>
<td>-</td>
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</tbody>
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B

| Row | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | Days after acquisition |
| 1 | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 |
| 2 | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | 3 |
| 3 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 5 |
| 4 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 7 |
| 5 | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | 9 |
| 6 | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | 1 |
| 7 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 3 |
| 8 | + | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | 5 |
| 9 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 7 |
| 10 | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 9 |

C

Each square in rows 1-5, columns 1-5 represents a haemolymph sample from 1 nymph and each square in rows 1-5, columns 6-10 represents a gut sample from 1 nymph.
Samples were taken every 2 days as for A & B and all samples were negative.
Virus standards in row 6, columns 1-4: 0.05, 0.50, 0.95 & 9.50 mg/ml
<table>
<thead>
<tr>
<th>Column number</th>
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<tbody>
<tr>
<td>1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20</td>
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<td>9</td>
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<tr>
<td>10</td>
<td></td>
</tr>
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</table>

**A**

**B**

**C**
No virus was detected in 5 controls from each acquisition time. The number of nymphs with virus in the haemolymph at each acquisition period was not significantly different \( \chi^2_{0.05,4} = 4.821, .25 < P < .50 \), therefore the results at each acquisition period were combined for further analysis. The number of nymphs with virus in the gut at each acquisition time was not significantly different \( \chi^2_{0.05,4} = 2.25, .25 < P < .50 \), therefore the results at each acquisition period were also combined for further analysis. This indicates that although the intensity of staining i.e. the concentration of virus, in samples from nymphs which had acquired virus for 3000 min was greater than for those which had acquired for 100 min, the overall number of samples with virus was not significantly different.

There was no significant decrease in the number of nymphs with virus in their haemolymph over the 9 days \( \chi^2_{0.05,4} = 8.44, .05 < P < .10 \). There was a significant decrease in the number of nymphs with virus in their gut over the 9 days \( \chi^2_{0.05,4} = 54.64, P < .001 \).
2. Distribution of VTMoV in *C. nicotianae*, and its relationship to transmissibility

To test the distribution of virus in the vector, and its relationship to transmissibility, 40 nymphs were given access to a virus source for 3000 min, and at the end of the acquisition period, haemolymph was taken from each nymph and applied to a nitrocellulose filter (Ch.2.IV.10). Instead of being sacrificed for a gut sample, as in section 1 above, nymphs were immediately transferred singly to a test plant for a 2 day inoculation period. The nitrocellulose filter with the haemolymph samples was stored in the dark at room temperature for 2 days. At the end of the inoculation period, 27 nymphs were still alive and each of these was removed from its test plant, killed with ethyl acetate and a gut sample taken (Ch.2.IV.10). Ten nymphs given acquisition access to healthy plants were treated as described above. Samples were tested for virus antigen using the HRP dot-immunobinding assay.

Plants on which the 27 nymphs which survived the haemolymph sampling were placed, and those on which the "non-infective" controls had fed, were observed for up to 1 month for symptom expression. No antigen was detected in the haemolymph or gut of control nymphs and their test plants remained healthy.
The 27 surviving nymphs were grouped according to the site of virus detection and their ability to transmit (Table 14).

Table 14: Classification of nymphs according to site of VTMoV detection and ability to transmit

Total number of nymphs tested = 27

<table>
<thead>
<tr>
<th></th>
<th>Number of nymphs transmitting in each category</th>
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<tbody>
<tr>
<td>Virus detected in haemolymph</td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>8</td>
</tr>
<tr>
<td>no</td>
<td>10</td>
</tr>
<tr>
<td>Virus detected in the gut</td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>11</td>
</tr>
<tr>
<td>no</td>
<td>7</td>
</tr>
</tbody>
</table>

All but 2 nymphs with virus in the haemolymph transmitted VTMoV, and 10 nymphs without virus in the haemolymph transmitted VTMoV. This suggests that either virus does not need to be circulating in the nymph for transmission to occur, or the HRP dot-immunobinding assay was not sensitive enough to detect low levels of virus in the haemolymph. Not all nymphs with virus in the gut transmitted VTMoV. Nymphs were given access to test plants before the gut was tested for virus, so virus may have cleared from the gut during this period. That is, although some nymphs which did not have virus in the gut transmitted VTMoV, they may have contained virus in the gut at the beginning of the inoculation period.
III Conclusions

The HRP-dot immunobinding assay was used to detect low levels of virus in mirid body samples. Detection of virus in haemolymph was limited by the volume extracted from each insect but direct application to the filter allowed antigen detection in sample volumes of about 1 ul. Salivary gland isolation and application to filters was a simple procedure but endogenous peroxidases in the glands limited the use of this assay. Endogenous peroxidases have also been reported in the salivary "sheath material" of the rose aphid and in the haemolymph and saliva of the peanut trash bug (Miles and Sloviak, 1970).

VTMoV was detected in the haemolymph, faeces and gut of infective C. nicotianae. Detection of virus in the gut and faeces is not surprising because infected plant sap accumulates here during feeding, but the detection of virus in the haemolymph confirms that VTMoV circulates in its vector. The results in Fig. 13 show that nymphs with virus in the haemolymph contained virus in the gut, which suggests that virus moves between the gut and the haemolymph. If this was a simple diffusion process, one would expect that most nymphs with virus in the gut would also contain virus in the haemolymph, but this was not the case (Fig. 13). Either there is a more complicated process involved in transport of virus into the haemocoele, as suggested by Gildow (1985) for the transport of BYDV into the haemocoele of the aphid vector, or the HRP dot-immunobinding assay was not sensitive enough to detect low levels of virus in the haemolymph.
It has already been shown by ELISA (Ch.6.2), that more nymphs contained detectable virus than could transmit, so it is possible that the partitioning of virus between gut and haemolymph may be related to transmissibility. That is, mirids may be able to transmit VTMoV only if virus is able to reach the haemolymph, regardless of whether it is in the gut.

VTMoV was not detected in the salivary glands of nymphs. There is some evidence to suggest that VTMoV is transmitted by C. nicotianae in a circulative manner, but such a mechanism of transmission is generally thought to involve the salivary glands, at least with heteropteran vectors which, unlike the coleoptera, do have salivary glands. The lack of detection of virus in the salivary glands of mirids may be interpreted in two ways. Either these insects transmit virus by an undescribed mechanism involving circulation of virus and inoculation to plants in a way that does not involve the salivary glands, or low levels of virus present in the salivary glands were not detected by the immunoassays used here. Since there is no evidence to suggest the salivary glands are involved in transmission, an alternative hypothesis will be investigated in chapter 8, but it is acknowledged that a single mechanism may not explain all the observed transmission parameters.

When clearance of virus from the gut and haemolymph was monitored over 9 days, there was a significant decrease in the number of nymphs with virus in the gut but not in the haemolymph. Virus in plant sap probably moved through the gut at a rate determined by nymph feeding rates. As nymphs fed on virus immune tomato, non-infected sap replaced infected sap in the gut as the latter was defecated. Virus is probably not cleared from the haemolymph by any active "excretion"
process, therefore it is not surprising that clearance is slower and perhaps more random than clearance from the gut.

When transmissibility was related to the presence of virus in the haemolymph and gut, it was found that mirids transmitted VTMoV regardless of whether virus was detected in the haemolymph or not. This cannot be said for the gut since these samples were taken after an inoculation feed, during which time virus may have cleared from the gut. This result shows that virus does not need to be circulating at detectable levels in the haemocoele of the nymph before transmission can occur.

The gut and haemolymph may serve as a reservoir for virus, especially during long-term retention, a phenomenon which has also been suggested for beetle transmission (Harris, 1981a). The mechanisms of beetle transmission bear a resemblance to mirid transmission since virus has been detected in the haemolymph, faeces and regurgitant of beetles but as with mirids, the mechanism for virus transferral to the plant during inoculation is unknown (Fulton et al, 1980). The situation is especially confusing since some non-beetle transmitted viruses have also been detected in the haemolymph, faeces and regurgitant of beetles (see chapter 1, section IV), and similar experiments should be done with non-mirid transmissible viruses to determine their distribution in the mirid.

Although it is unclear how mirids transfer virus to the plant, mechanisms do exist that allow transmission up to 10 days after acquisition. It has been shown that virus remains in the gut and haemolymph for this period. Thus virus can be stored in the mirid for a long period to possibly "supply" virus to a putative transmission pathway which includes feeding at least in the final stages.
VTMoV could not be detected in the salivary glands, so an alternative explanation is required to accommodate the observations made so far. It is possible that insect secretions, specifically faeces, may be one route through which virus is inoculated to the plant. If such a pathway exists, this mechanism of transmission may explain, in part, the observed transmission by a few mirids for up to 10 days. A similar hypothesis has been proposed as one explanation for mite transmission of WSMV. Mites can transmit through a moult and retain infectivity for up to 9 days (Slykhuis, 1965). Virus has been shown to accumulate in the midgut (Paliwal, 1980) and it is thought that one way that the virus may be transmitted is by excretion through the anus and subsequent inoculation through feeding punctures (Paliwal and Slykhuis, 1967). This possibility is tested in chapter 8.
Chapter 8

Contribution of Faeces and Saliva to Infectivity

I Introduction

The similarity between virus transmission by beetles and VTMoV transmission by C. nicotianae, has been discussed in chapter 7. It has been suggested that virus moves from the beetle haemocoel into the midgut (Slack and Scott, 1971; Fulton et al., 1975) and that mouthparts are contaminated by regurgitation (Fulton et al., 1975; Harris, 1981a; Walters, 1969). However Selman (1973) considered that infective regurgitant was not necessarily the natural mechanism of transmission. Harris (1981a) suggested that virus may be passed to the exterior by defecation, but viruses which are acquired but not transmitted, are also detected in beetle faeces and this observation has been taken to indicate that virus in faeces is not correlated with any mechanism of transmission (Fulton et al., 1975). The mechanism by which virus is inoculated to plants by beetles remains unknown.

Eriophyid mites have virus transmission characteristics similar to those described for C. nicotianae. Eriophyes tulipae transmitting WSMV retained infectivity for up to 9 days (Slykhuis, 1965) and could transmit through a moult (Orlob, 1966). Large amounts of intact virus particles persisted in the posterior midgut for up to 5 days after removal of the mites from the virus source (Paliwal, 1980), and transportation of virus from the alimentary canal was thought to involve backflow to the mouthparts during feeding as well as elimination of infective virus through the anus. Virus particles from faeces were thought to enter the feeding
punctures. The action of the anal sucker and the setae which are used by mites to cling to the leaf surface could also cause abrasion to introduce virus (Paliwal and Slykhuis, 1967). While Paliwal (1980) does not rule out this mode of transmission, WSMV was detected in the salivary glands of the mite vector E. tulipae (Paliwal, 1980), which suggests that virus can also be inoculated to plants through the saliva.

Regurgitation of infective plant sap is considered important in virus transmission by aphids and leafhoppers. Leafhopper transmission of noncirculative, semipersistent viruses, such as maize chlorotic dwarf virus and the rice tungro virus(es) are reported to involve an ingestion-ejection mechanism, in combination with the ability of virus to accumulate and persist in the anterior portion of the vector's alimentary canal (Harris and Bath, 1973; Harris et al, 1981). For some of the circulative viruses, the anterior portion of the midgut appears to be a major site of virus accumulation (Shikata and Maramorosch, 1967a; Harris and Bath, 1972) and regurgitation of gut contents might be important in the transmission of some noncirculative, semipersistent, and circulative viruses (Harris and Bath, 1973).

In a review of insect secretions in plants it was reported that regurgitation by plant bugs does not occur (Miles, 1968), but Miles (1968) observed the expulsion of very small volumes of liquid by both Heteroptera and aphids and considered that at least the column of liquid filling the food canal up to the sucking pump can be rejected by plant bugs. Virus transmission studies reported in the literature, have provided some evidence to suggest that these insects can egest plant sap (Ch.1.IV). Although there is some disagreement as to whether
sucking insects can regurgitate at all, it seems unlikely that insects
could regurgitate from as far back as the midgut. For mirid
transmission of VTMoV, a more probable transmission pathway is through
defecation, as suggested for mites and beetles.

Previous experiments have shown that mirids can retain
infectivity for up to 10 days (Ch.4.II.4) and virus has been detected
in gut contents for up to 9 days (Ch.7.II.1c). These observations
cannot be explained by the ingestion: salivation mechanism for
circulative viruses (Harris 1981b), because VTMoV was not detected in
the salivary glands (Ch.7.II.1b). One alternative mechanism for VTMoV
transmission by C. nicotianae is inoculation by contamination of
mouthparts or ingestion, after probing through infective regurgitant
and faeces on the leaf surface. To determine whether such a mechanism
could exist for mirid transmission; the requirement for probing in
transmission, the presence of infective virus in mirid secretions and
excretions, the length of time virus remains infective in the faeces,
the ability of mirids to inoculate plants through infective virus
deposits on the leaf surface, and the time taken for ingested material
to pass through the gut has been investigated.

In testing such a mechanism, the contribution of regurgitant
and faeces to infectivity should be assessed separately and in this
chapter, the contribution of faeces is tested. This is not to suggest
that the regurgitant is considered to be less important in
transmission, but the constraints of time precluded experiments using
regurgitant alone, although in one experiment, the infectivity of
mirid excretions and secretions are tested.
II Experimental

1. The requirement for stylet probing in transmission of VTMoV

An experiment was done to determine whether nymphs that cannot feed, can transmit VTMoV to a test plant, after walking on a virus infected leaf or on a leaf painted with a virus suspension. Nymphs that could feed were allowed to walk and feed on leaves treated as described above, and transmission of VTMoV was tested. These nymphs were included to test the ability of nymphs to acquire and inoculate virus from a healthy leaf painted with virus.

In this section, nymphs which were rendered unable to feed, are defined as "non-feeders" and nymphs which were able to feed normally are defined as "feeders". Non-feeders were prevented from feeding by damage to the labium and stylets. The labium of C. nicotianae is shown in Fig. 17. Infective non-feeders were prepared by giving 30 nymphs a 24 h acquisition period on a virus source plant and then immobilising them on a frigistor block mounted on a microscope stage. The lower section of the labium and stylets of each nymph was removed using a scalpel. To test the effect of the chilling treatment on transmission and survival on feeders, another 25 non-infective nymphs given access to a healthy plant for 24 h, were each immobilised on the frigistor block for 30 sec but the labium was not damaged.
Fig. 17: Mouthparts of *C. nicotianae*

Scanning electron micrograph of a 5th-stage nymph showing the labium which encloses the stylet.

Bar represents 200um.
The treatments used were listed:

Treatment 1:

Three healthy *N. clevelandii* leaves were placed on damp filter paper in a plastic petri-dish and 10 non-infective feeders were placed on the leaves.

Treatment 2:

Leaves from a systemically infected plant with 15 infective non-feeders placed on them.

Treatment 3:

Three healthy leaves painted with 200 ul of a 0.25 mg/ml purified preparation of VTMoV using a camel hair brush, and with 15 non-infective feeders placed on them.

Treatment 4:

Leaves were prepared as for treatment 3, and with 15 infective non-feeders placed on them.

In all treatments, the petri-dishes containing leaves and nymphs were kept for 16 h at 25°C. Single nymphs were then transferred individually to test plants for a 2 day "inoculation" period.

After mirids were transferred to test plants, the excised leaves used in each treatment were stained with erythrosine (Ch.2.I.2) to detect probing punctures on the leaf surface. Any wounds on the leaf surface stained red, including wounds made by forceps, so in addition to probing wounds made by feeders (Fig. 18a), any mechanical damage inflicted by non-feeders could be seen. Leaf surfaces exposed to non-feeders and stained using erythrosine did not show any mechanical damage (Fig. 18b).
Fig. 18: *N. clevelandii* leaf surface stained with erythrosine to show nymph probing punctures

a Probing punctures stained red on a leaf following feeding by nymphs.

b Absence of visible probing punctures on the surface of a leaf following access by nymphs prevented from feeding by removal of the lower section of the labium and stylets.

Bars represent 500 um.
The results shown in Table 15 indicate that no transmission occurred when infective nymphs were unable to feed but had been in contact with virus particles placed on the leaf surface of the acquisition host. Non-infective feeding nymphs which had access to virus particles painted onto a healthy leaf surface, did transmit VTMoV (Table 15). This result also shows that nymphs exposed to chilling on the frigistor block, could still transmit VTMoV.

Table 15: The requirement for probing by mirids, in the transmission of VTMoV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-inoculation access to:</th>
<th>Rate of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeders</td>
<td>Healthy leaf</td>
<td>0/10a</td>
</tr>
<tr>
<td>Non-feeders</td>
<td>Infected leaf</td>
<td>0/15</td>
</tr>
<tr>
<td>Feeders</td>
<td>Leaf coated with virus</td>
<td>9/15</td>
</tr>
<tr>
<td>Non-feeders</td>
<td>Leaf coated with virus</td>
<td>0/15</td>
</tr>
</tbody>
</table>

a numerator indicates number of plants infected; denominator, number of test plants used

The results show that (1) feeding is required for mirid transmission of VTMoV, (2) virus particles contaminating a leaf surface can be acquired by feeding and can then be transmitted to a healthy plant, (3) nymphs having access to virus while unable to feed, did not transmit VTMoV to healthy plants.
2. Presence of infectious VTMoV in the secretions and excretions of *C. nicotianae*

Mirid secretions and excretions were assayed for infectivity by mechanical inoculation to test plants. Although this experiment did not test infectivity of individual components of mirid secretions and excretions, e.g. faeces and saliva, the deposits left on parafilm that had been exposed to mirids were large and yellow-brown, and were probably faecal deposits.

Two hundred mirids were given access to a virus source in groups of 20 insects, for 2 days at 25°C. Ten, 10 cm diameter plastic petri-dishes were each lined with damp filter paper and covered by a sheet of parafilm. Mirids were removed from the virus source and transferred to the petri-dishes (20 mirids per petri-dish) and left for 24 h at 25°C. Forty control mirids which had fed on healthy plants were also maintained in 2 petri-dishes for 24 h.

Yellow-brown deposits were clearly observed on the parafilm from both infective and non-infective groups after 24 h when mirids were removed from the dishes. Forty infective and 10 non-infective mirids selected from this group, were given inoculation access to test plants for 2 days, to determine whether mirids subjected to these experimental conditions were still infective.

To each petri-dish was added 20 ul of 20 mM PB and this was smeared over the parafilm using a paint-brush. The preparation from each petri-dish was mechanically inoculated to 1 test plant per petri-dish, beginning with the controls. The amount of inoculum per petri-dish was only enough to inoculate 4 leaves of 1 test plant.
One test plant died soon after inoculation but all of the remaining 9 plants, inoculated with secretions from infective nymphs became infected. Immunodiffusion tests verified the presence of VTMoV in these plants. Control plants did not show symptoms and there was no reaction to VTMoV antiserum. No control mirids transmitted VTMoV and 14/40 mirids transmitted virus from the infective group.

These results show that the secretions and excretions of C. nicotianae contain infectious virus. It appears that virus can be cleared from the mirid in secretions and excretions, but it is not yet known to what extent clearance of virus from the faeces, for example, contributes to patterns of clearance as detected by ELISA. Nevertheless, the presence of infectious virus in secretions and excretions deposited on the surface of leaves, may contribute to transmission by probing through viruliferous deposits, and this possibility was therefore tested.

3. Transmission from infectious sap deposited on the leaf surface

An experiment was done to test whether non-infective mirids could cause inoculation of plants on which infectious sap had been previously placed. Infectious sap was prepared from 4 systemically infected leaves which had been ground in 100 ul distilled water and the extract filtered through 4 layers of cheesecloth. In treatment 1, 2 leaves of each of 10 test plants were treated by placing 3, 1 ul drops of infectious sap on each leaf and allowing it to dry. Two non-infective nymphs were caged on each plant for 24 h at 25°C. In treatment 2, 5 test plants were treated as described above and maintained without nymphs in the glasshouse. In treatment 3, 2 test
plants were mechanically inoculated with infectious plant sap to confirm the presence of infectious virus. In treatment 4, a healthy sap extract was also prepared as above and deposited on 2 leaves of each of 5 test plants and 2 nymphs were caged on each plant for 24 h at 25°C. In treatment 5, 2 test plants were treated as above but were maintained without nymphs and in treatment 6, 2 plants were mechanically inoculated with healthy sap. Test plants were observed for up to 1 month.

Deposits of infectious sap, in the absence of mirids did not cause infection of test plants, but when nymphs were present 4/10 plants became infected (Table 16). Placing of nymphs on leaves with deposits of non-infectious sap did not lead to transmission of VTMoV (Table 16).

Table 16: Transmission by non-infective nymphs feeding on test plants with deposits of infectious and non-infectious (healthy) sap applied to the leaf surface

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectious sap deposits + nymphs</td>
<td>4/10</td>
</tr>
<tr>
<td>Infectious sap deposits - nymphs</td>
<td>0/5</td>
</tr>
<tr>
<td>Mechanical inoculation of infectious sap</td>
<td>2/2</td>
</tr>
<tr>
<td>Healthy sap deposits + nymphs</td>
<td>0/5</td>
</tr>
<tr>
<td>Healthy sap deposits - nymphs</td>
<td>0/2</td>
</tr>
<tr>
<td>Mechanical inoculation of healthy sap</td>
<td>0/2</td>
</tr>
</tbody>
</table>

a numerator indicates number of plants infected; denominator, number of test plants used
These results indicate that non-infective nymphs can inoculate plants when virus is placed directly on the leaf. This is possibly achieved either by probing through viruliferous deposits or by mechanical inoculation associated with movement in the vicinity of the deposit. It seems unlikely that nymphs could have ingested the sap directly, since droplets appeared to dry on the leaf surface before nymphs were placed on the test plants. To distinguish between transmission by feeding or by mechanical inoculation, an appropriate control would have been to render nymphs unable to feed due to stylet damage, and then to have placed them on a healthy plant with viruliferous deposits on the leaves. If transmission had not occurred under these conditions, then transmission in treatment 1 would have been as a result of probing.

To assess the relevance of this transmission pathway in virus retention and transmission by mirids over long periods, the longevity of virus infectivity in the gut has been investigated. If virus in the gut, as detected by ELISA, was not infective after 5 or 6 days when it is deposited by either regurgitation or defecation, then the transmission pathway proposed here, would not be possible.
4. Longevity of virus infectivity in the gut

Faeces removed from "infective" and non-infective nymphs either immediately after, or 6 days after, acquisition, were assayed for virus infectivity by mechanical inoculation to test plants. To remove faeces, each nymph was first killed in ethyl acetate and then placed under the microscope at 12 x magnification on a 1 cm parafilm square. The abdomen was gently pressed until a faecal drop was deposited on the parafilm. Four nymphs were expressed in this way on each of square of parafilm. Ten ul of distilled water was mixed with the faeces on the parafilm and 5 ul per leaf was mechanically inoculated to each of 2 leaves of a test plant.

In treatment 1, 80 "infective" nymphs were given an acquisition access period of 2 days, and were then transferred to tomato at 10 per plant, and maintained at 25°C for 6 days. At the end of this period, nymphs were collected by aspiration from the tomato plants, and faeces were removed as described, and mechanically inoculated to 20 test plants. In treatment 2, 16 non-infective nymphs fed on healthy plants were treated as described in treatment 1, and their faeces inoculated to 4 test plants. In treatment 3, 40 "infective" nymphs were given an acquisition access period of 2 days, faeces were removed immediately after acquisition as described previously, and inoculated to 10 test plants. In treatment 4, 16 non-infective nymphs were treated as described in treatment 3, and their faeces inoculated to 4 test plants.
To test the effect of temperature on virus infectivity in faeces over 6 days, 80 "infective" nymphs were given an acquisition access period of 2 days, and at the end of this period, their faeces were removed and deposited onto 20 parafilm squares (4 faecal deposits per square) as described. In treatment 5, 10 of these parafilm squares were sealed in plastic tubes and stored at -20°C and in treatment 6, 10 parafilm squares were sealed in plastic tubes containing a piece of damp filter paper and stored at +20°C for 6 days. Four tubes each containing a parafilm square with faeces, from 4 non-infective nymphs, were also stored at -20°C (treatment 7), and 4 tubes at +20°C (treatment 8). For each treatment, and after storage for 6 days, 10 ul of distilled water was mixed with the faeces on each parafilm square and 5 ul inoculated onto each of two leaves of 1 test plant.

Faecal samples collected from "infective" nymphs immediately after acquisition, were infectious (Table 17). Samples collected at the same time but stored at ± 20°C for 6 days, were also infectious (Table 17), indicating that VTMoV remained infective in the vector for at least 6 days. A lower proportion of faecal samples collected from nymphs 6 days after acquisition, contained infectious virus (Table 17).
Table 17: Longevity of virus infectivity in the gut and the effect of temperature on stored virus, as determined by infectivity assay of expressed faecal deposits

<table>
<thead>
<tr>
<th>Nymphs given acquisition access to virus</th>
<th>No. of days after acquisition: that faeces were expressed</th>
<th>No. of days after acquisition: that faeces were stored before assay</th>
<th>Temperature at which faeces kept before assay</th>
<th>Rate of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>6/10(^{a})</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>6</td>
<td>-20</td>
<td>8/10</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>6</td>
<td>+20</td>
<td>10/10</td>
</tr>
<tr>
<td>+</td>
<td>6</td>
<td>0</td>
<td>-</td>
<td>6/20</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>0/4</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>6</td>
<td>-20</td>
<td>0/4</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>6</td>
<td>+20</td>
<td>0/4</td>
</tr>
<tr>
<td>-</td>
<td>6</td>
<td>0</td>
<td>-</td>
<td>0/4</td>
</tr>
</tbody>
</table>

\(^{a}\) numerator indicates number of plants infected; denominator, number of test plants used.

These results show that the virus detected in nymphs by ELISA and by the HRP dot-immunobinding assay would have been infectious for at least 6 days, and possibly up to the 9 day endpoint. As the virus also remains infective when stored at \(+20^\circ\text{C}\), clearance of virus from the insect with time is probably largely due to elimination from the mirid and not to degradation of accumulated virus \textit{in situ}. 


Faeces sampled in this experiment were forcibly expressed from nymphs, so it is not known whether mirids can naturally defecate infectious virus over the same period. An experiment was done to determine the passage time of food through the gut.

5. Defecation behaviour and implications for observed clearance rates

To determine the passage time of food through the gut, nymphs were fed dye through a membrane and faeces were observed over time. The dye solution (0.4% Nigrosin (Sigma Chemical Co., U.S.A.) in 5% sucrose) was fed to 20 fifth-stage nymphs for 24 h at room temperature. Four nymphs were fed 5% sucrose only. Following acquisition, dye could be clearly observed in the gut of 13/20 nymphs (12 x magnification). Each of these 13 nymphs and the 4 controls, were placed singly, in a 4 cm diameter plastic petri-dish lined with damp filter paper. The filter paper was covered by a 2 cm square of nitrocellulose filter and on top of this was placed 3, 4 mm diameter leaf discs (Fig. 19). Nymphs were left for 24 h at room temperature and transferred daily to new petri-dishes for 10 days. Each day, plates were observed at 12 x magnification and faeces were recorded as purple or green (Fig. 19).

The pattern of defecation of dyed food in nymphs, is represented by a histogram in Fig. 20. Control nymphs deposited green faeces only. All nymphs fed on the dye solution, and which defecated on day 1, deposited purple faeces only. On day 2, some nymphs deposited purple only, some deposited green faeces only, and 3 nymphs deposited a mixture of purple and green faeces.
Fig. 19: Passage time of dyed (purple) food through the gut of *C. nicotianae*

Four of the petri-dishes used to contain a single dye-fed nymph for 24 h. Faeces are indicated by arrows. One day after acquisition, only purple faeces were deposited (a). Four days after acquisition, a mixture of purple and green faeces were deposited by some nymphs (b). Five days after acquisition, most nymphs deposited only green faeces (c) and 6 days after acquisition, 1 nymph deposited 1 purple faecal sample as well as green faeces (d).
Fig. 20: Pattern of defecation in nymphs fed dye solution through a membrane.

Numbers above bars represent the number of nymphs not depositing faeces:

- Rectangular bar: Number of nymphs depositing purple faeces only
- Cross-hatched bar: Number of nymphs depositing green faeces only
- White square: Number of nymphs depositing purple and green faeces
From days 3 to 5, most nymphs deposited green faeces, but on each day, 1 or 2 nymphs deposited either all purple or a mixture of purple and green faeces. One nymph deposited a mixture of purple and green faeces on day 6 (Fig. 19d), but from days 7 to 10, all nymphs deposited green faeces only.

Four nymphs deposited green faeces on one day and on the following day deposited a mixture of purple and green faeces. This indicates that gut contents can be mixed in the insect and "first in is not necessarily first out" which may explain in part, why mirids can retain virus in the gut over long periods.

III Conclusions

The results in this chapter show that mirid secretions and excretions were infective when inoculated to test plants and that infectious virus was recovered from faeces 6 days after nymphs left the virus source. The rate of clearance by defecation is similar to the rate of clearance detected in ELISA trials (Ch.6.II.2), supporting the view (Ch.8.II.4), that clearance is generally due to elimination of virus from the gut rather than inactivation of accumulated virus.

Nymphs fed on a dye solution eliminated dye for up to 6 days after ingestion although most nymphs eliminated the dye after 2 days. In a small number of nymphs, elimination of green faeces was followed by elimination of faeces with dye, which indicates that food is well mixed in the gut and can be retained by some nymphs for up to 6 days. Non-successive elimination of ingested material has been reported in other animals, for example, lizards eliminated coloured beads in a different order to that in which they were fed (Christian, personal
communication). Determining the passage time of dyed food can be used to follow the probable movement of virus in the mirid and agrees with the rates of clearance of antigen in nymphs assayed by ELISA and the HRP dot-immunobinding assay. The evidence is consistent with the view that virus is cleared by defecation rather than by inactivation within the insect.

A mechanism of persistence of infectivity can therefore be proposed. Infectious virus in the mirid gut can be retained for up to 6 days after ingestion, and is eliminated during normal feeding and defecation behaviour. As plants which had viruliferous deposits on the leaf surface were inoculated by non-infective nymphs, it is likely that infectious virus in faecal deposits can be inoculated as a result of feeding and movement of the nymphs on the leaf surface.

Although it has been shown that probing is required for transmission by mirids (Ch.8.II.1), inoculation by way of viruliferous faeces may, in part, explain the persistence and intermittent transmission for up to 10 days after acquisition.

The incidence of both virus retention up to 10 days and of defecation of infectious virus up to 6 days, is less than 10% and the time WMoV remains infective on the leaf surface may determine the apparent retention period.

A similar mechanism of transmission has been proposed for the inoculation by aphids of TMV that had been spread on the surface of leaves (Teakle and Sylvester, 1962). Leaves of test plants sprayed with any of 3 noncirculative, nonpersistent viruses (alfalfa mosaic virus, cucumber mosaic virus, or tobacco etch virus), were inoculated when fed on and probed by aphids (Pirone, 1971). Other experiments have shown that aphids can inoculate virus by clawing leaf surfaces sprayed with TMV (Bradley and Harris, 1972; Pirone, 1972; Harris and Bradley, 1973a; Harris and Bradley, 1973b).
Chapter 9
General Discussion

I Characteristics of transmission of VTMOV by \textit{C. nicotianae}

1. Introduction

Virus transmission by a mirid has not been studied in detail before, and a number of unusual features have been observed. The aim of this chapter, is to discuss the results obtained and then propose a likely mechanism of transmission which is consistent with the data.

2. A summary of results obtained using feeding trials and an attempt to categorise the VTMOV-mirid association using accepted criteria.

Table 1 (chapter 1), lists parameters used to categorise virus-vector associations as noncirculative or circulative. Some of these parameters have been tested to categorise VTMOV transmission by \textit{C. nicotianae}. Table 18 lists parameters of VTMOV transmission and the category(s) they fit into. It was found that some parameters were characteristic of a noncirculative association, some were characteristic of a circulative association and others were characteristic of both.
Table 18: Parameters of transmission of VTMoV by C. nicotianae and the category defined by those parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Observation</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>acquisition threshold</td>
<td>less than 1 minute</td>
<td>noncirculative</td>
</tr>
<tr>
<td>acquisition period increases</td>
<td>transmission rate</td>
<td>noncirculative or</td>
</tr>
<tr>
<td></td>
<td>increases</td>
<td>circulative</td>
</tr>
<tr>
<td>inoculation threshold</td>
<td>1 - 2 hours</td>
<td>circulative</td>
</tr>
<tr>
<td>inoculation period increases</td>
<td>transmission rate</td>
<td>noncirculative or</td>
</tr>
<tr>
<td></td>
<td>increases</td>
<td>circulative</td>
</tr>
<tr>
<td>persistence</td>
<td>10 days</td>
<td>noncirculative* or</td>
</tr>
<tr>
<td></td>
<td></td>
<td>circulative</td>
</tr>
<tr>
<td>transstadial transmission</td>
<td>yes</td>
<td>circulative</td>
</tr>
<tr>
<td>virus in haemocoele</td>
<td>yes</td>
<td>circulative</td>
</tr>
<tr>
<td>infective after injection with virus</td>
<td>yes</td>
<td>circulative</td>
</tr>
</tbody>
</table>

* persistence for 10 days may occur in a noncirculative, semipersistent association (Table 1, chapter 1)

It can be seen from Table 18, that the VTMoV-mirid association is not adequately defined by one transmission category alone. Further work was therefore done to determine whether VTMoV propagated in the vector, using infectivity assays and ELISA.
3. Evidence against propagation of VTMoV in C. nicotianae

Infectivity studies showed that mirids stopped transmitting between 5 and 9 days after acquisition and that mirids assayed by ELISA, contained detectable antigen 8 days, but not 9 days after acquisition (Ch.6.II.2).

Using ELISA, it was shown that antigen cleared from mirids 9 days after a 3000 min acquisition period, but after a 10 min acquisition period, antigen cleared from mirids after only 1 day. Thus, persistence seems to be dose dependent and the loss of infectivity and the loss of antigen as detected by ELISA, indicates that VTMoV does not propagate in the mirid vector.

4. Location of VTMoV in C. nicotianae and its relationship to transmissibility

An HRP dot-immunobinding assay permitted detection of antigen in low sample volumes, and antigen was detected in the haemolymph, gut and excreta but not in the salivary glands of individual mirids (Ch.7.II.1ab). Haemolymph and gut samples were assayed from mirids maintained on tomato after acquisition. The number of mirids with antigen in the gut decreased significantly over 9 days however the number of mirids with antigen in the haemolymph did not. The rate of antigen clearance from the gut was similar to that detected in the whole mirid by ELISA, which suggests that the antigen detected in ELISA trials was from the insect gut.
Nymphs with antigen in the haemolymph did not always transmit VTMoV and some nymphs without detectable antigen in the haemolymph transmitted VTMoV. This indicates that either virus does not need to be present in the haemolymph before transmission can occur, or that the detection assay used was not sensitive enough to detect very low levels of antigen in the haemolymph.

Similar results were obtained for the gut, but more mirids contained detectable antigen, as detected by ELISA, than could transmit (Ch.6.II.2). Thus, it is not surprising that nymphs with virus in the gut did not always transmit VTMoV. Samples were taken from the gut after nymphs had fed on healthy test plants so it is possible that nymphs without detectable antigen in the gut, but which were still able to transmit VTMoV, actually lost virus from the gut during inoculation. Thus, it was not possible to determine whether nymphs without detectable antigen in the gut could transmit VTMoV. To overcome this problem excreta could have been sampled before inoculation as a test for gut antigen, however previous assays indicated that some nymphs without detectable antigen in the excreta actually did have antigen in the whole gut, therefore excreta was not considered to be a reliable indicator of antigen in the gut.

Thus, virus was detected in the haemolymph and gut of "infective" mirids. VTMoV was not detected in the salivary glands, but this may have been because the HRP dot-immunobinding assay was not sensitive enough to detect low levels of virus in the salivary glands. Future work could use nucleic acid probes to seek VTMoV sequences at a sensitivity in the range of 1-10 picograms.
Although the VTMoV-mirid association has characteristics of a circulative association (Table 18), it does not appear from the results obtained here, that the ingestion-salivation mechanism of circulative transmission (Harris, 1981b) is operating in this association. Further work on virus in faeces provided information as to the role of virus eliminated by defecation in transmission.

5. Viability and retention of virus in faeces and its relationship to transmission

Secretions and excretions deposited on parafilm by "infective" mirids were infectious when mechanically inoculated to test plants. Faeces expressed from nymphs 6 days after acquisition contained infectious virus. Faeces collected 1 day after acquisition and stored at +20°C for 6 days, also contained infectious virus, which indicates that a temperature similar to that in the mirid did not result in inactivation of the virus. It is concluded that loss of virus from mirids was due to elimination from the gut during defecation rather than to inactivation of accumulated virus.

Mirids prevented from feeding but given access to virus were not able to transmit virus when transferred to test plants. Mirids able to feed normally were able to transmit VTMoV under the same conditions, which suggests that probing is required for transmission of VTMoV.

One ul amounts of infectious plant sap were placed on the leaves of healthy test plants and non-infective mirids were given access to them. Some of these plants became infected, which indicates that mirids can infect healthy plants either by ingesting virus
particles and regurgitating them during probing, by probing through the infectious deposit and inoculating virus from contaminated mouthparts or by mechanical inoculation, resulting from movement in the vicinity of these infectious deposits. Infectious plant sap deposited on the leaf, could be likened to faecal deposition on the leaf surface and these results show there is a pathway for virus transmission from infectious deposits on the leaf surface to the plant itself.

Nymphs fed a dye solution through a membrane, eliminated dye for up to 6 days after ingestion, but most nymphs eliminated the dye within 2 days. ELISA (Ch.6.II.2), revealed a similar pattern of virus loss which is a further indication that virus is lost by defecation and not inactivation.

Thus, infectious virus retained in the mirid gut for up to 6 days after acquisition can be defecated onto the leaf surface during feeding. Viruliferous deposits, that is faecal deposits, on the leaf surface of healthy plants can be inoculated by non-infective nymphs feeding on those leaves.
II Possible mechanisms for the transmission of VTMoV by C. nicotianae

1. Noncirculative transmission

Noncirculative (nonpersistent and semipersistent), virus transmission is thought to result either from direct stylet contamination with virus or from insect regurgitation of virus laden material and subsequent inoculation during feeding; an ingestion-egestion mechanism of virus transmission (Harris, 1977). Noncirculative, semipersistent viruses are thought to resist rapid elimination by accumulating in the vector's alimentary canal and selectively adsorbing or attaching to the cell membrane linings. Virus is subsequently inoculated into plants when the virus-contaminated food material is regurgitated (Harris and Bath, 1973; Harris, 1977).

Garret (1973) showed that aphids can egest material from the foregut during brief probes into plants and Treur and Harris (1976) cited in Harris (1977) found that leafhoppers egest material following prolonged periods of ingestion. Harris (1977) cited other examples of egestion in non and semipersistent viruses but he did not rule out the possibility that a virus which circulates in its vector, may be transmitted by egestion of infectious plant sap, especially since for some of the circulative viruses, the anterior portion of the midgut appears to be a major site of virus accumulation (Shikata and Maramorosch, 1967a; Harris and Bath, 1972). It is not known whether or not aphids can egest material from the stomach during deep feeding probes (Harris, 1977) however Harris and Bath (1973) considered that regurgitation of gut contents might be important in the transmission
of some noncirculative, semipersistent and circulative viruses.

The VTMoV-mirid association has characteristics of noncirculative transmission (Table 18), and the ingestion-ejection mechanism may be important in explaining these characteristics. Although the role of regurgitation in transmission, has not been investigated in this thesis, it may be important in explaining the short acquisition threshold and the increase in transmission rates with increasing acquisition and inoculation times. Retention of infectivity for up to 10 days could be explained by regurgitation of infectious plant sap from the gut, but there is no evidence as yet to support this mechanism for mirid transmission.

An alternative mechanism to explain retention of infectivity is described in the next section, but it is not intended that this be considered a more appropriate explanation than the ingestion-ejection mechanism. It is possible that both mechanisms may be operating in mirid transmission of C. nicotianae.

2. An ingestion-defecation model of mirid transmission as a possible explanation for retention of infectivity

VTMoV transmission by C. nicotianae has some characteristics of circulative transmission (Tables 19). Unlike transmission of the luteoviruses for example, reviewed by Gildow (1987), VTMoV was not detected in the salivary glands of mirids. The virus does not propagate in its vector, but it can be retained for over a week in the insect and can be transmitted intermittently for up to 10 days. Detection of virus in the gut and haemolymph of some nymphs for up to 9 days indicates that these sites could act as
reservoirs for long term infectivity.

Although more sensitive assays may be able to detect virus in the salivary glands of *C. nicotianae*, a transmission mechanism consistent with the results found in this study cannot implicate the salivary glands in transmission. Insufficient evidence has been obtained to propose a transmission mechanism which would account for all of the observed characteristics of mirid transmission, however a model can be proposed to provide a basis for future experiments.

An ingestion-defecation model of insect transmission is proposed here as one explanation for the virus retention and extended transmissibility phase of the mirid-virus association, in which the salivary glands appear not to be implicated. Virus is ingested in plant sap during feeding and accumulates in the gut where it does not propagate but is retained for up to 9 days. It also enters the haemocoele, and virus in the gut and haemolymph "resist" elimination and serve as a reservoir for long-term transmission.

Evidence supporting this model is as follows: mirids can defecate membrane-fed dye up to 6 days after ingestion and therefore virus may also be retained in the gut for long periods before it is eliminated during normal defecation. Once in the gut, virus is infectious and may remain so after it is defecated onto the leaf surface. The duration of infectivity of virus in faeces outside the insect may determine the maximum time over which mirids can transmit VTMoV.

On the leaf surface, virus may be inoculated as mirids probe faeces during feeding, but it is not known whether mirids do this by ingesting infectious faeces and then regurgitating them during inoculation or by contaminating the mouthparts while probing. It is
unlikely that mirids could inoculate plants by walking over the leaf, since non-feeding mirids given access to virus, could not then inoculate a healthy test plant (Ch.8.II.1).

Transstadial transmission (Ch.4.II.5) may be consistent with this mechanism, if virus in the midgut was retained during ecdysis and could be inoculated after elimination in faeces. Paliwal and Slykhuis (1967), considered that accumulation of virus in the midgut may account for transstadial transmission by mites since the linings of the midgut are not shed during moulting and thus the virus reservoir is maintained.

Transmission by mirids following injection may occur if there is a pathway of virus elimination from the haemolymph through the gut wall and into the gut lumen.

The intermittent nature of transmission over 9 days is similar to the intermittent defecation pattern of dye-fed nymphs (Ch.8.II.5).

As discussed in the previous section, the acquisition and inoculation thresholds are characteristic of a noncirculative, semipersistent association and cannot be explained using this model. Mirids can acquire sufficient virus in less than one minute to transmit and can inoculate virus in 2 h. For both acquisition and inoculation, the rate of transmission increases with time. The WTV-mirid association has characteristics of both a noncirculative and circulative mechanism of transmission and is perhaps best explained by both the ingestion-excretion (Harris, 1977), and ingestion-defecation models of transmission.
III Virus-vector associations that are similar to the WMoV-C nicotianae association

Beetle transmission has some characteristics in common with mirid transmission and although virus has been localised in beetle vectors, the transmission mechanisms are unknown. Virus has been localised in the regurgitant, haemolymph, gut and faeces of beetles (Walters, 1969; Harris, 1981a), however non beetle-transmissible viruses have also been detected in the haemolymph and faeces of beetles (Harris, 1981a). Although beetle larvae do not transmit through a moult, virus can be detected in the regurgitant after injection (Harris, 1981a), and beetles can transmit intermittently for up to 20 days (Freitag, 1956; Walters and Henry, 1970).

Slack and Scott (1971) recovered virus from the beetle gut up to 8 days after acquisition and on one occasion found virus in the midgut 20 days after acquisition. The authors proposed that due to the 19 day retention period, virus in the haemolymph may act as a reservoir for sporadic and long-term periods of transmission. Walters (1969) suggested that virus in the haemolymph is introduced into the digestive tract for regurgitation and this could account for occasional long-term transmissions.

Harris (1981a) proposed that virus in the beetle haemocoele could exit the vector and become available for transmission in a number of different ways. It may pass into the gut lumen and subsequently be passed to the exterior by regurgitation or defecation or both and faecal contamination of plant areas damaged by feeding would seem possible.
VTMoV is both beetle (Greber, 1981) and mirid transmissible, i.e., by both chewing and stylet-feeding insects, therefore it is possible that the gut-virus association between a beetle and a mirid may be the same.

A similar mechanism of transmission has been proposed by Paliwal (1980) for mite transmission of WSMV. Mites can retain infectivity for up to 9 days (Slykhuis, 1965) and can transmit through a moult (Orlob, 1966). Virus was detected in the midgut 5 days after acquisition (Paliwal, 1980) and in the hindgut 4 days after acquisition (Takahashi and Orlob, 1969). Paliwal (1980) clearly outlined the possible modes of transmission by mites and suggested that although the virus is circulative and is mainly inoculated to the plants via salivary secretions during feeding, other modes of transmission are possible.

Paliwal and Slykhuis (1967) proposed that virus which accumulated in the mite gut may flow back towards the mouthparts and be introduced into plant cells during feeding. However due to the large concentration of virus particles in the hindgut some of the virus may be defecated and introduced into the leaf cells through feeding punctures, as described here for the mirid, or through the action of the anal sucker and the setae.

Orlob (1968) reported that a tetranychid mite defecating high levels of TMV did not inoculate the plant during feeding, but when purified TMV was sprayed on the leaves, mites inoculated the plants. Paliwal (1980) noted that tetranychids defecate a solid waste which is not easily smeared on a leaf surface, but the eriophyids, like mirids, defecate a liquid waste which is easily smeared on the leaf surface.
IV Problems associated with accepted criteria for categorising virus-vector associations

A classification system proposed by Watson and Roberts (1939), categorised transmitted viruses as nonpersistent, semipersistent or persistent depending on how long vectors retain virus. One major problem with this classification system is that retention time for a given virus-vector combination can vary with both temperature and vector feeding behaviour (Harris, 1977).

Kennedy et al. (1962) used the system in which viruses were classified as stylet-borne or circulative, and viruses which multiplied in their vectors were termed circulative-propagative. One problem with this system is that there is no category for semipersistent viruses (Harris, 1977).

Harris (1977), categorised aphid-borne viruses as noncirculative (nonpersistent and semipersistent) and circulative (persistent). Circulative includes circulative-propagative and noncirculative is an inclusive term to encompass stylet-borne transmission and an ingestion-ejection mode of transmission.

The use of ingestion-ejection (Harris, 1977) and ingestion-salivation (Harris, 1981b) is a useful classification for both aphid and leafhopper-borne viruses. These terms however relate to stylet-bearing insects and are inappropriate for mandibulate insects such as beetles (Harris, 1981a). However, even some aphid-borne viruses are not transmitted in a way that falls into any of these categories. Examples of this are dahlia mosaic virus (Brierly and Smith, 1950) and cauliflower mosaic virus (Caldwell and Prentice, 1942). Chalfant and Chapman (1962) referred to this atypical mode of
transmission as bimodal.

Harris (1977), states that no single mechanism is likely to be valid for all vector-virus-plant combinations and an example which illustrates this, is his ingestion-egestion hypothesis in which transmission is linked with a factor as flexible and unpredictable as aphid probing behaviour. The proposed mechanism of transmission of VTMoV by the mirid also includes unpredictable events such as acquisition, defecation and probing behaviour linked to a noncirculative transmission mechanism.

A more appropriate classification system may be one in which viruses are classified as noncirculative or circulative and within each category, a range of transmission mechanisms are accommodated. This system would better reflect the flexibility and unpredictability inherent in biological systems, especially those in which a pathogen, host and vector are interacting.

V Concluding remarks

The VTMoV-mirid association is complex and further work is required before a verifiable transmission mechanism can be proposed. A number of parameters should be investigated to determine whether they can be explained by the ingestion-defecation model.

Transstadial transmission - virus localisation in newly moulted mirids to determine accumulation sites, especially in the midgut. The presence of virus in the midgut would indicate that there is a source of infectious faeces following moulting.

Injection of virus into the haemocoele - assay for virus in the gut of mirids which have been injected with virus, to determine
whether there is an elimination pathway of virus from the haemolymph to the gut. If such an elimination route existed, injected virus could accumulate in the gut and be defecated onto the leaf surface.

Localisation of virus in the mirid - to determine the relationship between transmission and the presence of virus in the gut and haemolymph, mirids could be given access to a virus that they don't transmit, and then this virus could be localised in the mirid.

Inoculation of infectious faeces - mirids may inoculate infectious deposits on the leaf surface in a number of different ways. Mirids may ingest virus on the leaf surface and regurgitate it during feeding, and in so doing inoculate the plant, or they may inoculate plants mechanically by walking over these deposits. One experiment to study this, would be to place deposits of infectious sap onto the leaves of a healthy plant and then put mirids that cannot feed, onto these plants. If such mirids transmitted VTMoV, then this would suggest that virus can be inoculated mechanically. To test whether mirids actually ingest virus from these deposit, or if they just contaminate their mouthparts, mirids given access to deposits of virus on the leaf surface could be assayed by removing the head and legs and assaying the whole body. If virus was not detected in the gut i.e. body, one could suggest that inoculation was through probing wounds or mechanical inoculation.

The significance of other transmission mechanisms also require further investigation. For example, it is possible that an ingestion-ejection mechanism may be involved in mirid transmission which may account for the short acquisition periods and the intermittent transmission over 10 days. To study this further, experiments concerning probing behaviour are required. For example,
mirids could be given access to a labelled solution through a membrane and then given access to an unlabelled sucrose solution also through a membrane. Label in the second solution would indicate that mirids were able to regurgitate during feeding.

The possibility that the salivary glands are involved in transmission by mirids also requires further investigation. Techniques such as electron microscopy and molecular hybridization with nucleic acid probes could be used to examine the salivary glands for the presence of low levels of virus.

While the ingestion-defecation model does not answer all aspects of the VTMoV-mirid association, it provides direction for further experiments. Transmission models should not only accommodate the unpredictable nature of transmission but should also provide the basis for approaching old problems in new ways. Virus-vector associations are complex and may require a number of flexible models to explain them. The ingestion-egestion and ingestion-defecation mechanisms may be just two of a number of models required to explain the transmission of VTMoV by *C. nicotianae*. 
Appendix

Published Work:

Isolation of velvet tobacco mottle virus capable of replication with and without a viroid-like RNA

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Isolation of Velvet Tobacco Mottle Virus Capable of Replication with and without a Viroid-like RNA

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Field isolates of velvet tobacco mottle virus (VTMoV) induce severe symptoms in Nicotiana clevelandii and encapsidate viroid-like RNA reported to be essential for virus infection. An isolate of the virus producing only mild symptoms on N. clevelandii and devoid of viroid-like RNA has now been isolated from a plant inoculated by a single viruliferous Cyrtopeltis nicotianae, the mirid vector. However, after adding viroid-like RNA isolated from normal VTMoV to the inoculum, the new isolate was shown to support the synthesis of, and encapsidate the viroid-like RNA, thereby reverting to virulence characteristic of the normal isolate. This indicates that the viroid-like RNA can behave as a satellite RNA of VTMoV. The data are discussed in relation to previously published conclusions that viroid-like RNA is essential for the infectivity of VTMoV.

Velvet tobacco mottle virus (VTMoV) (1), solanum nodiflorum mottle virus (SNMV) (2), lucerne transient streak virus (LTSV) (3), and subterranean clover mottle virus (SCMoV) (4) have many properties characteristic of Sobemoviruses (5). However, in addition to a linear single-stranded (ss) RNA (RNA 1) of about 4500 nucleotides, each of the viruses also encapsidates circular (RNA 2) and linear (RNA 3), viroid-like RNAs between 324 and 388 nucleotides long (6, 7, J. Haseloff and R. H. Symons, personal communication).

Gould et al. (8) reported that infection of plants by VTMoV and SNMV was dependent on the presence of both RNA 1 and RNA 2 in the inoculum. Moreover, they observed that heterologous mixtures of the RNAs from VTMoV and SNMV were non-infective. Contrary to this, Jones and Mayo (9) were able to infect plants with highly purified SNMV RNA 1 and showed that the infected plants contained virus particles devoid of RNAs 2 or 3. They also showed that addition of RNA 2 had no significant effect on the infectivity of RNA 1, but the progeny virus contained RNAs 1, 2, and 3. These data support the view that RNA 2 of SNMV is a satellite (sat)-RNA. It has also been shown that RNA 2 of LTSV is a sat-RNA (10). Moreover, LTSV RNA 2 lacks specificity in that it can also use as its helper virus either SNMV (11), sowbane mosaic virus (12) or southern bean mosaic virus (13). We now report the isolation of a VTMoV devoid of RNA 2 which can, however, support the replication of and encapsidate the viroid-like RNA.

VTMoV induces local necrotic lesions and severe mosaic symptoms on inoculated and systemically infected Nicotiana clevelandii A. Gray leaves, respectively (1). However, in the course of an experiment on VTMoV transmission by its mirid vector, Cyrtopeltis nicotianae, (Konigsberger) we observed that one N. clevelandii plant infected with the virus (isolate R17), on which a single insect had fed, developed unusually mild mosaic symptoms. Upon mechanical transmission to other N. clevelandii plants, virus isolated from this plant did not produce any lesions on the inoculated leaves, and only mild mosaic was observed on the systemically infected leaves.

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The new virus isolate (K1 VTMoV) was purified by our standard procedure (5) and shown to be serologically indistinguishable from wild-type VTMoV (R17 isolate) in immunodiffusion tests (1). However, when K1 VTMoV RNA was isolated by phenol extraction and subjected to polyacrylamide gel electrophoresis in the presence of urea (14), no viroid-like RNA was detected. When K1 VTMoV was passaged a further six times in N. clevelandii by mechanical inoculation over a period of 4 months, the mild symptoms remained unchanged and no viroid-like RNA was detected in virus preparations analyzed after each passage.

In order to determine if K1 VTMoV could support the synthesis of viroid-like RNA from wild-type (R17) VTMoV, the following experiment was done. RNA from purified virus of both the K1 and R17 isolates of VTMoV were prepared by phenol extraction (14). RNA 2 from a portion of the R17 VTMoV RNA was separated by preparative polyacrylamide gel electrophoresis in the presence of urea (14) to yield a source of viroid-like RNA devoid of RNA 1. Although only the RNA 2 bands were excised from the gels, the preparations were shown to also contain substantial amounts of RNA 3 but no RNA 1 when reelectrophoresed and stained with toluidine blue.

Four inocula were prepared from the RNAs and inoculated to groups of eight N. clevelandii plants as indicated in Table 1.

Three leaves of each plant were inoculated with 2 μl of the appropriate preparation. Results of the experiment (Table 1 and Fig. 1) show that the plants inoculated with K1 VTMoV RNA alone (inoculum A), all developed mild symptoms characteristic of that virus isolate. The plants inoculated with viroid-like RNA alone (inoculum B), all remained healthy. However, the plants inoculated with a mixture of K1 VTMoV RNA 1 and the viroid-like RNA (inoculum C; containing 1/10 the concentration of viroid-like RNA to that in inoculum B), all developed necrotic lesions and severe mosaic indistinguishable from that on plants inoculated with R17 VTMoV RNA (inoculum D).

The viruses from plants in treatments A, C, and D (Table 1) were purified, the RNAs were isolated by phenol extraction and analyzed by polyacrylamide gel electrophoresis. Viroid-like RNA was detected in virus from treatments C and D but not from treatment A (Fig. 2).

The data presented above demonstrate that K1 VTMoV RNA 1 can replicate without the aid of viroid-like RNA to produce virus particles indistinguishable from those of the wild-type virus. It has also been shown that the RNA 1 can support the replication and encapsidation of the viroid-like RNA from wild-type virus resulting in increased virulence. Thus it appears that the viroid-like RNA behaves like

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<th>Inoculum*</th>
<th>Symptoms induced:</th>
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<td>Necrotic lesions and severe mosaic*</td>
</tr>
<tr>
<td>A K1 VTMoV RNA (50 μg/ml)</td>
<td>0/8</td>
</tr>
<tr>
<td>B R17 VTMoV RNAs 2 and 3 only* (250 μg/ml)</td>
<td>0/8</td>
</tr>
<tr>
<td>C K1 VTMoV RNA (50 μg/ml) + R17 VTMoV RNAs 2 and 3 (25 μg/ml)</td>
<td>8/8</td>
</tr>
<tr>
<td>D R17 VTMoV RNAs 1, 2, and 3 (50 μg/ml)</td>
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* Three leaves of each plant were inoculated, each with 2 μl of the RNA preparation indicated.
* Symptoms produced by all previously known isolates of VTMoV including the R17 isolate used in this study.
* Symptoms produced by VTMoV isolated from a plant infected by a single mirid (Clytus pallidus) described in this paper (K1 isolate).
* Numerator indicates the number of plants with symptoms and denominator, the number of plants inoculated.
* RNAs separated from VTMoV (R17 isolate) by polyacrylamide gel electrophoresis (8).
a sat-RNA (15). It would seem that the K1 virus isolate arose from a VTMoV particle or particles transmitted by the mirid vector which contained only RNA 1. To confirm this, attempts were made without success to generate a virus isolate like K1 VTMoV by inoculating plants with preparations of RNA 1 purified from R17 VTMoV RNA by repeated cycles of gel electrophoresis. The failures probably stem from the difficulties of completely eliminating RNA 2 from RNA 1 preparations because of its presence in capsids as a series of multimers (16) as well as its extremely high specific infectivity. In preliminary experiments, we have found that addition of as little as 300 pg/ml of purified RNAs 2 and 3 to inoculum of K1 VTMoV RNA resulted in infections characteristic of wild-type virus (unpublished results).

From data presented in this paper we conclude that RNA 2 of VTMoV is a sat-RNA which is consistent with data concerning the relationship of a number of viroid-like RNAs encapsidated by other Sobemoviruses (9–13). However, it is contradictory to that reached in our laboratory by Gould et al. (8) whose data indicate that RNA 1 cannot replicate without RNA 2. This conclusion was supported by two types of experiments. In the first, VTMoV RNAs 1 and 2 separated by electrophoresis in gels were inoculated alone or in combination on opposite half-leaves of N. clevelandii. Necrotic lesions were observed only on half-leaves inoculated with a mixture of both RNAs. Observations reported here (Figs. 1 and 2) indicate that lesion production is a function of RNA 2 and hence absence of lesions following inoculation with RNA 1

**Fig. 1.** Appearance of *N. clevelandii* plants 12 days after inoculation with RNA preparations from VTMoV isolates as described in Table 1. (A) K1 VTMoV RNA (50 μg/ml). (B) R17 VTMoV RNAs 2 and 3 only (250 μg/ml). (C) K1 VTMoV RNA (50 μg/ml) + R17 VTMoV RNAs 2 and 3 (25 μg/ml). (D) R17 VTMoV RNAs 1, 2, and 3 (50 μg/ml). (Inoculation details are described in text and Table 1.) Note the prominent lesions on the inoculated leaves of plants C and D and their severely distorted young leaves.
alone, should not have been interpreted as a lack of infection. However, the results of the other type of experiment done by Gould et al. (8) cannot be dismissed as not supporting their conclusion and its significance remains unexplained. N. clevelandii plants were inoculated with RNA 1 alone, RNA 2 alone, and with a mixture of RNAs 1 and 2. The plants were observed for symptom development and nucleic acid extracts were probed with the appropriate cDNAs for the presence of RNAs 1 and 2. All the plants inoculated with RNAs 1 and 2 produced typical VTMOV symptoms and both RNAs were readily detected. All the plants inoculated with RNA 2 alone remained symptomless and neither RNA 1 nor RNA 2 was detected. However, whereas the majority of the plants inoculated with only RNA 1 failed to produce symptoms, a few did. Moreover, neither RNA 1 nor RNA 2 was detected in the symptomless plants but both RNAs were shown to be present in those with symptoms. It was concluded that those plants which did become infected, did so because the RNA preparations were contaminated with traces of RNA 2. Slight contamination was indeed confirmed by $R_{ot}$ analysis (8).

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