STUDIES ON A *NICOTIANA* HYBRID INFECTED WITH A PLANT RHABDOVIRUS

BY

IMAD DAWWOUD ISMAIL

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University of Glasgow

Department of Botany

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DEDICATION

I would like to record my sincere gratitude to my wife, Hanaa, and to my children, Dima & Sahmyr, for their continued patience, encouragement and good spirit as always and especially during the period of my research. To them this work is dedicated.

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LIST OF GENERAL ABBREVIATIONS

APS	Ammonuim persulphate
ATP	Adenosine triphosphate.
BLCV	Beet leaf curl virus.
BNYV	Broccoli necrotic yellows virus.
BSA	Bovine serum albumin.
BYSMV	Barley yellow striate mosaic virus.
cDNA	Complementary DNA.
CEV	Citrus exocortis viroid.
CGMMV	Cucumber green mottle mosaic virus
c.p.m.	Counts per minute
d	Days.
dATP	2'-Deoxy-adenosine-5'-triphosphate.
dCTP	2'-Deoxy-cytidine-5'-triphosphate.
dGTP	2'-Deoxy-guanosine-5'-triphosphate.
dTTP	2'-Deoxy-thymidine-5'-triphosphate.
DI	Defective interfering.
DMSO	Dimethylesulphoxide.
DNA	Deoxyribonucleic acid.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetraacetic acid.
EGTA	Ethyleneglycol-bis(β-amino-ethyl ether)N,N'-tetraacetic acid.
ELISA	Enzyme-linked immunosorbent assay.
EM	Electron microscopy.
EMDV	Eggplant mottle dwarf virus.
g	Gravity.
GTP	Guanosine triphosphate.
h	Hours.
HEPES	(N-2-Hydroxyethylpiperazine-N'-2-ethanesulforic acid.
HMW	High molecular weight.
KD	Kilodalton.

Kb	Kilobase.
Kbp	Kilobase pair.
LNYV	Lettuce necrotic yellows virus.
LV	Long particle.
m	Minutes.
mRNA	Messenger RNA.
MW	Molecular weight.
NC-ELISA	Nitrocellulose-ELISA.
NCMV	Northern cereal mosaic virus.
PAGE	Polyacrylamide gel electrophoresis.
PEG	Polyethylene glycol.
Poly(A)$^+$RNA	Polyadenelated RNA.
PVCV	Pittosporum vein clearing virus.
PSTV	Potato spindle tuber viroid.
PVP	Polyvinylpyrrolidone.
PYDV	Potato yellow dwarf virus.
RNA	Ribonucleic acid.
RNP	Ribonucleoprotein.
r.p.m.	Revolutions per minute.
rRNA	Ribosomal RNA.
RTYV	Rice transitory yellowing virus.
SCV	Strawberry crinkle virus.
SDS	Sodium dodecyl sulphate.
SV	*Sonchus* virus.
SYNV	Sonchus yellow net virus.
SYVV	Sowthistle yellow vein virus.
TEMED	N'N'N'N'-Tetramethylethylenediamine.
TCA	Trichloroacetic acid.
TRV	Tobacco rattle virus.
TYMV	Turnip yellow mosaic virus.
VSV	Vesicular stomatitis virus.
WSMV	Wheat striate mosaic virus.
WSSMV	Wheat spindle striate mosaic virus.

LIST OF ABBREVIATIONS FOR THE FIGURE LEGENDS OF THE ELECTRON MICROGRAPHS

CH	Chloroplast.
CW	Cell wall.
CY	Cytoplasm.
ER	Endoplasmic reticulum.
FR	Fibrillar ring.
IE	Inner nuclear envelope.
M	Mitochondrion.
MB	Microbody.
N	Nucleus.
NU	Nucleolus.
OE	Outer nuclear envelope.
P	Plasma membrane.
Pd	Plasmodesmata.
PL	Plasmalemmasomes.
S	Starch.
SV	Vesicle.
T	Tonoplast.
V	Virus particle.
VN	Nucleocapsid.
VP	Viroplasm.
GB	Golgi body
IC	Intracellular space

ERRATA

For inner or outer nuclear envelope read inner or outer nuclear membrane throughout.

SUMMARY

The morphology of Sonchus yellow net virus (SYNV) has been studied by electron microscopy in both negative stained and ultrathin sectioned samples. Depending on the stain, bullet-shaped or bacilliform particles could be observed. However, the incubation of grids containing the samples with anti-SYNV antiserum prior to negative staining preserved the integral bacilliform morphology of the virus particles.

The yield of the virus during the purification-steps was estimated by enzyme-linked immunosorbent assay (ELISA). The loss of the virus, using the standard procedure for purification was about 24%.

Systemic movement of SYNV to leaves and roots was first detected by protein immunoblotting and ELISA 24 h after mechanical inoculation. Virus levels rose to a maximum ten days after inoculation: the highest levels, between 4.0 and 7.3 μg/g tissue, were in leaves which were not yet fully expanded. Electron microscopy of tissue sections revealed that when the virus content of tissues was greatest, virtually all leaf and root cells were infected. Most of the virions were in the perinuclear space, with only a few scattered particles in the cytoplasm. Nuclei contained large viroplasms associated with viral nucleocapsids, the matrix of these viroplasms reacted strongly to anti-SYNV antiserum in immunogold labelling experiments. Between 10 and 20 days after inoculation, levels of virus antigen and viral RNA fell to about 20% of their maximum. By 20 days after inoculation, no more than 10% of cells contained virus particles and almost all the virions were within the cytoplasm. Virions were almost never observed in most tissues of plants infected for longer than 60 days. These results suggest that SYNV spreads systemically until most or all cells are infected. The plants then undergo a recovery phase during which virions disappear from the nuclei of infected cells and vesiculate into the cytoplasm.

The effects of SYNV on the nucleus, chloroplasts, mitochondria, endoplasmic reticulum, plasma membranes and the cell walls in mechanically inoculated *N. edwardsonii* were studied at

various times after inoculation. Immunogold labelling was used to localize the viral protein(s) in infected cells.

During the acute phase of the virus infection, nuclei showed obvious abnormalities, most strikingly, the development of nuclear viroplasms containing viral nucleocapsids and granular or fibrillar matrix. The association of the nucleocapsids with viroplasms and the strong reaction, in immunogold labelling experiments, of gold particles to these viroplasms suggest that viroplasms are the sites of nucléocapsid assembly.

Chloroplasts of infected cells exhibited a number of ultrastructural abnormalities. In immunogold labelling experiments, antiserum to purified SYNV bound extensively to the thylakoids and stroma of chloroplasts from infected cells at all stages of infection, but not to the vesicles or inclusion bodies. Mitochondria of infected cells also exhibited a number of ultrastructural abnormalities. Neither nucleocapsids nor virus particles were observed in association with diseased mitochondria and in immunogold labelling experiments, no label was bound to mitochondria. Changes in the endoplasmic reticulum (ER) network were observed. Nucleocapsids and virus particle were associated with this ER.

Infected cells showed alteration in the plasma membranes including the formation of plasmalemmasomes, multivesiculated plasmalemmasomes and plasmalemmasome-like structures. Tubular channels interconnecting adjacent cells and often containing virions developed. Similar channels containing nucleocapsids were observed within the nucleus and interconnecting the nucleus and the cell wall. Immunogold labelling indicated the presence of viral proteins associated with the cell wall or associated structures. These channels may be involved in movement of virus from cell to cell.

Virus particles were not detectable by electron microscopy in chronically infected plants. However, virus proteins G & N plus a novel immunologically cross-reacting polypeptide of 41 KD (p41) were detectable in immunoblots. Immunogold labelling experiments revealed the presence of considerable quantities of free virus protein in the nucleus and cytoplasm. In leaf discs labelled with ^{35}S-methionine, synthesis, *in vivo*, of all four virus structural proteins was detectable. Proteins N, M1 & M2 were detected in the *in vitro*

translation products of poly(A)$^+$mRNA from these plants. Thus proteins G & N accumulate in leaves but M1 & M2 fail to do so, presumably as a result of rapid turnover or specific degradation. The origin of protein p41 is not clear. It may be an additional non-structural viral protein, a modified form of one of the other structural proteins or a cross-reacting host protein induced by chronic infection.

Plants were examined by electron microscopy 5 months after inoculation with SYNV. No virions were observed in leaf or root cells, but cells in sections of calyx contained large numbers of virus particles. Most particles were only 73-86% of the length of standard SYNV but reacted with anti-SYNV antiserum in immunogold labelling. Plants inoculated with sap extracted from calyx became systemically infected but exhibited chlorotic mottling, instead of the normal vein-clearing symptoms. Most virus particles in these plants were short, and when purified, sedimented more slowly than standard SYNV. Purified short particles were not infective, but plants inoculated with a mixture of short and standard particles developed mottling symptoms and yielded predominantly short particles. Proteins from short particles were electrophoretically and antigenically identical to those from standard virus. RNA from short particles was about 77% the size of RNA from standard SYNV and hybridized to cloned SYNV cDNA. These short particles have all the characteristics of defective-interfering particles.

When plants were infected using inocula derived from chronically infected plants, nucleocapsids were observed within chloroplasts. Western blots of protein from chloroplasts isolated from these plants revealed the presence of the virus nucleocapsid protein N and possibly protein L.

CHAPTER 1

INTRODUCTION

1.1 THE VIRUS, HISTORY, DEFINITION, AND CLASSIFICATION:

Historically, the earliest known pictorial records of virus-infected plants are the broken tulips, often depicted in paintings from the 17th century Dutch school of art (Stevens, 1983). However, the science of plant virology may be thought to have originated in 1886 when Adolf Mayer described a mosaic disease of tobacco and demonstrated that the mosaic symptoms could be transferred to healthy tobacco by rubbing with sap from the mosaic plants. Ivanowsky (1892) showed that the disease producing agent retained activity even after passing through a bacteria proof filter; he identified the pathogen as a "toxin-producing entity" Beijerinck (1898) repeated and expanded the work of Ivanowsky by showing that the mosaic agent multiplied in plant tissue and could not therfore be a toxin; he named the agent a "contagium vivum fluidum". For more details on the history of viruses see Matthews (1981) and Gibbs and Harrison (1976).

As more information has accumulated concerning the chemical and physical characteristics as well as replicative features of viruses, so changes have taken place in the definition of a virus. Bawden (1964) defined a virus as an obligate parasitic pathogen with dimensions of less than 200nm. Matthews (1981) defined a virus as follows: a virus is a set of one or more nucleic acid template molecules, normally encapsidated in a protective coat or coats of protein or lipoprotein, which is able to organize its own replication only within a suitable host cell. Within such cells virus productions is (1) dependent on the host's protein synthesizing machinery, (2) organized from pools of the required materials rather than by binary fission and (3) located at sites which are not separated from the host cell content by a lipoprotein bilayer membrane. Stevens (1983)

defined the viruses as submicroscopic particles made of one or more pieces of a single species of nucleic acid (RNA or DNA), surrounded by proteins, these particles replicate alone or in the presence of similar structures, but only in living cells, using at least some of the host cell enzymes.

With increasing numbers of viruses, virologists found themselves in need of system of nomenclature and classification. Early workers generally gave a virus a name derived from the host plant in which it was found and the most conspicuous disease symptoms. However, by the early 1930s virologists faced the fact that different strains of viruses can exist, each of which may cause very different symptoms in the same host plant. Different viruses may cause very similar symptoms on the same host plant and some diseases may be caused by a mixture of two unrelated viruses. Several systems have been used in the past to classify viruses (Johanson, 1927; Johanson & Hoggan, 1935; Smith, 1937; Holmes, 1939; Lwoff *et al.*, 1962). However, at the International Congress for Microbiology (1966), an organization was set up for developing an internationally agreed taxonomy and nomenclature for all viruses. The organization is now known as the International Committee for Taxonomy of Viroses (I.C.T.V) and meets at each International Congress For Virology to vote on new taxonomic proposals, (see reports by Wildy, 1971; Fenner, 1976; Matthews ,1979, 1982). In addition several other attempts have been made to overcome classification problems (Bellett, 1967a, b; Gibbs, 1968, 1969; Gibbs & Harrison, 1968).

Grouping is still mainly based on particle morphology and size, but further criteria are whether the nucleocapsids are naked or enveloped, the number of virion types and of genome fragments (multipartite

viruses or viruses with split genome), and type (RNA or DNA) and strandedness of nucleic acid (single or double stranded). On this basis plant viruses are, at present, classified into 28 groups (Matthews, 1982), (table 1-1). For further details on virus classification see Lwoff & Tournier (1971), Gibbs & Harrison (1976), Edwardson & Christie (1978), Kurstak (1981), Hamilton *et al.* (1981), Matthews (1981), Bos (1983) & Stevens (1983).

Table 1-1 shows plant virus classification with some properties

Group name	Type member	Particle shape	Nucleic acid Type
Tobravirus	tobacco rattle virus	E	sR
Tobamovirus	tobacco mosaic virus	E	sR
Hordeivirus	barley stripe virus	E	sR
Potexvirus	potato virus X	E	sR
Carlavirus	carnation latent virus	E	sR
Potyvirus	potato virus Y	E	sR
Closterovirus	beet yellows virus	E	sR
Maize chlorotic dwarf virus		I	sR
Tymovirus	turnip yellow mosaic	I	sR
Tombusvirus	tomato bushy stunt	I	sR
Sobemovirus	southern bean mosaic	I	sR
Tobacco necrotic virus		I	sR
Luteovirus	barley yellow dwarf	I	sR
Comovirus	cowpea mosaic virus	I	sR
Nepovirus	tobacco ringspot virus	I	sR
Pea enation mosaic virus		I	sR
Dianthovirus	carnation ringspot virus	I	sR
Cucumovirus	cucumber mosaic virus	I	sR
Bromovirus	brome mosaic virus	I	sR
Ilarvirus	tobacco streak virus	I	sR
Alfalfa mosaic virus		B	sR
Plant rhabdoviruses (Rhabdoviridae family)	lettuce necrotic yellows	BEd	sR
Phytobunyaviruses**	tomato spotted wilt virus	PEd	sR
Plant reovirus, (Reoviridae family)			
* phytoreovirus genus	wound tumor virus	I	dR
* fijivirus genus	fiji disease virus	I	dR
Geminivirus	maize streak virus	I	sD
Caulimovirus	cauliflower mosaic	I	dD
Furovirus	soil born wheat mosaic	E	sR

E : elongate B : bacilliform R : RNA D : DNA
I : isometric Ed: enveloped P : pleiomorphic
s : single stranded
d : double stranded. * : subfamily ** : Haan & Peters (1987).

1.2 RHABDOVIRIODAE FAMILY:

1.2.1 Introduction:

This family of bacilliform, enveloped viruses is of particular interest because members infect both animals and plants and are generally transmitted by arthropods, (Hummeler, 1971; Wagner, 1975; Brown *et al.*, 1979; Matthews, 1981). Assignment of viruses to the taxon of rhabdoviruses was originally based entirely on particle morphology. However, this classification is supported by up-to-date biochemical studies which reveal remarkable uniformity among these structurally similar viruses isolated from extremely diverse hosts. Wagner (1975) stated six important characteristics of rhabdoviruses:

1 - Rhabdoviruses are rod-shaped particles which vary considerably in length (60 - 400 nm) but are of fairly uniform width (60 - 85 nm).

2 - Animal rhabdoviruses tend to be bullet-shaped in appearance, flat at one end and a tapered sphere at the other (but see section 1.2.2.2). Plant rhabdoviruses are usualy bacilliform in shape, quite elongated and with two round ends.

3 - All rhabdoviruses appear to be surrounded by a membranous envelope with protruding spikes. All these viruses contain lipids and are, therefore, susceptible to disruption by ether and detergent.

4 - The nucleocapsid inside the envelope of rhabdoviruses is a ribonucleoprotein (RNP) core which gives the appearance of striations when viewed by electron microscopy. All rhabdoviruses examined contain one

molecule of single-stranded RNA which is not by itself infectious and does not serve as a messenger. Therefore, rhabdoviruses are generally classified along with the myxoviruses, paramyxoviruses and bunyaviruses as negative-strand viruses.

5 - Many, if not all, rhabdoviruses contain an RNA-dependent-RNA polymerase (transcriptase) as part of the nucleocapsid, which renders it infectious in the absence of the envelope.

6 - A common characteristic of animal rhabdoviruses, conceivably also of plant rhabdoviruses, is the frequent occurence of defective interfering particles (DI) which are noninfectious because a considerable segment (one third to two-thirds) of the RNA genome is deleted. This will be discussed later (section 1.7).

So far, two genera in the family have been defined; vesiculovirus and lyssavirus, with vesicular stomatitis virus (VSV) and rabies virus as their respective type species; none of the plant infecting members has been assigned to either genus (Matthews, 1979), although Peters (1977) subdivided the plant rhabdoviruses into two groups on the basis of site of assembly. Rhabdoviruses can be divided into two groups on the basis of their host range. Those that infect animals and those that infect plants. The two groups share many morphological, physical and biochemical properties (Hummeler, 1971; Francki, 1973; Wagner, 1975; Francki & Randles, 1980; Francki *et al.*, 1981).

Table 1-2 summarizes and compares the basic morphological properties of the most extensively studied of two animal and two plant

rhabdoviruses (VSV and rabies; lettuce necrotic yellows (LNYV) and , potato yellow dwarf (PYDV) viruses respectively).

Table 1-2 Morphological properties of rhabdoviruses .

Property	VSV	Rabies	PYDV	LNYV	Reference
Morphology	Bullet*	Bullet	Bacilli-form	Bacilli-form.	a/b/c/d
Dimensions (nm)	175x68	180x75	290x75 ts 179x73 ns	227x66 ts 360x52 ns.	a/b/c/d/e
Ribonucleo-capsid width (nm)	47	40	40	35	f/b/g/h
Cross-striation periodicity.	4,5	4,5	5,5	4,5	b/g/d/.
Surface projection length (nm)	10	6-7	+	6	a/b/e/g.

+ = Present . ns = Negative stains ts = thin section.
a = Howatson & Whitmore, (1962) . e = Chambers *et al.*, (1965)
b = Hummeler *et al.*, (1967) . f = Howatson, (1970).
c = MacLeod *et al.*, (1966) . g = MacLeod, (1968).
d = Harrison & Crowley, (1965). h = O'Loughlin & Chambers, (1967)
* = but see section (1.2.2.2).

Although, there seem to be significant differences in the length of the virions, Knudson (1973) justified this discrepancy by the fact that the plant rhabdoviruses are bacilliform, However, if the bullet-shaped virions that are occasionally seen in preparations of PYDV are measured, the dimensions obtained are compatible with these generally quoted for VSV. Perhaps cross-striation periodicity, nucleocapsid width and surface projection length are not points of particular significance, but they reinforce the notion that,

morphologically, these viruses are very similar. It should be mentioned here that, vesicular stomatitis virus also has true bacilliform particles (Ornstein *et al.*, 1976) and that bullet-shaped particles seen in purified preparation are most likely the result of artefacts induced by fixation procedure.

Table 1-3 summarizes and compares some physical properties of plant and animal rhabdoviruses. The sedimentation coefficients suggest that the plant rhabdoviruses are about one-third larger, whereas the molecular weight of the virions imply that they are twice as large. The buoyant densities of the viruses are the first indication that they may be chemically similar. Plant rhabdoviruses (e.g. LNYV and broccoli necrotic yellow virus (BNYV)) share with animal rhabdoviruses (e.g. VSV) the presence of an RNA-dependent-RNA polymerase internal to the virus envelope.

Table 1-4 summarizes and compares some chemical properties of animal and plant rhabdoviruses. Rhabdoviruses, as complex viruses contain lipid and carbohydrate as well as the usual viral constituents, nucleic acid and protein. The lipid composition for VSV and PYDV has been reported, and for VSV its composition is host-dependent reflecting the plasma membrane of the host (McSharry & Wanger, 1971). The nucleic acid of rhabdoviruses is single stranded RNA and comprises less than 3% of the mass of the animal rhabdovirus, but this ratio is about 1% for plant rhabdoviruses (Peters, 1981). Wagner *et al.* (1972) tried to standardize the nomenclature for the structural proteins of rhabdoviruses, (their recommendation is followed in table 1-4). The molecular weight of the structural proteins as assessed by sodium dodecyl sulphate-polyacrylamide gel

electrophoresis (SDS-PAGE) for VSV, rabies, PYDV, and LNYV are shown (Table 1-4) .

Table 1-3 Physical properties of animal and plant rhabdoviruses.

Property	VSV/a,b.	Rabies/a,b.	PYDV/c,d.	LNYV/e,f,g.
VIRIONS				
Sedimentation coefficient(s)	625	600	880	945
Buoyant density (gm/cm^2)	1.18	1.20	1.17	1.20
NUCLEOCAPSIDS				
Sedimentation coefficient(s)	140	200	250	260
Infectivity.	Yes	Yes	Yes	Yes
RIBONUCLEIC ACID.				
Sedimentation coefficient.	36-45	45	45	43
Infectivity.	No	No	No	No
Molecular weight($x10^6$ dalton)	3.6-4.5	4.6	4.6	4.2

a = Francki, (1973).
b = Wagner, (1975).
c = Black, (1970).
d = Reeder *et al.*, (1972).
e = Francki & Randles, (1970)
f = Chambers *et al.*, (1965).
g = Francki & Randles, (1973)

Table 1-4 Chemical properties of animal and plant rhabdoviruses .

Property		VSV	Rabies"	PYDV	LNYV	Reference
CARBOHYDRATE		13%	+	+*	*	a/b/c.
LIPID.		20%	+	20%*	+*	a/d/e/f/g
PROTEIN.						
Percentage.		64	nr	nr*	nr*	a
Structural	L	161	+	+	171	h/h/i/c.
	G	64	58.5	78	71	
	N	52	50.5	56	56	
	NS	42		-	38	
	M	24			19	
	M1		33	33		
	M2		23	22		
NUCLEOCAPSID .						
Type.		RNA	RNA	RNA	RNA	j/k/l/m.
Strandedness.		Single	Single	Single	Single	

* = Plant rhabdoviruses contain 70% protein, (Peters, 1981)
25% lipid,
4% carbohydrate,
1% single stranded RNA. .

+ = Present. - = Not present. nr = Not reported .
a = McSharry & Wanger, (1971). h = Wagner, (1975) .
b = Sokol *et al.*, (1971) . i = Francki & Randles, (1981).
c = Knudson & MacLeod, (1972) . j = Huang & Wanger, (1966).
d = Kuwert *et al.*, (1968) . k = Sokol *et al.*, (1969).
e = Sokol *et al.*, (1972) . l = Reeder *et al.*, (1972).
f = Ahmmed *et al.*, (1964) . m = Francki & Randles, (1972).
g = Harrison & Crowley, (1965).
" = protein mol. wts. based on sequencing data (Tordo *et al.*, 1986).

L =Large protein, may not be an aggregate or precursor of the other proteins and possibly associated with nucleocapsid .

G = Glycosylated protein, probably the spike protein .

N = Nucleoprotein, binds to the RNA and thus represents the major structural protein of the nucleocapsid .

NS = Minor nucleocapsid protein, originally thought to be non-structural protein associated with VSV infections, but evidence now suggests that it is a constituent of the nucleocapsid.

M = Matrix, or membrane protein, either represents a protein that helps to bind the ribonucleocapsid to the envelope or functions as a constituent of membrane.

Rhabdoviruses may be subgrouped as plant and animal rhabdoviruse. However, Peters (1977) outlined a system for rhabdoviruse classification based on a number of genetically inherited stable characters which cannot be influenced by the host such as the presence of proteins L and NS, the presence of one or two matrix proteins (M or M1+M2), the involvment of the nucleus in the replication of virus, and the occurrence of a detectable transcriptase activity. On this basis the family may be divided into two subgroups. Those viruses which contain two M proteins (M1 + M2), no detectable defined minor proteins (L + NS) or transcriptase activity, and which involve the nucleus in the replication constitute one subfamily with rabies virus as the type member. The plant viruses PYDV, sowthistle yellows vein (SYVV), Sonchus yellow net (SYNV), and eggplant mottle dwarf (EMDV) may be members of this group . The other group is formed by VSV as type member with LNYV, BNYV and sonchus viruses (SV) being virus members infecting plants. This group has one M protein and a L and NS proteins, contains transcriptase activity, and the nuclei seem not to be involved in their replication. Recent studies (Tordo *et al.*, 1986) have indicated that the rabies M1 protein is phosphorylated and appears to be analogous to the NS protein of VSV in function and gene location. This may be true for the other virus within the subgroup.

1.2.2 The Plant Rhabdovirus Group:

1.2.2 Member of the group, Geographical Distribution and Host Range:

The number of known plant rhabdoviruses has increased from 16 confirmed and 3 possible members of the group listed by Francki (1973) to 38 confirmed rhabdoviruses plus 30 possible members listed by Peters (1981) and 43 members by Jackson *et al.* (1987). Because of the scanty data available, there can be no assurance that the same virus has not been entered under different names just because it has been observed in cells of a different host plant. It is true that in many descriptions of plant rhabdoviruses, differences in reported particle dimensions are given as evidence that two viruses are distinct. However, because an individual measurement of rhabdovirus particles can be misleading. Francki and Randles (1980) considered that the morphology of two rhabdoviruses in negatively stained preparations can be taken as distinct only when significant differences are evident in a mixture of the viruses.

Plant rhabdoviruses and rhabdovirus-like viruses have been reported from most parts of the world including tropical, subtropical and temperate regions Peters (1981). Some viruses such as maize mosaic (MMV), raspberry vein chlorosis (RVCV) and strawberry crinkle (SCV) are fairly widespread. Many individual rhabdovirus, such as Sonchus yellow net virus (SYNV) seem to have restricted distribution; this probably reflect the distributions of their vectors.

The host range of most individual members are narrow. However rhabdoviruses, in general, infect a wide of plants including both

monocotyledons and dicotyledons (Francki, 1973; Peters, 1981; Jackson, 1981; Jackson *et al.*, 1987). There is direct evidence that some plant rhabdoviruses multiply in their vectors. This is probably a characteristic of all members of the group.

1.2.2.2 Morphological Properties and Structure :

Although two morphologically distinct types of particles, bacilliform and bullet-shaped are normally found, both in thin sectioned and negatively stained preparations, it is generally agreed that the true morphology of the mature forms are bacilliform (Howatson, 1970; Hummeler, 1971; Knudson, 1973; Francki, 1973; Martelli & Russo, 1977a; Francki & Randles, 1980; Francki *et al.*, 1981; Jackson *et al.*, 1987). Hence bullet-shaped elements are either immature variants in various stages of development, or they originate from the bacilliform ones as a consequence of preparative artifacts (Martelli & Russo, 1977a). RTYV represents a noteworthy exception, for its particles are reported to be dominantly bullet-shaped both in the host cells and in free preparations. Only occasional bacilliform virions can be observed (Chen & Shikata, 1971). In this respect, RTYV comes close to animal rhabdovirus whose particle shape was thought to be bullet-like (Wanger, 1975). However, evidence has been obtained that animal rhabdoviruses e.g. VSV also have true bacilliform particles (Ornstein *et al.*, 1976) It should be mentioned that particle morphology may easily be altered during manipulation, in particular in negative stain mounts for electron microscopy. Striking examples of particle variability due to preparative artefacts have been reported for LNYV, and the explanation of their possible origin have been given (Wolanski & Fracki, 1969; Fracki, 1973). Preparative

methods designed to preserve particle integrity have been used to study the real morphology of plant viruses (McLeod, 1968; Peters & Kitajima, 1970; Ahmed *et al.*, 1970; Lin & Campbell, 1972; Russo & Martelli, 1973).

The size of the particles varies a great deal not only among different members of the group but also for the same virus, depending on methods of measurement and sample preparation for electron micrscopy. However, rhabdovirus particles appear smaller in sections of infected cells than in negatively stained preparations, presumably due to shrinkage during fixation and embedding, and flattening during air drying. Since there appear to be a number of unknown factors able to affect particle size during various preparative procedures, it is difficult to make valid comparisons between published morphometric data (Francki, 1973). Despite this, Francki and Randles (1980) reported larger particles in thin section for LNYV, WSMV and BNYV reflecting the difficulty in determining absolute sizes. However, particles are normally between 200 and 350 nm long and betwwen 70 and 95 nm in diameter and sediment at 1000 to 1200S (Peters, 1981).

Structurally, a typical rhabdovirus particle consists of an outer envelope enclosing a long strand of nucleoprotein, the nucleocapsid, wound into a helix of low pitch (fig. 1-1a). The envelope is a membrane with projections. Electron microscopy of negatively stained, or sectioned rhabdoviruses reveals that the surface projections line the particle contour and protrude 6-10 nm from the envelope (Martelli & Russo, 1977a; Francki *et al.*, 1981). There is evidence for VSV that the projections are not superficial structures but are associated with the nucleocapsid, thus penetrating the whole thickness of the enveloping membrane (Brown *et al.*, 1974).

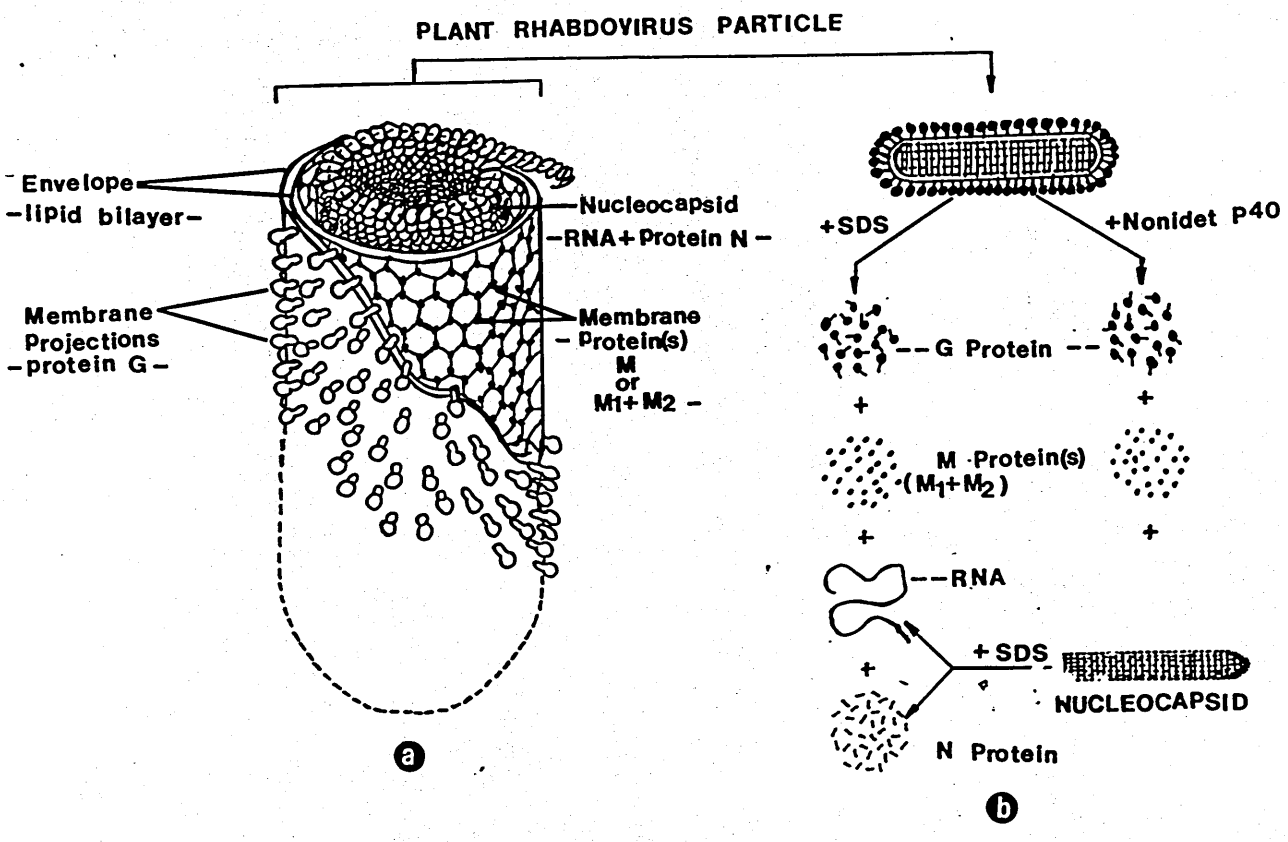


Figure 1-1:

(a) Model of plant rhabdovirus particle cut open to show the various internal structural components. (b) Disassembly of (a) with SDS and Nonidet p40 detergents (from Francki *et al.*, 1981).

The spikes are hexagonally arranged and cover the whole surface of the virion (Francki, 1973; Hull, 1976), they are composed of glycoproteins (protein G), each projection possibly being made up by two glycopolypeptides (Hull, 1976). In VSV the projections seem to represent the only source of glycosylated proteins (Wagner, 1975), whereas this may not apply to SYVV, where L protein is also reported to be glycosylated (Ziemiecki & Peters, 1976a,b). It is conceivable that protein G is the primary source of glycopolypeptides in both animal and plant rhabdoviruses (Martelli & Russo, 1977a). The surface projections in animal rhabdoviruses (eg. VSV) are involved in the initiation of infection, G protein seems to be required for particle attachment to the plasma membrane of the host cells (Wagner, 1975). A similar function was postulated for plant rhabdoviruses with regard to their attachment to the plasma membrane of insect vector cells in which they multiply (Francki, 1973; Gaedigk *et al.*, 1986; Adam & Gaedigk, 1986) but their function with regard to plant host is unknown since *in vitro* enzymatic cleavage of spikes does not appear to destroy infectivity towards plants (Adam & Gaedighk, 1986).

The viral envelope of plant rhabdoviruses varies depending on the site of maturation. Some of the viruses derive their envelopes from the inner membrane of the nuclear envelope, some from the endoplasmic reticulum, and yet others from cytoplasmic viroplasms induced by infection (Francki & Randles, 1980; Francki *et al.*, 1981). The envelope constitutes a bilayered membrane about 10nm thick (Martelli & Russo, 1977a) when viewed by electron microscopy in section or negative stain. Virus particle can be structurally studied by stepwise disassembly with non-ionic detergents such as Nonidet-P40, and ionic detergents such as sodium dodecyl sulphate (SDS) (Fig.

1-1b) followed by fractionation of the products on polyacrylamide gel electrophoresis. Chemical analysis of PYDV, WSMV, MMV and SYNV has indicated the lipid content to be about 20, 24, 40 and 18% of the weight of the particle, respectively (Ahmed *et al.*, 1964; Sinha & Becki, 1972; Lastra & Acosta, 1975; Selstam & Jackson, 1983). The viral-envelope protein consist of the matrix (M) protein (type I), which may occur as two species M1 and M2 (type II) with different molecular weight. Recent studies indicate that in the case of rabies virus (type II) there is only one matrix protein (M2), and M1 is found to be a phosphoprotein analogous to NS (Tordo *et al.*, 1986). This is most likely the case for type II plant rhabdoviruses, since the sequencing data of Heaton *et al.* (1987) and Heaton (personal communication) on the genomic RNA of SYNV showed that the gene for SYNV-M2 protein maps at the same position for phosphoproteins of the animal rhabdoviruses VSV-NS and rabies-M1. The matrix protein is believed to form a tubular structure, a hexamer layer surrounding the nucleocapsid, on which it exerts a stabilizing fuction (Knudson, 1973; Hull, 1976) .

The nucleocapsid, a nucleoprotein helix forming a hollow cylinder constitutes the internal component of rhabdoviruses. The nucleic acid is a molecule of single-stranded RNA with molecular weight ranging from 4.0 to 4.6×10^6 Daltons (Francki & Randles, 1980; Francki *et al.*, 1981). It has been reported that the ss-RNA of WSMV has molecular weight of only 2.2×10^6 Daltons (Sinha *et al.*, 1976). Francki and Randles (1980) state that this data needs confirmation before it is accepted since it can be calculated that in order to code for the four WSMV proteins, an RNA with molecular weight of at least 2.8×10^6 Daltons would be required (Stevens & Lee, 1977).

Estimates of the percentage of RNA vary a great deal and were reported to be 0.6% for PYDV (Knudson, 1973), 5% for WSMV (Sinha & Becki, 1972) and 0.47% for SYNV (Jackson & Christie, 1977). The viral RNA is believed to be negative stranded and not infectious by itself, although its complementerity to mRNA has been directly demonstrated only for SYNV (Milner & Jackson, 1979). The presence of an RNA-dependent RNA polymerase (transcriptase) associated with the virion capable of transcribing the viral RNA *in vitro* has been characterized and shown to be an integral part of VSV (Wagner, 1975; Bishop & Flamand, 1975). In the case of VSV an active transcriptase complex involves the nucleocapsid protein N, the NS phosphoprotein and the L transcriptase; in the case of rabies the M1 protein is apparently fulfill an analogous function to NS. Enzymatic activity has been convincingly demonstrated in preparations of LNYV (Randles & Francki,1972; Francki & Randles, 1973; Toriyama & Peters, 1981) and BNYV (Toriyama & Peters, 1981). In the case of SYNV a type II plant rhabdovirus, there have been reports of low levels of transcriptase activities, but they have not been independently confirmed (Peters *et al*, 1978). Since both PYDV and SYNV have many characteristics in common with LNYV, it would appear that either these viruses contain a transcriptase activity which is difficult to detect *in vitro*, or they must depend for their replication on enzymes from their host plants (Francki & Randles, 1980; Stevens, 1983). The detection of a viral mRNA probably coding for L protein in SYNV-infected tobacco (Milner & Jackson, 1983; Rezaian *et al.*, 1983) supports the former supposition.

Nucleocapsid protein N is a structural protein tighty bound to the RNA filament. If the envelope is removed *in vitro*, the

conformation of the nucleocapsid changes to yield a narrower structure (Wolanski *et al.*, 1967; Peters & Kitajima, 1970; Conti & Plumb, 1977). A model for this transition has been reported by Francki (1973). The helical organisation of the nucleocapsid in the intact particle is responsible for the typical cross striations seen in rhabdovirus particles in negative stain or in sections which have been appropriately stained. The precise arrangement of the nucleocapsid strand and the structure of the envelope at the hemispherical ends of rhabdovirus particles are not yet clear and various possibilities have been suggested (Francki, 1973; Peters & Schults, 1975; Hull, 1976).

Electron micrographs of plant rhabdoviruses in cross section show a series of concentric rings corresponding to the nucleocapsid and envelope with its projections (Francki, 1973). A central electron-dense spot is also usually seen. However, Francki (1973) states that there is no evidence of structure internal to the nucleocapsids, and that central darkly-staining region in cross-sections are artefactual.

1.2.2.3 Purification of Plant Rhabdoviruses:

Most purification procedures for plant viruses involve clarification of crude plant extracts either by heating at 55°-60°C or by treatment with organic solvents which coagulate cell membranes and proteins (Francki, 1972). These procedures cannot be used for the purification of plant rhabdoviruses as they have thermal inactivation temperatures around 50°C and have envelopes that are readily destroyed by organic solvents. The rapid loss of infectivity of rhabdoviruses *in vitro* contributes to difficulties in their purification (Francki,

1973; Jackson *et al.*, 1987). Moreover, the concentration of plant rhabdoviruses in infected plants is usually lower than that of many other plant viruses (Matthews, 1981; Jackson *et al.*, 1987).

A variety of methods, for purification of plant rhabdoviruses, have been used (Francki, 1973; Jackson *et al.*, 1987). These procedures share several points, heating and organic solvents are avoided, all are carried out at temperature of 0 to 4°C, crude plant extracts are clarified through a celite pad and sucrose gradients are used as an extra purificatory step.

The yield of purified virus varies from virus to virus, and from purification to purification of the same virus. Yield depends on the host or cultivar, the age of the plants at the time of inoculation, the environmental conditions and the time after inoculation at which the leaves were harvested (Jackson & Christie, 1979).

1.2.2.4 Serology of Plant Rhabdoviruses:

Antisera have been prepared against at least 17 members of the group (Jackson *et al.*, 1987). These antisera have been used for several purposes such as to study the relationship between plant rhabdoviruses, detection of the virus in plants or vectors and identification of viruses in infected plants or vectors. Techniques used have included gel-diffusion test, ring precipitin test and enzyme-linked immunosorbent assay (ELISA).

Up-to-date information gained by serological tests have been well discussed and documented (Jackson *et al.*, 1987), and the serology of plant rhabdoviruses has been discussed in a number of

reviews (Martelli & Russo, 1977a; Francki, 1973; Francki & Randles, 1980; Francki *et al.*, 1981; Peters, 1981).

Recently, Adam *et al.* (1987) have used two different serological techniques electro-blot-immunoassy and immunosorbent electron microscopy to compare three different isolates of EMDV Serological tests of plant rhabdoviruses should be expanded to cover all other members of the group in order to investigate the relationships between them.

1.2.2.5 Replication of Plant Rhabdoviruses:

This topic will be discussed later in REPLICATION STRATEGY section 1.4.2.

1.3 SONCHUS YELLOW NET VIRUS:

Sonchus yellow net virus (SYNV) was originally found in central and south Florida, USA infecting Sowthistle (*Sonchus oleraceous*) and *Bidens pilosa* (Christie *et al.*, 1974). Symptoms were distortion and general yellowing of leaves. Subsequently SYNV-infected lettuce (*Lactuca Sativa*) with bright yellow interveinal spotting of old leaves has been found in Florida (Falk *et al.*, 1986). SYNV has not been reported elswhere. Strains of SYNV have not been reported.

Christie *et al.* (1974) were the first to transmit the virus, both by an aphid (*Aphis coreopsidis*), and mechanically, to several dicotyledonous hosts such as, *Nicotiana edwardsonii* (*N. clevelandii X N. glutinosa*) which was shown to be most susceptible to infection with sap, *S. oleraceous, B. pilosa, N. glutinosa, N. clevelandii, Zinnia elegans* and *L. sativa* by triturating naturally infected leaf with a reducing agent (0.5% Na_2SO_3). Repeated attempts to transmit SYNV to Turkish Tobacco (*N. tabacum* L.), *Datura stramonium* L. *Gomphrena globosa, Chenopodium quinoa*, and *C. amaranticolor* failed. Subsequently *C. quinoa* (Jackson & Christie, 1977), *C. amaranticolor* (Van Beek *et al.*, 1985b) and *N. benthamiana* have been shown to be hosts. More recently cowpea protoplasts have been shown to support replication of SYNV when infected in the presence of polyethylene glycol as a mediator (Van Beek et al., 1985a, 1985b, 1986). There is no evidence that SYNV can infect the intact cowpea plant.

SYNV was first purified by Jackson and Christie (1977) using celite pad filtration and sucrose density gradient centrifugation. The yield of purified virus was 200-445 µg/100g fresh weight of leaves

(Selstam & Jackson, 1983). However it was very dependent on plant age at inoculation, light, temperature and time after inoculation. The infectious preparation of SYNV particles sedimented at 1044S in linear-log gradients, banded at 1.183g/ml in sucrose-equilibrium density gradients, and the molecular weight of the virion estimated from size and density, was about $9x10^8$ (Jackson & Chrictie, 1977).

The SYNV virion is a bacilliform particle measuring 94 x 248 nm after fixation in glutaraldehyde and negative staining. The particle itself has internal cross-striations with a periodicity of 4.1nm and an outer envelope through which 6nm long surface prejections protrude (Jackson & Christie,1977). SYNV consists of RNA, proteins, lipids and is thought, like other rhabdoviruses, to have carbohydrate associated with G protein (Jackson,1978).

Following disruption of SYNV particles with SDS, the nucleic acid sedimented in sucrose gradients at 44S and had a molecular weight of 4.42 x 10^6 (13 kb) as estimated by polyacrylamide gel electrophoresis (Jackson & Christie, 1977). Several lines of evidence have demonstrated the single strandedness and negative sense of SYNV RNA

(i) SYNV RNA is susceptible to ribonuclease (RNase) under high ionic strength conditions, (Jackson & Christie, 1977).

(ii) SYNV RNA can act as a template for cDNA synthesis (Rezain *et al.*, 1983)

(iii) SYNV RNA hybridizes to polyribosomal RNA from tobacco infected with SYNV (Milner & Jackson, 1979; Milner & Jackson, 1983).

(iv) SYNV RNA is neither infectious nor translatable *in vitro* (Milner et al., 1979).

The RNA comprises only about 0.47% of the mass of the virion assuming that virions of about 9 x 10^6 daltons contain a single copy of RNA (Jackson & Christie, 1977).

Purified SYNV virions contain four major and some minor electrophoretically distinguishable polypeptides (Jackson, 1978). A similar electrophoretic pattern was reported by Dale and Peters (1981) using different electrophoretic conditions, but with some small differences in molecular weight. Recent sequencing studies of the SYNV genome by Zuidema *et al.* (1987) and Heaton *et al.* (1987) have demonstrated the true sizes of N protein and M2 protein to be 50.641 and 38.332 KD respectively. The published sizes of SYNV-proteins reported by Jackson (1978), Dale and Peters (1981) and Van Beek *et al.* (1986) are shown in table 1-5.

Table 1-5 Reported sizes of SYNV-proteins.

Reported by	Proteins(a)				
	HMW	G	N	M1	M2
Jackson (1978).	+	76.8	63.8	45.5	39.5
Dale & Peters, (1981)	+	82	59	34	31
Van Beek *et al.* (1986)**	+	82	56	41	35
from sequencing studies	*	*	53.6'	*	38.3"

a= Nomenclature according to Wagner, *et al.*, (1972).
HMW=High molecular weight. ** = see section (1.4.2).
+= present. * = MW not determined. a = MW x 10^{-3}.
'= Zuidema *et al.* (1987). "= Heaton *et al.* (1987).

A recent abstract (Heaton *et al.*, 1987) reports the presence of six open reading frames on the SYNV genome, the sixth possibly coding for an additional non-structural polypeptide with no homologue in the animal rhabdoviruses.

SYNV particles have been reported to contain in addition to the four major structural proteins, several high molecular weight proteins, of which one is presumably the L protein, and a protein with an electrophoretic mobility slightly faster than that of N protein (Jackson, 1978; Dale & Peters, 1981). The G protein is reported to be glycosylated (Jackson, 1978; Dale & Peters, 1981; Van Beek *et al.*, 1986), and M1 protein is reported to be phosphorylated (Van Beek *et al.*, 1986).

A recent study of the lipid composition of SYNV (Selstam & Jackson, 1983) showed that the lipid fraction represents about 18% of the virion. Table 1-6 shows the lipid composition of purified SYNV particles. SYNV-lipids comprise 62% phospholipids, 31%sterols and 7% triglycerides. Selstam and Jackson (1983) reported that, phospholipids are the major constituents of the polar lipid fraction consists of phosphatidyl, choline, ethanolamine, serine, inositol and glycerol, in molar ratios of approximately 13:5:3:3:1 respectively, plus a small amount of an unidentified acyl lipid. The fatty acids of SYNV were more unsaturated than those of animal rhabdoviruses such as VSV. These differences probably reflect differences between the plant and animal membranes from which the viral envelopes are derived (Selstam & Jackson, 1983; Harwood, 1980; Mazliak, 1977; Compans & Klenk, 1979).

Table 1-6 The lipid composition of SYNV particles.

Lipid	Viral protein* (μg/ml)	Weight percentage of total	Viral protein (nmol/mg)	Mole percentage of total
Total phospholipids	156±26"	62	186±31	47
Free sterols(a)and esterified sterols	68±14	27	173±41	43
Sterol glycosides** and esterified sterol glycosides	10±7	4	17±11	4
Triglycerides	18[¶]	7	22[¶]	6

* = Values are mean of four analyses.
" = Standard error of the mean.
(a) = Calculated as if the fraction were composed entirely of free sterols.
* = Calculated as if the fraction were composed entirely of sterol glycosides.
[¶] = Mean of two determinations.

1.4 REPLICATION STRATEGY:

1.4.1 Replication of Animal Rhabdoviruses:

The replication of VSV has been the most extensively studied of all rhabdoviruses and is discussed below as a model of rhabdovirus replication.

1.4.1.1 Replication and Transcription:

Virus entering the cell is uncoated and releases its nucleocapsid (NC) in the cytoplasm. This nucleocapsid contains a negative polarity RNA strand which serves initially as a template for transcription of five subgenomic monocistronic mRNAs, each of which is translated to give a different protein. Later in infection, full-length positive RNA strand is synthesized (Fig. 1-2). Primary transcription of the incoming genome and translation of the resulting mRNAs generates the proteins necessary for replication (N, NS, L). Emerson (1982) has reported that the polymerase complex (L, NS) has a single entry site at the 3' end of the genome.

In the replication mode, the polymerase proceeds to the 5' end, ignoring all internal termination, polyadenylation and reinitiation signal sites along the RNA strand (Dubois-Dalcq *et al.*, 1984). The resulting antigenome then becomes the template for synthesis of full-length negative strand genomes which, in turn, are used to generate more RNAs (secondary transcription). Replication requires protein synthesis (Hill *et al.*, 1981) and may be enhanced in the presence of N protein which encapsidates genomic RNA (Blumberg *et al.*, 1983).

In the transcription mode, the polymerase will first synthesize the leader RNA, terminate, and without dissociating from

the template, reinitiate transcription at the start of the next gene. This results in the sequential synthesis of five mRNAs (Roy & Bishop, 1973; Testa *et al.*, 1980; Emerson, 1982). Transcription requires a complete NC with its three proteins N, NS, and L .

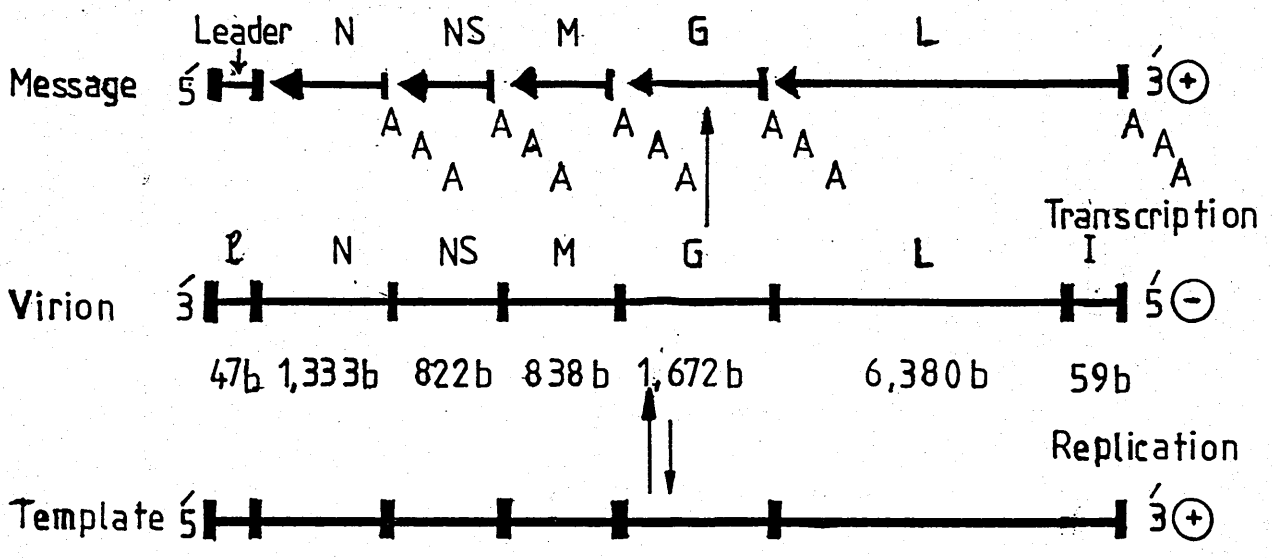


Figure 1-2:
Diagrammatic representation of transcription and replication of the animal rhabdovirus (e.g. VSV), (from Dubois-Dalcy *et al.*, 1984). See text for details.

1.4.1.2 Intracellular Synthesis of Virus Components:

VSV has five functional proteins. The first protein, following infection, to be detected by immunological and biochemical methods is the N protein, which is detectable about 30 minutes before the other proteins. N protein forms scattered foci throughout the cytoplasm, that are not closely associated with the rough ER. Later in infection, N protein is found on a large number of linear structures showing that N is synthesized on free ribosomes and is first found as a cytoplasmic soluble protein before being incorporated into the NC (Knipe *et al.*, 1977; Hsu *et al.*, 1979).

NS protein is also synthesized on the free ribosomes and is abundant in the cytoplasm of infected cells. Most of the NS antigen is colocalized with N antigen. How and where NS is associated with the virus genome is unknown. Infected cells contain a large soluble pool of NS, whose function is obscure .

M protein is synthesized on free polysomes (Knipe *et al.*, 1977); it is found distributed diffusely throughout the cytoplasm of infected cells, and does not colocalize with any other protein early in infection. Later in infection N, NS and M all show some accumulation close to the membrane; thus it is likely that M protein interacts with NC only close to the assembly site at the membrane.

G protein is synthesized on membrane-bound ribosomes and its insertion in the rough ER. as well as co-and post-translational glycosylation and transport to the cell surface have been described in detail (Rothman & Lodish, 1977; Etchison & Summers, 1980; Rothman *et al.*, 1980; Lodish & Rothman, 1980; Morrison, 1980; Bergmann *et al.*, 1981; Rose & Bergmann, 1982; Wehland *et al.*, 1982).

Little is known about L protein synthesis.

1.4.1.3 Assembly of Virus Components :

Encapsidation, the association of virus protein with the viral genome, is a stepwise process starting with the initial condensation of N protein with viral RNA (Hsu *et al.*, 1977). The nature of the interactions between N protein and viral RNA during encapsidation of plus or minus RNA strand have been studied *in vitro* (Blumberg & Kolakofsky, 1981; Blumberg *et al.*, 1983). It appears that the aggregating property of the N protein is essential for the formation of a helical NC. Assembly of N protein with the genomic RNA appears to be a highly cooperative process in which there is linear addition of N protein starting at the leader RNA which prevents the establishment of a secondary structure, which could hinder assembly (Blumberg *et al.*, 1983). In addition, the synthesis of RNA is probably closely coordinated with N binding of the nascent product molecule preventing the polymerase from moving very far ahead of the assembling NC.

From *in vitro* studies on isolated NCs, it appears that at least two factors, salt concentration and M protein, can influence the NC helical organization and, perhaps, the transcription process. In the absence of M, NC can form a helix in the presence of 1M NaCl (Heggeness *et al.*, 1980), this helix has half the diameter and twice the periodicity of that observed in the budding and complete virion. De *et al.* (1982) reported that addition of M protein to purified NCs *in vitro* can increase NC compaction at low ionic strength. Moderate ionic strength (0.1 to 0.2M NaCl) dissociates M protein, unfolds the NC and probably favours RNA chain elongation, reinitiation and transcription *in vitro*. How the NCs move to their site of packaging in the viral envelope and how budding works is poorly understood.

Depending on the cell type, G protein may play a more or less important role in attracting NCs to the infected cell membrane. Lodish & Porter (1980) reported that the presence of G protein on the surface of many cells is not a prerequisite for virion maturation, but it may facilitate the extent of budding. When VSV-infected cells are treated with monensin, which blocks the transport of G protein to the cell surface, virus buds are not detected at the cell surface (Johnson & Schlesinger, 1980). Roth *et al.* (1979) and Roth and Compans (1981) reported that the polarity of VSV budding is maintained after tunicamycin treatment, indicating that glycosylation of G protein is not a determinant of the virus budding site in this system. Rather, the carboxyl-terminal region of G may be the essential element allowing selective incorporation of the virus glycoprotein in a specific membrane site.

The interactions between NC and envelope protein have been proposed and three assembly scenarios have been discussed (Dubois-Dalcq *et al.*, 1984):

1- M protein may first bind to NC forming a M-NC complex which probably takes place in the membrane and might recognise the carboxyl-terminal end of G protein, inducing clustering of G molecules and the formation of spikes. Thus, there would be two binding sites on M, one to NC and one to G, but the latter binding site would be less specific, since certain host membrane proteins can enter the viral envelope when G is absent.

2- M protein first forms a patch in the membrane by self aggregation and binds to G molecules and/or cellular membrane proteins. Subsequently, a NC recognizes this patch

of associated M-G and induces further clustering (Jacobs & Penhoel, 1982).

3- NC recognizes and binds directly to the carboxyl-terminal end of G, and M only plays a role in coiling of NC (Odenwald *et al.*, 1984).

During these transmembrane assembly events, an increasing number of NC coils are incorporated into the bud, and the virus envelope is growing, tightly packed with virus protein molecules and excluding most host cell protein. Changes in molecular conformation might occur at that time. However, the inner leaflet of the budding virus envelope shows fine granular material instead of the usual intramembrane particles seen on the rest of the membrane (Brown & Riedel, 1977; Dubois-Dalcq *et al.*, 1979). Such morphological changes probably reflect exclusion of host proteins in the virus bud (Dubois-Dalcq *et al.*, 1984). Generally, the most frequent site of rhabdovirus budding are the plasma membrane, viroplasm, inner nuclear envelope and ER.

1.4.1.4 Virus Release:

Once the rhabdovirus bud has reached its final shape and contains the entire coiled NC, it is released from the cells as a bacilliform or bullet-shaped particle. The NC length, number of turns, and the virus length and shape vary somewhat with the type of rhabdovirus. However, the shorter the genome, the fewer turns and the shorter the virus. Figure 1-3 illustrates the replication and assembly of a rhabdovirus (modified from Dubois-Dalcq *et al.*, 1984).

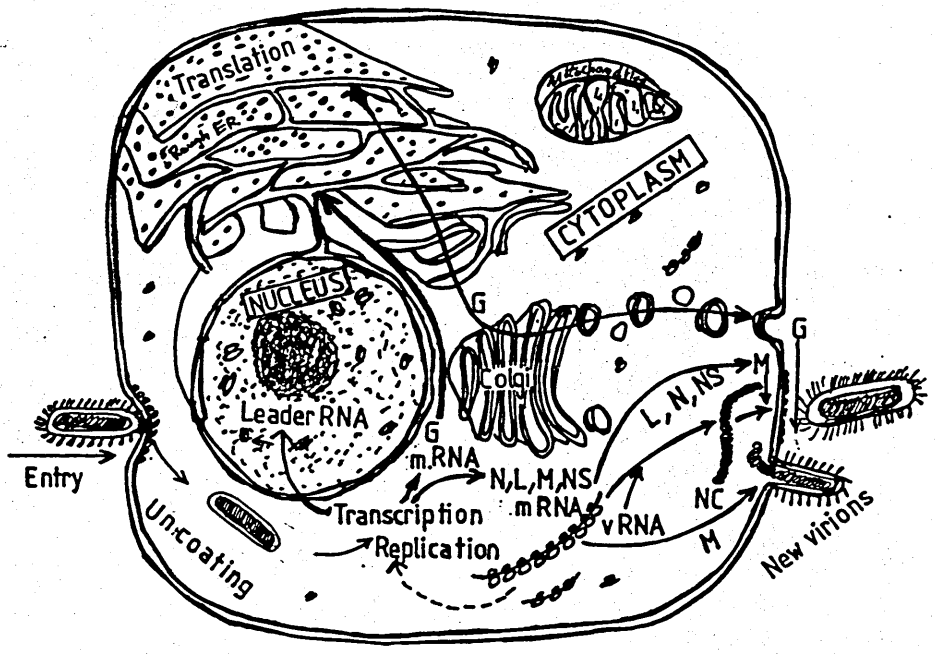


Figure 1-3:

Diagrammatic representation of the entry, replication and assembly of a rhabdovirus that replicates in the cytoplasm of infected cell e.g. VSV, LNYV (modified from Dubois-Dalcq *et al.*, 1984).

1.4.2 Replication of Plant Rhabdoviruses:

Several plant rhabdoviruses have been studied in infected cells using the electron microscope. The information is given in some detail in the reviews of Howatson (1970), Francki (1973), Knudson (1973), Martelli and Russo (1977a), Francki and Randles (1980), Francki *et al.* (1981), Peters (1981) and Jackson *et al.* (1987). There appears to be much variation in their sites of assembly and accumulation. Despite the number of studies, little is known about the sites and mechanism of replication in rhabdovirus-infected plants.

An attempt was made by Wolanski and Chambers (1971) to study events following the infection of leaves with LNYV in the presence of [^{3}H]-uridine. The results led them to suggest that the cell nucleus is involved in the early events leading to replication of this virus, although, at the later stages the cytoplasm also becomes involved. They also reported that no nucleocapsids were observed in the nucleus nor any budding from the inner nuclear membrane up to about 6 days after infection, but from the seventh day, complete virus particles were observed in perinuclear and cytoplasmic vesicles. Some membrane-free virus particles were also found associated with electron-dense material.

It is not yet clear how the virus naturally enter its host plant. However the G protein of the SYDV serotype of PYDV is reported to be necessary for infection of the insect cells, and might play a role during either the adsorption or fusion processes or both (Geadigk *et al.*, 1986; Adam & Geadigk, 1986). G protein may not be necessary for infecting plants as the nucleocapsid of LNYV is infectious on its own (Randles & Francki, 1972). Immunological studies show that antigens of PYDV (Chiu *et al.*, 1970) and SYVV

(Peters & Black, 1970) are at first confined to the nucleus of leafhopper and aphid cells in culture.

The assembly of some plant rhabdoviruses may differ in plant cells and in aphid cells. Working with SYVV Richardson and Sylvester (1968) and Sylvester and Richardson (1970) reported that in plants this virus was associated with the host cell nuclei, and was usually bullet-shaped, whereas in aphids smaller mostly bacilliform particles were found in the nuclei and cytoplasm suggesting that the virus particles were first assemblied in nuclei and then moved into the cytoplasm. The virion of BNYV (Garrett & O'Loughlin, 1977) appeared to be exclusively confined to the cytoplasm in cauliflower whereas in its aphid vector they were observed largely in the nuclei. One can draw the conclusion that the attempts by Hull (1970) and Knudson (1973) to group the plant rhabdoviruses according to their morphogenesis may therefore be unjustified.

Of all rhabdoviruses infecting plants, the replication of SYNV has been most extensively studied. Investigations into the replicative mechanism of the virus have centred on RNA's, which have been isolated from polysomes of SYNV-infected tobacco, and which have been found to contain sequences complementary to SYNV RNA (Milner *et al.*, 1979). The viral-complementary RNA (vcRNA) consists of several subgenomic species complementary to more than 90% of the viral genome (Milner & Jackson, 1979), and because it has several characteristics of messanger RNA (mRNA) it is believed, in fact, to be mRNA. Four electrophoretically distinguishable viral-complementary RNA species have been found on polyribosomes from SYNV-infected tobacco (Milner & Jackson, 1983). Heaton (1987) has analysed cDNA clones derived from poly(A)$^+$mRNA's and viral RNA. These studies indicate that the four

major electrophoretically distingushable vcRNA's comprise six distinct species sc1 to sc6. The three smallest poly(A)$^+$RNA's sc4, sc5, and sc6 co-electrophoresis in an agarose gel. Antibodies to fusion protein, produced in *Eschrichia coli* were used to identify the translation products of sc3 as N protein (Zuidena *et al.*, 1987) and sc5 as M2 protein (Heaton *et al.*, 1987). On the basis of size sc1 and sc2 presumably code for L and G, respectively, while sc4 and sc6 code for M1 and an additional protein. The function of the extra, non structural, polypeptide is unknown.

Tobacco infected with SYNV contains 130-144 b short transcripts complementary to the 3' terminus of the SYNV-RNA genome (Zuidema *et al.*, 1986) which are similar to, but somewhat larger than, leader RNA's associated with several animal rhabdoviruses.

The development of a system for infecting plant protoplasts with rhabdoviruses (Van Beek *et al* ., 1985a, 1985b, 1985c, 1986) has allowed Van Beek and others to study the *in vivo* morphogenesis and protein synthesis of SYNV. The time course of SYNV-replication has been studied in infected cowpea protoplasts using electron microscopy (Van Beek *et al* ., 1985b). The following replicative events have been reported: Polysomes increased sharply in number by 8-9 hours after inoculation indicating an acceleration of translational activity; the first nucleocapsids were found in the nuclei at the edge of granular matrix by 10 hours after inoculation; at the same time the first virus was observed budding from the inner nuclear envelope; by 12 hours after inoculation virus particles were found accumulating in the perinuclear space and particles were found entering the lumen of the endoplasmic reticulum; particles with a loosely fitting membrane were found in the cytoplasm 12 to 20 hours after inoculation and by 24

hours nucleocapsids were detected lying free in the cytoplasm, often very close to the virus particles scattered throughout the rough endoplasmic reticulum (RER).

Synthesis of SYNV-proteins has been also studied in cowpea protoplasts (Van Beek *et al.*, 1986). Infected cowpea protoplasts incorporated ^{35}S-methionine into a variety of host polypeptides, plus the four major structural proteins G, N, M1 and M2 with molecular weights of 82, 56, 41 and 35 KD respectively. In addition to these proteins, a minor protein with a molecular weight of 45 KD occasionally, a protein with a molecular weight of 38 KD and a protein with a molecular weight of about 12 KD, incorporated label together with an additional five proteins in the lower molecular weight range (22, 20, 16 and 12 KD). Proteins immunoprecipitated from *in vivo* synthesised proteins in SYNV-infected cowpea protoplasts (Van Beek *et al.*, 1986) were similar to those above. In addition several other proteins were consistently observed in the high molecular weight region, as well as, a protein with a molecular weight of 52 KD which was precipitated from both infected protoplasts and healthy controls, and which most likely represent a host protein which co-purified with the virus.

1.5 VIRUS TRANSMISSION AND MOVEMENT:

1.5.1 Virus Transmission:

Transmission of plant viruses has been extensively discussed in a number of reviews (Gibbs & Harrison, 1976; Matthews, 1981; Stevens, 1983). The ways in which the plant rhabdoviruses spread from plant to plant (field spread) is also discussed (Francki, 1973; Francki & Randles, 1980; Francki *et al.*, 1981; Peters, 1981) .

Naturally, plant rhabdoviruses are spread by aphid or leafhopper vectors (see the above references). The plant rhabdovirus-vector relationships are highly specific. Viruses seem to replicate in their vectors; therefore, culture of insect cells can be used to study e.g. the replication of plant rhabdoviruses (for review see Adam, 1984).

Experimentally, a few plant rhabdoviruses can be transmitted mechanically to plant hosts. Since most of the plant rhabdoviruses are unstable *in vitro* (Francki,1973), a suitable buffer is essential for each virus (Francki, 1973; Peters, 1981).

1.5.2 Virus Movement in Infected Plant.

Immediately after inoculation of a plant, only a very small number of cells become infected. The virus replicates in the primarily infected cells and the progeny move to neighbouring healthy cells. It is universally believed that virus genome moves in infected plants either as mature virions, or possibly in the case of positive stranded RNA viruses in form of free RNA, and as a nucleocapsids in the case of negative stranded RNA viruses.

During the primary infection of the plant, virus particles penetrate through microinjuries into the cells of the epidermis and probably into occasional cells of the mesophyll (Sulzinski & Zaitlin, 1982). Further systemic spread of infection takes place in two ways (Matthews, 1981; Atabekov & Dorokhov, 1984): (i) slow cell-to-cell movement (short-distance transport) through the plasmodesmata, and (ii) rapid migration over long distance via the vascular tissues (long-distance transport). The transport for short and long distance has been extensively discussed in a number of reviews (Bennett, 1956; Esau, 1956; Schneider, 1965; Gibbs & Harrison, 1976; Matthews, 1981; Atabekov & Dorokhov, 1984). It is widely believed that the plasmodesmata play the role of the transport channels through which the infective agent is transferred from cell to cell. The most direct evidence for such movement of viruses through plasmodesmata comes from electron microscope studies (Esau *et al.*, 1967; Davison, 1969; Kitajima & Lauritis, 1969; Gill & Chong, 1981; Gibbs & Harrison, 1976). Virus infection may lead to modification in the fine structure of the plasmodesmata (Esau *et al.*, 1967; Davison, 1969; Kitajima & Lauritis, 1969; Esau & Hoefert, 1972; Kim & Fulton, 1973; Chamberlain *et al.*, 1977) opening the gates for the migration of the virus genetic materials to the healthy cells (Atabekov & Dorokhov, 1984).

The long distance movement in infected plants is normally much more rapid than cell-to-cell movement. Defective viruses lacking a functional coat protein gene such as the NM form of tobacco rattle virus (TRV), as well as their normal counterparts both appear to move in this manner (Matthews, 1981). The time at which infectious agent moves from inoculated leaf to the rest of the plant is highly dependent on host species and virus, age of host, temperature and

method of inoculation (Matthews, 1981). Such movement may occur after weeks or after a period as short as 24 hours. It is generally accepted that phloem and xylem tissue are involved in long distance movement (for review, see Gibbs & Harrison, 1976; Matthews, 1981). In the case of plant rhabdoviruses, it has been reported that the infectivity of LNYV can be found associated with xylem sap (Francki & Randles, 1970). Özel (1973) detected by electron microscopy SYVV particles in roots of *S. oleraceus* within 48 hours of aphid-mediated inoculation of leaves. He suggested that the virus moved initially into the roots through the phloem and subsequently, after multiplication, moved from roots to leaves through the xylem .

There is recent evidence concerning the role of a virus-specific transport protein. This transport factor may be able to bind specifically to virions in order to help their transit through plasmodesmata (Langenberg, 1986). Another possibility is that the transport factor modifies the wall or plasmodesmata between adjacent infected and non infected cell making them permeable to the infectious agent (Stussi-Garaud *et al.*, 1987).

Immunoelectron microscopy with gold-labelled antibodies showed that homologous capsid proteins or virions are associated with cylindrical inclusions of Wheat streak mosaic (WSMV) and of Wheat spindle streak mosaic (WSSMV) viruses *in vitro* (Langenberg, 1986) and such association is thought to be in correlation with cell-to-cell movement of virions through the plasmodesmata.

The majority of P3, a non-structural protein of alfalfa mosaic virus (AlMV), has been shown to accumulate in the cell wall fraction of *N. tabacum* following viral infection and replication (Godefroy-Colburn *et al.*, 1986). The ultrastructural location of this protein was

visualized immunocytochemically in the middle lamella of the walls of either infected parenchymal or infected epidermal cells (Stussi-Garaud *et al.* 1987). These findings support the concept that P3 protein is the transport factor of AlMV and is involved in the movement of the viral infection from cell-to-cell .

The 30K protein, a non-structural protein of TMV, has been identified as a transport factor for viral infection (Leonard & Zaitlin, 1982; Ohno *et al.*, 1983). There is evidence that this protein binds to the cell wall of infected *N. tabacum* (Godefroy-Colburn *et al.*, 1987).

The rate of virus movement in the infected tissue appears to be determined by the number of plasmodesmata connecting neighbouring cells (Wierimga-Brants, 1981). Atabekov and Dorokhov (1984) state that, special virus-specific ribonucleoprotein particles (vRNP) were found formed in TMV-infected plants. The vRNP's were different from the virion in structure and contained of substantial amounts of subgenomic and a relatively small quantity of genomic viral RNA's. In addition, vRNP contains virus-specific proteins (Dorokhov *et al.*, 1983). Evidence favours the idea that vRNP plays the part of the transport form of viral infection (Dorokhov *et al* ., 1984) and contains the subgenomic RNA coding for transport protein as well as probably the transport protein itself.

1.6 SYMPTOMOLOGY AND CYTOPATHOLOGY OF INFECTED PLANT

1.6.1 Symptomology:

Among 68 definitive and tentative members of the plant rhabdovirus group listed by Peters (1981), only a few are known to cause diseases of economic importance and these were usually distributed in limited geographical areas (Francki *et al.*, 1981). Of those diseases, the most serious are those caused by beet leafcurl (BLCV), LNYV, PYDV and SCV viruses. Also, eleven rhabdovirus diseases of *Gramineae* have been listed by Jackson *et al.* (1979). It should be mentioned that SYNV has been reported recently to be of potential economic importance, causing diseases of Florida lettuce (Falk *et al.*, 1986).

External symptoms, which occur as a host plant response to the virus infection, are very varied and the factors controlling the nature and occurrence of symptoms include, type and strain of virus, type and variety of host plant, physiology of the host, the presence of other viruses and pathogens and environmental and climatic conditions (Stevens, 1983). Lettuce plants infected with LNYV exhibit chlorosis with varying degrees of necrosis. In some cases the plants actually die, with survivors producing useless hearts with a characteristic sweet taste (Randles & Crowley, 1970). Most of the *Gramineae* infected with plant rhabdoviruses become stunted and develop chlorotic spots and strips or streaks on leaves; more susceptible host varieties sometimes die (Jackson, 1979). It can be said, that there are no unique or characteristic symptoms on plants that can be considered as typical of infection by rhabdoviruses (Peters, 1981). Therefore, symptomology has no value in plant rhabdovirus identification (Jackson *et al.*, 1987).

1.6.2 Cytopathological Changes, and Inclusion Bodies Observed, in Infected Plant Tissue:

Most of our knowledge about cytopathological changes in plants infected with rhabdoviruses has been gained from electron microscopy.

1.6.2.1 Nuclear changes:

In some cases of virus infection, the cell nuclei may stain less readily, as with EMDV (Martelli & Russo, 1973). The appearance of clear areas around the nucleolus, and possible clumping of chromatin are the first signs of degeneration in the nucleus of *N. glutinosa* infected with LNYV. Also, chromatin disappears and the nucleolus loses its granular texture and becomes fibrous; the degenerate nucleus finally resembles an empty, membrane-bound vesicle (Wolanski, 1969). Most plant rhabdoviruses appear to replicate in the nucleus of the infected plant, and virus particles often accumulate in the perinuclear space (for reviews see Jackson *et al.*, 1987; Francki *et al.*, 1985; Peters, 1981). It has been found also, that the inner nuclear membrane is the site of maturation of such viruses, e.g. SYNV, PYDV, EMDV, respectively (Christie *et al.*, 1974; Black, 1970; Martelli & Russo, 1973). Mature virions cluster in monomembranous-inclusion bodies either in the nucleus or in the cytoplasm, or often on both sides (Francki, 1973; Martelli & Russo, 1977b).

Van Beek *et al.* (1985b) observed densely stained matrix in nuclei of cowpea protoplasts infected with SYNV after being treated with actinomycin D. Such materials were occasionally found associated with viral nucleocapsids of SYNV. MacLeod *et al.* (1966) foud that PYDV particles accumulate in cytoplasmic invaginations located in the

nuclei. These inclusions may contain cytoplasmic organelles such as mitochondria or may appear full with virus particles only.

1.6.2.2 Cytoplasmic Changes:

Chloroplasts: Electron micrographs of chloroplasts of *N. glutinosa* infected with LNYV show displacement and disorganization of lamellar membranes, the appearance of vacuoles, osmiophilic granules, and at a late stage, the disappearance of chloroplast membrane and starch grains (Wolanski, 1969). Ramsey wheat (*triticum durum*) leaves infected with wheat striate mosaic virus (WSMV) showed dramatic changes in chloroplasts of infected cells when the grana formed a concentric pattern (Lee, 1967).

Randles and Coleman (1970,1972), working on *N. glutinosa* infected with LNYV, found that the chloroplast ribosomes declined in numbers, within one day of symptoms appearance and were completely undetectable one to three days later. They, also, found that there were parallel reductions in the ribosomal RNA synthesis, concentration of fraction I protein, and size of the chloroplast. To my knowledege no such study has been carried on any other plant rhabdovirus.

Mitochondria: Mitochondrial changes have been reported with BNYV (Hill & Campbell, 1968). In BNYV-infected *N. glutinosa* cells, mitochondria were swollen and contained few cristae. *N. glutinosa* infected with Sonchus virus (SV) showed a large percentage of modified mitochondria containing one or several clusters of an electron dense material, and very distorted mitochondria were observed containing fibroid material (Vega *et al.*, 1976). It is generally reported that normal mitochondria or degenerated ones may aggregate during virus infection of cells (Stevens, 1983; Matthews, 1981). No particles of

any plant rhabdovirus have been found either in mitochondria or in chloroplasts (Peters, 1981).

Other cytoplasmic changes: Cells infected with either barley yellow striate mosaic virus (BYSMV), or northern cereal mosaic virus (NCMV) develop extensive membrane-bound viroplasms in the cytoplasm from which virus particles bud and accumulate in the vacuole-like space, (Conti & Appiano, 1973; Toriyama, 1976).

Plant rhabdoviruses whose maturation is associated with endoplasmic reticulum, e.g. LNYV (Wolanski & Chambers, 1971), accumulate almost exclusively in vesicles formed from this endoplasmic reticulum.

1.6.2.3 Changes in the Cell Wall:

Ultrustructural changes in cell walls of virus-infected cells have been well reviewed (Matthews, 1980, 1981).

In mechanically inoculated *Pittosporum tobira* (Thumb) but not in plants naturally infected with pittosporum vein clearing virus (PVCV), a plant rhabdovirus, some large particles were reported to be located in the centre of cell wall protrusions, and identical particles were reported to be located in the cytoplasm (Di Franco *et al.*, 1980).

1.7 DEFECTIVE-INTERFERING PARTICLES:

1.7.1 Introduction:

Defective interfering (DI) particles were first recognized in preparations of influenza virus, an orthomyxovirus, propagated *in vivo*. Von Magnus (1954) was the first to state a clear definition of DI-particles by showing that homologous interference exerted by yields from serial undiluted passages of influenza virus in eggs was due to the replication of "incomplete particles" which showed interfering ability but not infectivity. In fact, Henle and Henle (1943) were the first to report a "paradoxical behavior" in which late harvest virus from eggs showed lower infectivity in mice when undiluted than when diluted one thousandfold. They concluded that interference was due to "inactivated" infectious virus, as the agent of interference had resistance to UV light and heat; their results were almost certainly due to the presence of DI particles (Holland *et al.*, 1980).

DI particles have been reported in both positive- and negative-stranded animal viruses (see Holland *et al.*,1980). It has been known that repeated passage of such animal viruses results in attenuation of virulence. This attenuation often follows the generation of DI particles (Huang & Baltimore, 1977). DI particles have the same protein components as the standard virus, require the presence of standard virus for replication and interfere with the replication of the standard virus (Huang, 1973). Defective viruses can be generated naturally during infection and have been implicated,

for example, in the aetiology of several slow virus diseases of animal and man (Yougner & Preble, 1980).

The generation of DI-particles of VSV has been well studied (Huang & Baltimore, 1977). Short particles accumulate following repeated passage of the virus at high multiplicities of infection. Their genomic RNA sequences differ from those of the parental virus as a result of deletion and sequence rearrangements. These rearrangements involve the termini and sometimes internal regions of the genome (Faulkner & Lazzarini, 1980).

DI-particles are defective, in that the genes for one or more of the virus proteins are absent or functionally inactive and they rely, for replication, on complementation by standard particles which must be present as helper. The presence of DI-particles interferes with the replication of standerd particles, reducing yields of latter and in addition modulates the cytopathogenicity of the standard particles resulting in persistent rather than cytolytic infections (Johnson & Lazzarini, 1980).

1.7.2 Defective Interfering Particles of Plant Viruses:

Many defective strains of plant viruses have been reported. These may be generated by point mutations, deletions, or in the case of viruses with multipartite genomes, loss of entire genome segments of the parental virus. The NM form of TRV, a natural isolate, arises from the complete loss of RNA 2 which contains the coat protein gene (Harrison, 1970). Similarly, repeated sap-transmission of wound tumour virus results in loss of insect transmissibility, accompanied by loss of genome segments (Black, 1979). Naturally occurring defective isolates of soil-borne wheat mosaic and tomato spotted wilt

viruses both result from deletions of part of a genomic RNA segment (Shirako & Ehara, 1986; Verkleij & Peters, 1983). However, none of these examples can be considered to fulfil the criteria for DI-particles since they are all capable of replication in the absence of standard virus, albeit in an altered manner and they do not interfere with the replication of standard virus, although in the latter case, the defective isolate of tomato spotted wilt may be an exception.

Authentic DI-particles have not been definitely reported in plant-virus preparations; this is possibly a consequence of the difficulty in obtaining a high multiplicity of infection in plants (Jackson *et al.*, 1987). Adam *et al.* (1983) reported the isolation of a defective strain of PYDV following repeated passage by sap transmission to *N. rustica*. They propagated PYDV under conditions shown to result in most rapid development of symptoms in tobacco. After about 30 successive mechanical passages at high multiplicities, recovery of virus in purification trials decreased markedly. In such preparations particles with slower sedimentation and a lower buoyant density than normal virus particles were found. Also, plants failed to develop lesions typical of infection with normal PYDV when inoculated with the low density particles isolate and appeared not to be infectious. However, when mixed with normal virus, the infectivity of the latter was drastically reduced. When the preparation of the new particles was diluted and inoculated to healthy plants, the number of viral particles recovered appeared to be greately reduced.

Although no detailed structural data on the putative DI-particles were presented, the particles appeared to be derived from PYDV, since they contained a normal complement of PYDV protein (Adam

et al., 1983; Adam, 1984). These preliminary findings suggest that plant rhabdoviruses have the potential for forming DI-particles. The details of such evolutionary alterations might provide useful information concerning the infectious process and the ability of plant to recover from infection (Jackson *et al.*, 1987).

1.8 AIM OF THE RESEARCH:

Comparatively little is known about either the replication of plant rhabdoviruses or the mechanism by which they induce their effects on the host plants. The project involves an investigation into the multiplication of SYNV in *N. edwardsonii* and its interaction with the host plant. Of particular interest is the spread of virus through the host during the initial phase of acute infection and the subsequent effect of chronic infection on virus multiplication and host metabolism. The keypoint of the research was to answer the following questions:

1- How does SYNV spread throughout mechanically inoculated *N. edwardsonii* plants?. What is the time course?.

2- Where is the virus located within infected tissues?. Is this the same at various times after inoculation?.

3- What are the virus infection on the host at various times after inoculation?.

4- Do the initially infected plants remain infected at the later stage of infection?. If so, how does the virus behave?. What are the effects on both, the virus and the host?

5- Is there any possibility SYNV DI-particles are generated during any infection?.

CHAPTER 2

MATERIALS AND METHODS

2.1 SOURCE OF MATERIALS:

2.1.1 Source of Seeds:

N. edwardsonii (*N. Clevelandii x N. glutinosa;* Christie, 1969) seeds were originally obtained from Dr. A.O. Jackson, Purdue University, U.S.A in 1981. Subsequently, plants have been allowed to flower and used as a source of seeds.

2.1.2 Source of Inoculum:

Sonchus yellow net virus was isolated originally in Florida (Christie *et al.*, 1974), and maintained in *N. edwardsonii* by regular passage. The type isolate (ATCC PV-263) was imported to Glasgow in 1981, and maintained under licence by Dr. J.J. Milner, in the same experimental host (*N. edwardsonii*) by regular passage.

An isolate of SYNV, isolated from lettuce in Florida 1985 by Falk *et al.* 1986 and designated SYNV-L85 was a kind gift from Dr B. Falk. The inoculum had been transferred mechanically four times only (Dr B. Falk, personal comunnication).

2.1.3 Source of Chemicals:

Sources of specialist chemicals are stated in the text in this chapter. General laboratory reagents were obtained from various sources and were "Analytical" grades exept were stated.

2.2 GENERAL METHODS USED THROUGHOUT THIS PROJECT:

2.2.1 Sterilization of Equipments, Buffers and Solutions:

All autoclavable equipment, buffers and solutions were sterilized, when required, by autoclaving for 15 to 30 m at 15lb/in^2.

Heat labile equipment was rendered nuclease free with 0.5M NaOH. Equipment was soaked in 0.5M NaOH for 10 to 15 m, then rinsed thoroughly with sterile distilled water and air dried.

2.2.2 Siliconization of Plasticware:

For certain procedures, such as preparation of complementary DNA (cDNA) or for handlling very small quantities of single stranded DNA or RNA, eppendorf tubes were coated with a thin film of silicone according to the procedure described by Maniatis *et at.* (1982). The procedure was carried out in a fume-cupboard because dichlorodimethylsilane is toxic and highly volatile.

Items to be siliconized were placed inside a large desiccator, in which was centrally located a small beaker containing a 1ml of dichlorodimethylsilane (Sigma). The desiccator was attached to a vacuum through a trap, the vacuum was turned on for 5 m and then off. Air was quickly allowed into the desiccator to ensure a uniform dispersion of the gaseous dichlorodimethylsilane. Once again, the vaccum was turned on for 1 m, then turned off and the items left for 2 to 3 h. Air was allowed in, items were taken out of the desiccator, rinsed very well with sterile distilled water and autoclaved or dried at 60°C

2.2.3 Preparation of Dialysis Tubing:

Tubing (1cm in diameter) was cut into pieces 10-15 cm in length. These were boiled for 10 m in approximately 2 l 0f 2% (w/v) sodium bicarbonate and 1mM ethylenediaminetetraacetic acid (EDTA). After boiling, tubing pieces were rinsed thoroughly in sterile distilled water, boiled for further 10 minuts in 1mM EDTA, allowed to

cool to room temperature and then stored in 25% ethanol. Before use, tubing pieces were washed thoroughly inside and outside with sterile AnalaR water. Dialysis tubing was handled with gloves.

2.2.4 Preparation of Deionized Formamide:

Formamide was mixed with Amberlite MB-1 monobed resin (Sigma) in a ratio of 50ml/2g. The mixture was stirred on magnetic stirrer for 45 to 60 m at room temperature, and the resin removed by filtration through Whatman No.1 filter paper. Formamide was stored in brown bottle at 4°C and used within 14 d.

2.2.5 Preparation of Buffer Saturated Phenol:

The procedure was carried out wearing gloves in a fume-cupboard. About 100-500g of crystalline redistilled phenol was melted at 60-68°C, 8-hydroxyquinoline was added to a final concentration of 0,1% as an antioxidant, an equal volume of 0.1M tris-HCl (pH 8.0) was added, and the components were mixed vigorously. The mixture was left in the fume-cupboard until the two phases had separated, poured into a glass separatory funnel and left overnight at 4°C. The lower layer was collected and stored in a brown glass bottle and the upper layer (buffer) was discarded. An equal volume of 0,1M tris-HCl (pH 8.0) was added to the phenol and the phase separation was repeated three times. The phenol phase was stored in a brown glass bottle at 4°C below about a 5 cm thick layer of buffer (0.1M tris-HCl, pH 8.0).

Phenol/chloroform was prepared by mixing the phenol with an equal volume of chloroform before use. This mixture was stored at 4°C and used within a month.

2.3 GROWTH CONDITIONS AND INOCULATION OF *NICOTIANA EDWARDSONII* WITH SONCHUS YELLOW NET VIRUS:

2.3.1 Germination and Growth of *N. Edwardsonii*:

Prior to the seeding step, *N. edwardsonii* seeds were surface sterilized by soaking in 1% (v/v) bleach (Chlorox) for 12-16 h at 4°C, recovered by filtration through Whatman No. 1 filter paper, and air dried at room temperature.

Sterilized seeds were scattered on to the surface of sterile damp potting compost (S.A.I.) covered with a layer of sterile fine sand, and maintained at 23°-25°C under 14 h/d illumination. Seedlings appeared above the surface 4-6 d after sowing and were pricked out and individually grown in pots (90 mm or 200 mm diameter) containing sterile potting compost.

All plants were grown in the growth chamber under the above environmental conditions, unless otherwise stated. Each plant was watered every 1-3 d.

2.3.2 Inoculation of *N. edwardsonii* with Sonchus Yellow Net Virus.

Once the plants had the first four to six rosette leaves fully expanded (about 8-10 cm length), or when they were at the flowering stage (about 20-30 cm length), they were inoculated with the virus.

Prior to inoculation, the mortar and pestle, 1% Na_2SO_3 and 9-12 cm square pieces of muslin were sterilized and then chilled on ice. The following steps were carried out on ice.

Approximately 1g of either well-infected leaves (usually 10-12 d after inoculation), 1g of symptomless, chronically infected leaves (about 60-120 d after inoculation) or 1g of calyx from

chronically infected plants (about 125-150 d after inoculation) were ground up in 1% Na_2SO_3, in a ratio of 1g material to 1ml solution, in the mortar and pestle (Christie *et al.*, 1974). Three fully expanded leaves, from each plant to be inoculated, were lightly sprinkled with carborundum (silicon carbide, super fine, about 600 grit, BDH) which acted as an abrasive, helping the virus to gain entry into the epidermal cells. The inoculated leaves were, on occasion, marked with a tag. The inoculum was soaked up into a muslin pad and, was rubbed on to the carborundum-dusted-leaves.

The inoculated plants were then left to grow under the environmental conditions described above, or occasionally, in the greenhouse under conditions as near to the above as possible.

2.4 PURIFICATION OF SONCHUS YELLOW NET VIRUS PARTICLES, EXTRACTION OF VIRAL RNA, AND DETERMINATION OF THE VIRAL PROTEINS CONCENTRATION.

2.4.1 Purification of Sonchus Yellow Net Virus.

Virus was purified as described by Jackson and Christie (1977), with minor modifications (Milner and Jackson, 1979). During each step of the protocol all buffers and solution were kept on ice.

Systemically infected leaves showing normal vein-clearing symptoms were harvested 10-12 d after inoculation. The final yield of harvested leaves ranged from about 100 to 150g, depending on the number of plants previously infected, environmental conditions under which the plants were grown and the age at which the plants were inoculated. Harvested leaves were processed immediately, or stored at 4°C for not longer than five days. These were hand-cut into small pieces and placed in a large liquidizer with 1X viral extraction

buffer (1X VEB) in the ratio of 1g leaf to 2-3 ml cold 1X VEB (0.1M tris-HCl pH 8.4; 0.01M Mg acetate; 0.04M Na_2SO_3; 0.001M $MnCl_2$). VEB was prepared as 10X stock and stored at -20°C; the last two components were added just before use. Infected leaves were homogenized for 30-50 s and the homogenate was strained through four layers of muslin. The brei was then centrifuged at 2.500-3.000 r.p.m.(650-950g) in an MSE-18 centrifuge for 10 m. The supernatant was filtered through two layers of soft Kleneex tissue. The filtrate was layered into 25.4 x 89 mm polyallomer centrifuge tubes over discontinuous gradients formed from 8ml (bottom) and 5ml (top) of 60% (w/v) and 30% (w/v), respectively, sucrose made up in cold 1X viral maintenance buffer (1X VMB; 0.1M tris-HCl pH 7.5; 0.01M Mg acetate; 0.04M Na_2SO_3; 0.001M $MnCl_2$). VMB was prepared as 10X stock and stored at -20°C; the last two components added just before use. These discontinuous gradients were centrifuged in an AH629 rotor at 27.000 r.p.m.(97.000g) for 45 m in a Sorvall OTD-65B ultracentrifuge. Three layers were visible in each gradient. The green band between the pale yellow upper layers (30% sucrose layer) and the lower clear 60% sucrose layer, were collected, combined and diluted with an equal volume of 1X VMB..

A celite pad 0.6-0.75 cm thick was prepared as follows: sufficient celite (diatomaceous earth, Sigma) to fill three 50 ml beakers was suspended in 250ml 1X VMB. This was carefully poured over two layers of Whatman No.1 filter paper in a Buchner funnel and the liquid pulled through under gentle vacuum. The celite pad was washed with about 100-150ml 1X VMB and stored at 4°C

The crude preparation from the discontinuous gradient step was made up to 3-4% (w/v) with celite, mixed well and vacuum-filtered through the celite pad, followed by about 100ml 1X VMB. The light

brown filtrate containing the virus, was centrifuged in the Sorvall T865 rotor at 30.000 r.p.m.(67.000g) for 25 m to pellet the virus. The clear virus pellets were resuspended in 2ml 1X VMB. Rate zonal sucrose gradients were formed into 25.4 x 89mm polyallomer centrifuge tubes by pipetting (from bottom to top) 5.6ml, 11.2ml, 11.2ml,and 4.9ml of 30%, 20%, 10%, and 5%, respectively, of sucrose made up with 1X VMB. Gradients were allowed to diffuse at 4°C for about 16-18 h before use. Rate zonal gradients were occosionally prepared from an equal volumes of 30% and 5% sucrose, made up with 1X VMB, using a gradient maker (LKB). Virus suspension was layered on the top of the sucrose gradients and centrifuged in the Sorvall AH629 rotor at 25.000 r.p.m.(83.100g) for 20 m at 4°C.

Gradients were fractionated and scanned at 254nm with an ISCO Model 185 density gradient fractionator attached to an ISCO Model UA5 absorbance monitor. The light scattering virus bands were collected in a centrifuge tube, diluted with 1X VMB to the shoulder of the tube and centrifuged in the Sorvall T865 rotor at 30.000 r.p.m. (67.000g) for 30 m at 4°C. The pellet was gently rinsed with 1X VMB, resuspended in 0.5-1ml 1X VMB and stored in sterile 1ml Nunc cryotubes at -196°C.

2.4.2 Extraction of Sonchus Yellow Net Virus RNA.

Two methods have been used for extraction of SYNV RNA.

2.4.2.1 Extraction of RNA by phenol/chloroform:

The purified SYNV suspension was lysed by addition of one fifth volume of 10% sodium dodecyl sulphate (SDS). To this an equal volume of phenol/chloroform, (prepared as described previously,

section 2.2.5) was added, vortex-mixed and then centrifuged either in a microcentrifuge (11.600g) in the case of small volume preparations or in an MSE-18 centrifuge. After centrifugation for 10 m the upper aqueous phase was saved and transferred to a 1.5ml eppendorf. The remaining phenol/chloroform phase was re-extracted with 100µl AnalaR water. Once again, the upper aqueous phase was saved, the two aqueous phases were combined and re-extracted with an equal volume of phenol/chloroform. The aqueous phase was transferred to a siliconized eppendorf tubes and RNA was precipitated by addition of 2.5 volumes ethanol and 0.1 volume 4M potassium acetate pH 5.5 and leaving overnight at -20°C or occasionally at -70°C for about 3-4 h. The RNA precipitate was pelleted in the microcentrifuge, pellets were washed twice with 75% ethanol, dried under gentle vacuum, resuspended in 20-30µl AnalaR water and stored at -70°C.

The concentration of RNA was estimated spectrophotometrically at 260nm and 280nm using a Philips Pye Unicam SP8-500 UV/VIS spectrophotometer. The RNA content was calculated on the basis that:

true A_{260} = (observed A_{260} - observed A_{280}) X 2

A 40µg/ml solution of RNA has an A_{260}= 1.

2.4.2.2 Sucrose Gradient Method:

This method, described by Milner and Jackson (1979), was used to extract pure viral RNA free of contaminating ribosomal RNA (rRNA)

Stock solutions of 60% (w/v) sucrose and 10X STE buffer (1.8M NaCl; 0.2M tris-HCl pH 7.5; 0.02M EDTA) were made up in AnalaR water). All solutions, AnalaR water, glass test tubes, pipette tips, eppendorf tubes, 10% (w/v) SDS and centrifuge tubes were sterilized and chilled on ice before use. Stock dilutions of sucrose in 1X STE were prepared

and linear-log sucrose gradients (Brakke & Van Pelt, 1970) were formed in 14 X 95 mm polyallomer centrifuge tubes by layering (bottom to top) suitable concentration of each stock as shown in the following table 2-1 and allowing the gradients to diffuse overnight at 4°C.

Table 2-1

Sucrose mg/l	Pipetted volume (ml)
324	1.9
270	3.4
210	2.4
162	1.5
99.6	1.2
0	1.3

About 0.5 to 0.75ml suspension of purified SYNV was lysed by addition one tenth volume of 10% SDS and carefully layered on top of the gradient. TMV RNA (20µg in 0.5ml AnalaR water) to act as a standard for calculation of SYNV RNA concentration, was carefully layered on top of another gradient. The tubes were centrifuged at 40.000 r.p.m. (293.000g) for 5 h at 4°C in a Sorval TST 41.14 rotor. Gradients were fractionated and the absorbance at 254nm scanned using an ISCO Model 185 density gradient fractionator attached to an ISCO Model UA5 absorbance monitor. The tubes and syringe were soaked in a solution of 0.5M NaOH for 20 m and thoroughly rinsed with AnalaR water

before use. Fractions containing SYNV RNA were recovered, and the yield calculated by comparing the area under the peak with the area of the TMV RNA peak.

The SYNV RNA was ethanol precipitated (section 2.4.2.1), centrifuged in an MSE 18 centrifuge at 15.000 r.p.m. (23.100g) for 30 m at 4°C. The pellet was resuspended in 0.3ml AnalaR water and transferred to a 1.5ml eppendorf tube. SYNV RNA was phenol/chloroform extracted, ethanol precipitated, dried, resuspended and stored as described previously (section 2.4.2.1).

2.4.3 Determination of the Virus Protein Concentration:

The coomassie blue binding assay (Bradford, 1976) was used to determine the concentration of the virus protein. Bovine serum albumin (BSA) was used as a standard.

A stock of 2mg/ml BSA was prepared in sterile AalaR water. Concentrated dye-reagent (Bio-Rad) was diluted to 1:4 with sterile AnalaR water and filtered through Whatman No.1 filter paper.

To a series of tubes containing 1ml diluted dye reagent was added duplicate aliquots of 5µl or 10µl of purified SYNV or 2µl, 5µl, 10µl or 20µl of BSA stock solution (2mg/ml) and the final volume made up to 1.1ml with sterile AnalaR water. The A_{595} of each sample was determined against a blank sample containing 1ml diluted dye-reagent and 100µl AnalaR water. The A_{595} for BSA samples were plotted against concentration and used to determined the concentration of the SYNV protein sample.

2.5 PURIFICATION OF TOBACCO MOSAIC VIRUS RNA:

Virus was maintained and propagated on *Nicotiana tabacum* c.v. White Burley in the greenhouse. Virus was prepared by the method of Godding & Hebert (1967). Systemically infected leaves were harvested 15-20 d after inoculation. These were hand-cut into small pieces and placed in a large liquidizer with viral extraction buffer (0.1M potassium phosphate, pH 7.0) in the ratio of 1g tissue/2ml buffer. Infected leaves were homogenized for about 1 m and strained through four layers of muslin. The filtrate was emulsified with n-Butanol/chloroform (1:1) in the ratio of 1ml/1g starting material, and the mixture was stirred for 10 m. The brei was centrifuged for 10 m at 6000 r.p.m. (3.670g) in an MSE 18 centrifuge at 4°C. The upper aqueous phases of each tube were combined and centrifuged at 50,000 r.p.m. (186.000g) for 45 m in the Sorvall T865 rotor at 4°C. The supernatants were discarded and the pellets were resuspended in 0.01M potassium phosphate pH 7.0 (1ml/2g starting material). The tubes were allowed to stand on ice for about 10-20 m, then the pellet was homogenized using a ground glass homogenizor. The virus suspension was once again centrifuged as above and the pellets were homogenized with 0.01M potassium phosphate pH 7.0 in the ratio of 1ml/10g starting material. The virus suspension was clarified with a final low speed centrifugation [6000 r.p.m. (3.670g) for 10m and the supernatant containing the virus was either used for extraction of RNA or stored at -70°C in aliqouts of 1ml.

One tenth volume of 10% (v/v) SDS was added to about 1-2ml virus suspension and RNA was purified by extraction with phenol/chloroform, ethanol precipitated and the concentration

determined as previously described for extraction of SYNV RNA (section 2.4.2.1).

2.6. ISOLATION OF INTACT CHLOROPLASTS FROM *N. EDWARDSONII*:

Intact chloroplasts were isolated from SYNV-infected *N. edwardsonii* and from healthy leaf tissues according to the procedure of Stokes and Walker (1971) and Leegood and Malkin (1986). All glassware was thoroughly cleaned. All medium, buffers and solutions were stored frozen and partially thawed just before use. All the steps of the procedure were carried out on ice.

Usually 12-15g of leaf tissue was harvested, hand-chopped into small pieces, homogenized with 60-75ml of extraction medium (0.33M sucrose, 10mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 50mM K_2HPO_4, pH 6.8) for about 10 s using a Polytron PCU-2 homogenizor and quickly strained through ten layers of muslin. Filtrates were transferred into conical glass tubes and centrifuged at 3.000 g for 1-2 m in a bench-top centrifuge (MSE-minor 'S', swing-out). The supernatants were discarded, pellets were washed twice by resuspending them in 2ml of 50mM tricine (pH 8.0) and 5mM EDTA, and pelleting as above to remove cytoplasmic contamination.

Chloroplasts (washed pellets) were then lysed with 2ml lysis buffer (2mM $MgCl_2$, 10mM NaCl, 15mM hydroxyethylpiperazine-N'-2-ethanesulforic acid (HEPES)-KOH, pH 7.6), a further 10ml lysis buffer was added and samples were stood on ice for about 15-30 m to ensure complete lysis. Thylakoids (pellets) and stroma (supernatant) fractions were collected by 1-2 m centrifugation as above.

Stroma fractions were combined and dialyzed against 4 changes of 1l of buffer (10mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 50mM K_2HPO_4, pH 6.8)

over a period of 24 h. They were pelletted by 30 m centrifugation at 45.000 r.p.m. (151.000g) in the Sorvall T865 rotor at 4°C. The pellet was resuspended in 2ml of extraction medium. Thylakoid fractions were resuspended in 2ml of extraction medium and the chlorophyll concentration was detrmined as follows:

Fifty µl of thylakoid suspension was added to 10ml 80% acetone, mixed well for 5 m and centrifuged at 3.000 g for 1-2 m in the bench-top centrifuge. The absorbance of the supernatant was measured at 645_{nm} and 663_{nm} using 80% acetone as a blank. The chlorophyll concentration was determined assuming that:

$$1\mu g \text{ chlorophyll/ml} = (20.2 \times A_{645}) + (8.02 \times A_{663}).$$

Both thylakoid and stroma fractions were analysed by polyacrylamide gel electrophoresis, and protein was detected immunologically as described subsequently (sections 2.10.2 & 2.13.8). The rest of the samples were occasionally stored at -196°C until required.

2.7 ISOLATION OF TOTAL CELLLULAR RNA FROM *N. EDWARDSONII*:

Leaf tissues (15g) from either healthy or SYNV-infected *N. edwardsonii* were frozen in liquid nitrogen and ground in a mortar and pestle. Ground tissue, with an excess of liquid nitrogen, was carefully poured into a sterile flask containing a mixture of 6ml 5X buffer (0.25M NaCl; 25mM sodium acetate; 5mM EDTA; 5% (W/V) SDS; pH 5.0, autoclaved before use), 24ml sterile AnalaR water and 30ml phenol/chloroform (1:1). The resultant frozen extract was thawed gradually at 65°C in a water-bath with gentle shaking and by passing the liquid up and down a sterile glass pasteur pipette for 3-5 m. The

phases were separated by 20 m centrifugation at 5.000 r.p.m. (2,570g) in the 8 x 50ml rotor of an MSE-18 centrifuge at 4°C. The upper aqueous phase was saved, the remaining phenol/chloroform phase was re-extracted as above, but with the addition of a mixture of 3ml 5X extraction buffer and 12ml sterile AnalaR water. The two aqueous phases were combined and re-extracted with an equal volume of phenol/chloroform. RNA was precipitated by addition of 2.5 volumes ethanol and one tenth volume 3M sodium acetate pH 5.5. After incubation overnight at -20°C, or occasionally at -70°C for 2-3 h, the RNA precipitate was collected by centrifugation for 15 m at 12.000 r.p.m. (14.300g) in the MSE 18 centrifuge. Precipitates were washed twice with cold 75% ethanol, vacuum dried, resuspended in 2-3ml sterile AnalaR water and stored in 0.5ml aliquots at -70°C. Before storage, the concentration of RNA was determined spectrophotometrically as described previously (section 2.4.2.1).

2.8 PREPARATION OF POLYRIBOSOMES AND EXTRACTION OF POLYADENYLATED RNA

Polyribosomes from SYNV-infected *N. edwardsonii* and from healthy leaf tissues were prepared by the method of Jackson and Larkins (1976). All steps of the procedure were carried out at 0° to 4°C. All buffers were made up in AnalaR water, autoclaved and chilled on ice before use.

2.8.1 Preparation of Polyribosomal RNA:

About 8-15g of healthy or SYNV-infected leaf tissues was harvested and ground in a pre-chilled mortar and pestle with extraction buffer (0.2M tris-HCl pH 9.0; 0.4M KCl; 0.2M sucrose; 35mM

$MgCl_2$; 25nM ethyleneglycol-bis-(β-amino-ethy ether) N,N'-tetraacetic acid (EGTA)) in the ratio of 9ml buffer per 1g tissue. The extracts were transferred into sterile pre-chilled MSE-18 centrifuge tubes and centrifuged at 15.500 r.p.m. (24.700g) for 10 m at 4°C. The supernatants were carefully overlayered onto 8ml of sucrose pad buffer (0.04M tris-HCl pH 9.0, 1.75M KCl; 0.2M sucrose, 0.03M $MgCl_2$, 5mM EGTA) in centrifuge tubes and centrifuged in the Sorvall T865 rotor at 55.000 r.p.m. (225.000g) for 90 m at 4°C. The supernatants were aspirated off leaving the glassy-green polyribosome pellets.

In order to confirm that polyribosomes were undegraded one of the pellets, was resuspended in 200µl of resuspension buffer (0.04M tris-HCl pH 8.5; 0.2M KCl; 0.03M $MgCl_2$; 5mM EGTA) and layered on the top of sucrose gradients, which were prepared from (bottom to top) 1.9ml, 3.8ml, 3.8ml and 1.9ml of 60%, 45%, 30% and 15% respectively, (w/v) sucrose in gradient buffer (0.04M tris-HCl pH 8.5; 0.02M KCl; 0.01M $MgCl_2$), and left overnight (at 4°C) to diffuse. These were centrifuged at 37.000 r.p.m. (174.000g) for 75 m at 4°C in the Sorvall TST 41.14 swing-out rotor. Gradients were fractionated with an ISCO gradient fractionator (Model 185) and monitored at 254nm with an ISCO Model UA5 absorbance monitor.

The remaining polyribosome pellets were resuspended in 5-6ml binding buffer (0.5M NaCl; 10mM HEPES, pH 7.0; 0.5%(w/v) SDS) and used to prepare polyadenylated RNA [poly(A)$^+$RNA].

2.8.2 Isolation of Poly(A)$^+$RNA by Oligo(dT)-Cellulose Chromatography:

Oligo(dT)-cellulose chromatography was carried out by the method of Krystosek *et al.* (1975) with minor modifications (Milner and Jackson, 1979).

Oligo(dT)-cellulose (0.5g, Boehringer Mannhein GmbH) was swollen in 5ml elution buffer (10mM HEPES, pH 7.5). The resultant slurry was poured into a 10 cm x 1 cm siliconized column and the column was washed with 5ml elution buffer. The oligo(dT)-cellulose was equilibrated with 20ml binding buffer (section 2.8.1).

Approximately 5-6ml of polyribosomal RNA in binding buffer (section 2.8.1) was carefully applied to the surface of the column (at 24°-26°C) and recirculated through the column for approximately one hour at 15ml/h using a peristaltic pump (EYELA Micro Tube Pump, M.P.3). Unbound material was pumped off and the column was washed with 10ml binding buffer at 15ml/h. The pump tubing was disconnected, cleaned thoroughly with 0.5M NaOH followed by elution buffer, and then reconnected. Five ml elution buffer was very carefully layered on the surface of the column, the pump was connected to the lower end of the column, and the poly(A)$^+$RNA eluted. The poly(A)$^+$RNA was precipitated by adding 2.5 volumes of ethanol and 0.1 volume of 4M potassium acetate (pH 5.5) and incubated overnight at -20°C. Poly(A)$^+$RNA was pelleted by 10 m centrifugation at 15.000 r.p.m. (23.100g) in the MSE-18 centrifuge, dried and resuspended in 0.5ml sterile AnalaR water. Poly(A)$^+$RNA was further freed of contaminating protein by phenol/chloroform extraction, precipitated, washed, and the concentration determined spectrophotometrically as described previously (section 2.4.2.1). After use, the column was stored at -20°C, and thoroughly washed with 10ml 0.5M NaOH followed by equilibration (as above) before re-use.

2.9 ELECTRON MICROSCOPY:

2.9.1 Preparation of Thin Sections:

2.9.1.1 Standard Preparation:

Samples of healthy or SYNV-infected tissues (approximately 1-2 mm square pieces) were fixed in 3% (v/v) gluteraldehyde in 0.2M sodium cacodylate buffer pH 7.2 at room temperature for 12-16 h. Samples were initially infiltrated under vacuum to remove air and allow penetration of fixative. Samples were then thoroughly washed in 0.2M sodium cacodylate buffer pH 7.2 (four changes over 24 h) and post-fixed in 1% (v/v) OsO_4 in 0.2M sodium cacodylate buffer pH 7.2 for 3 h. Samples were washed in distilled water (3 changes over 30 m) and then block-stained in 2% (w/v) aqeuous uranyl acetate for 2 h.

Samples were dehydrated through a graduated ethanol series (25%, 50%, 75% for 2 h each and overnight in 100%), embedded in Spurr resin (Spurr, 1969) and polymerized at 60°C for 24 h. Sections of approximately 60 nm thickness were cut on an LKB III ultramicrotome using a glass knife and mounted on 300 mesh copper grids. Sections were stained with saturated uranyl acetate in 50% methanol for 20 m and lead citrate (Reynolds, 1963) for 5 m. They were then examined in a Philips EM 301 electron microscope at 60 and 80KV. Negatives were prepared on Kodak positive release 35mm film at various magnifications and printed on Kentmere photographic paper.

2.9.1.2 Preparations of Samples for Immunogold labelling:

Up to the post-gluteraldehyde buffer rinse, samples were treated identically to the standard preparation method (section 2.9.1.1), but post-fixation with OsO_4 and block-staining with uranyl acetate were omitted. Dehydration, embedding and staining were as in

the standard method (section 2.9.1.1), but sections were mounted on 300 mesh nickel grids.

2.9.2 Preparation of Samples for Negative Staining :

For rapid diagnoses of SYNV-infected plants, sap was expressed from leaves and examined for virions using either of the following two methods.

2.9.2.1 Negative Staining:

Samples (approximately 2mm square pieces) of various parts of the plant were individually crushed with the end of a slender glass rod in about 10-15µl of 0.1M tris-HCl buffer pH 7.5 on a glass slide. A formvar-carbon coated 200 mesh grid was touched to the resultant sap for 5-10 s, grids were jet rinsed with distilled water and drained on a small piece of Whatman No.1 paper. Grids were touched either to a droplet of 1% aqueous uranyl acetate or a droplet of 1% aqueous phosphotungstate for 5-10 s then rinsed with distilled water, drained, dried and examined in the electron microscope at 60 KV.

2.9.2.2 Decoration:

The use of "decoration" (Milne & Lesemann, 1984) identifies the particles very positively and renders them highly visible even with low concentrations of virus in the applied sample.

The procedure is identical to that normal negative staining, but once the samples had been applied to the grids and rinsed with water they were then incubated at room temperature for 30-60 m in a droplet of 25µl anti-SYNV antiserum (section 2.13.1) diluted to 1:10

with 0.1M tris-HCl pH 7.5. Grids were monitored, stained and examined as described above.

2.10 GEL ELECTROPHORESIS:

2.10.1 Electrophoresis of RNA on Agarose-Formaldehyde Gels:

RNA was fractionated on 1% agarose-formaldehyde gels as described by Gustafson *et al.* (1982). To prepare the gel, the following components were combined, boiled for 5 m and cooled to 60°C.

4ml · 10X runing gel buffer (0.4M HEPES; 0.1M sodium acetate; 0.01M EDTA, pH 7.8).

29.2ml AnalaR water.

0.4g agarose (Type I Sigma).

Once the agarose mixture had cooled to 60°C, 6.66ml of 37% formaldehyde was added, mixed well, and the gel cast in a BRL mini gel (5 X 7.5 cm) apparatus. Care was taken to avoid any trapped air bubbles either underneath the comb teeth or in the gel body.

RNA samples were diluted to 1mg/ml and freshly prepared buffer X (5:1:2, deionized formamide: 10X runing gel buffer: 37% formaldehyde respectively) was added in the ratio of 1 volume RNA sample to 5.7 volumes buffer X. Samples were denatured by incubation at 60°-65°C for 10 m and chilled quickly on ice. To these denatured samples, dye (0.2% (w/v) bromophenol blue in 1:5:4 10X runing gel buffer: glycerol: water) was added in the ratio of 1.5 volume dye to 13.5 volumes denatured RNA solution. Samples (12-25 µl each) were applied to the walls, and the gels were run at 100V in the submarine mode until the dye front had nearly reached the end of the gel.

Gels were stained for 30 m in 0.2% (w/v) toluidine blue in 0.4M acetic acid, 0.4M sodium acetate and destained either in water or 10% acetic acid.

2.10.1.1 Northern Blotting (RNA):

RNA was fractionated by agarose-gel-electrophoresis (section 2.10.1) and blotted onto Biodyne nylon membrane (Pall Ltd) using a similar procedure to that described for blotting of protein (section 2.13.8) except that 20X SSC (3M NaCl; 0.3M sodium citrate; pH 7.0) was used as transfer buffer. The RNA was fixed to the membrane by baking for 1-2 h at 80°C. Blots were hybridized to a suitable probe as described in section 2.14.2.

2.10.2 Electrophoresis of Proteins on Polyacrylamide Gels:

Proteins (virus proteins, total cellular plant proteins, *in vivo* labelled and *in vitro* synthesised proteins, and immunoprecipitated proteins) were fractionated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970).

Gels were formed between two glass plates (19.5 X 18 cm and 16.5 X 18 cm, separated by 1.0 mm thick Teflon spacers) which had been thoroughly washed with detergent (Decon 90), rinsed with distilled water, wiped with ethanol and air dried before use. The following stock solutions were prepared and stored at 4°C:

Acrylamide:	25%(w/v)	acrylamide (Koch-Light)
	0.66%(w/v)	N,'N methylene-bis-acrylamide
Stacking Gel Buffer:	0.5M	tris-HCl, pH 8.3
	0.4%(w/v)	SDS

Separating Gel Buffer:	1.5M	tris-HCl pH 8.8
	0.4%(w/v)	SDS
Electrolyte Buffer:	25mM	tris-base
	0.192M	glycine
	0.2%(w/v)	SDS
	pH adjusted to 8.3-8.4 with HCl	
Boiling Buffer:	62.5mM	tris-HCl pH 8.0
	2%(w/v)	SDS
	10%(v/v)	glycerol
	2%(v/v)	2-mercaptoethanol
	0.001%(w/v)	bromophenol blue

Gels were formed with either a 10% or 12.5% separating gel and 4.5% stacking gel using stock solutions as indicated in table 2-2.

Table 2-2

solution	separating gel		stacking gel
	10%	12.5%	4.5%
acrylamide	16ml	25ml	2ml
separating gel buffer	10ml	12.5ml	-
stacking gel buffer	-	-	3.1ml
distilled H_2O	14ml	12.5ml	7.4ml
10%(W/V) APS*	150µl	200µl	100µl
TEMED**	40µl	50µl	25µl

* = ammonium persulphate, freshly prepared.

**= N'N'N'N'-tetramethylethylenediamene.

Both separating and stacking gel solutions were assembled on ice. The separating gel solution was first poured between the pre-assembled plates, leaving about twice the length of the comb teeth to form the stacking gel. Four to six ml of either water, 50% (v/v) methanol in water or isopropanol were carefully applied to the top of the separating gel to form a flat interface. After the gel had polymerized, this interface was washed with distilled water and stacking gel was added and allowed to polymerize.

Polypeptides were denatured for gel electrophoresis by adding 1-2 volumes of boiling solution and boiling for 2-5 m.

Standard marker proteins of known molecular weight (MW) were run on each gel. For use with non-radioactive polypeptides the following were used:

High Molecular Weight Standard Mixture (Sigma)

Protein	Approximate MW
carbonic anhydrase from bovine erythrocytes	29.000
albumin, egg	45.000
albumin, bovine	66.000
phosphorylase b from rabbit muscle	97.000
β-galactosidase from *Escherichia coli*	116.000
myosin from rabbit muscle	205.000

Low Molecular Weight Standard Mixture (Dalton Mark VII-L™, Sigma)

Protein	Approximate MW
α-lactalbumin	14.200
trypsin inhibitor, soyabean	20.100
trypsinogen, PMSF treated	24.000

carbonic anhydrase from bovine erythrocytes	29.000
glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle	36.000
albumin, egg	45.000
albumin, bovine	66.000

Gels were either stained overnight with approximately 5 volumes of 0.2% Coomassie Brilliant Blue R250 in 10% acetic acid and 7% methanol and destained in 10% acetic acid for three changes over a period of 24 h, or were blotted onto a nitrocellulose membrane (section 2.13.8).

For use with radioactive polypeptides, the same molecular weight standards labelled *in vitro* with ^{125}I were kindly provided by Dr. J.J. Milner.

2.11 *IN VITRO* PROTEIN SYNTHESIS IN CELL-FREE WHEAT GERM SYSTEM.

The method used to prepare wheat germ cell-free extract was that of Davies *et al.* (1977). All operations were carried out at 0 to 4°C in thoroughly cleaned sterile equipment.

2.11.1 Preparation of S-30 Wheat Germ Extract.

Working in a fume cupboard, 75ml cyclohexane was mixed with 250ml carbon tetrachloride in a clean 500ml beaker. About 5g of wheat germ (obtained from General Mills, Vallejo, California) was stirred into the above mixture for about 5 m. That which floated was collected using a tea strainer (all germ sinks if left too long), spread out on 3MM Whatman filter paper and left to dry. Any obvious bits of non-embryo material were discarded. Only that which had a

pale yellow colour with little brown (chaff) or white (endosperm) material was used. A proportion of this germ was used for next stage, the rest was stored in a desiccator at 4°C until required.

A half gram of floated germ was ground with the tip of a sterile pasteur pipette in a pre-chilled sterile mortar and pestle for 15 s, then 1ml griding buffer (0.5mM HEPES pH 6.4-6.9; 12mM potassium acetate; 0.5mM magnesium acetate; 1mM dithiothreitol (DTT)) was added and ground for a further 1 m. Grinding buffer was prepared as 10X stock, and DTT was added just before use. A further 1ml grinding buffer was added to the thick paste and grinding was continued for 1 m more. The final slurry was mixed with an additional 1ml grinding buffer and transferred to an ice cold 15ml Corex centrifuge tube using a further 2ml grinding buffer to wash out the mortar. Wheat germ slurry was centrifuged at 16.000 r.p.m. (26.300g) for 10 m in an 8 X 50ml fixed angle rotor of an MSE-18 centrifuge. The supernatant was carefully transferred (avoiding the pellet as much as possible) to another 15ml ice cold Corex tube contained 0.05ml 0.5M HEPES pH 7.5 and centrifuged for further 15 m at 16.000 r.p.m. (26.300g) Once again, using a sterile long-tipped pasteur pipette, the supernatant, which had a clear yellow-green colour, was collected (avoiding the upper lipid pellicle and the pellet), transferred to a pre-chilled tube, and then to dialysis tubing (for preparation of dialysis tubing see section 2.2.3). The solution was dialysed overnight against two changes of 1l of ice-cold dialysis buffer (20mM tris acetate pH 7.6; 120mM potassium acetate; 5mM magnesium acetate; 1mM DTT). The dialysis was carried out in a sterile conical flask which had been buried in ice and set on a magnetic stirrer in the 4°C room. The dialysed wheat germ extract was transferred to a pre-chilled 15ml

Corex tube and centrifuged at 5.000 r.p.m. (2.570g) for 5 m in the MSE-18 centrifuge. The supernatant was, divided into 0.5ml aliquots and stored at -196°C.

2.11.2 *In Vitro* Translation Of Poly(A)$^+$RNA.

The following stock solutions were used:

S-30 Wheat germ cell-free extract (prepared as in section 2.11.1)

MIX "6" which was prepared from stock solutions table 2-3.

HKMS was prepared from stock solutions as in table 2-4.

Table 2-3 Preparation of MIX "6".

stock solution	volume added (ml)
30mM guanosine triphosphate(GTP)	0.05
1mM amino acids required for protein synthesis (minus methionine & leucine).	0.15
0.1M adenosine triphosphate (ATP)	0.1
0.8M creatine phosphate	0.045
8mg/ml creatine phosphokinase	0.005
5mM leucine	0.03
sterile AnalaR water	0.02

GTP and ATP were neutralized with 2M KOH (0.01ml/ml solution, 0.05ml/ml solution respectively) before use. MIX "6" was stored in 0.5ml aliquots at -20°C

Table 2-4 Preparation of HKMS.

stock solution	volume added	final concentration
0.5M HEPES (pH 7.6)	4 ml	0.2M
2M potassium acetate	2 ml	0.4M
0.2M magnesium acetate	0.2 ml	4mM
spermidine tris-HCl	10.5mg	4mM
sterile AnalaR water	3.8 ml	-

HKMS was stored in 0.5 aliquots at -20°C.

The *in vitro* translation incubation mixtures were assembled in an 0.5ml sterile eppendorf vial as shown in table 2-5. This preparation is sufficient for 8 reactions, and may be scaled up or down according to requirements.

Table 2-5.

component	µl required
S-30 Wheat germ extract	100
MIX"6"	20
HKMS	20
L-(^{35}S) methionine (NEN >1000 Ci/mmo)	100-200µci
sterile AnalaR water	to gave a final volume of 160µl

Five µl of each RNA at a concentration of 0.5mg/ml, was added to 20µl of the above translation incubation mixture in individual sterile eppendorf vials, mixed and then incubated for 90 m at 30°C.

2.11.3 Analysis of *In Vitro* Translation Products:

Translation products were analysed using three methods.

2.11.3.1 Determination of Incorporation of ^{35}S-Methionine into Protein:

The incorporation of ^{35}S-methionine into protein was determined by measuring trichloroacetic acid (TCA) precipitable radioactivily. Using a positive displacement micropipettor (BCL PDM8), duplicate aliquots of 2µl sample from each incubation vial were individually added to 1ml of freshly prepared bleach-stop reagent (0.33M NaOH; 1.66% H_2O_2 "100 volumes"; 0.33mg L-methionine). Samples were incubated at 37°C for 10-15 m. The proteins were precipitated by addition of 1ml of 25% (w/v) TCA followed by addition of 10µl 2% (w/v) BSA (pH 7.0) as carrier. To ensure complete precipitation, the samples were incubated on ice for 15-20 m. Precipitated proteins were collected by vacuum filtration on Whatman GF/C glass microfibre filters (2.5 cm diameter) which had been pre-washed with about 3-5ml 8% (w/v) TCA. After filtration of the protein, the filter pads with precipitates were washed twice with 10ml 8% TCA followed by 10ml ethanol, placed individually in scintillation vials and dried at 80°C for 10 m under vacuum. Four ml of liquid scintillation fluid (Koch-Light, Unisolve E) was added and radioactivity was measured in a Packard, TRI-CARB liquid scintillation counter.

2.11.3.2 Immunoprecipitation of Viral Proteins:

This was carried out using the method of Ziegler *et al.* (1985) with minor modifications. To a sterile microcentrifuge tube containing the *in vitro* translation reaction (usually about 10-15 µl) equivalent to 1-2x10^6 TCA precipitable counts/m (c.p.m.), 0.2ml PBSTDS (10mM $NaHPO_4$ pH 7.2; 0.9% (w/v) NaCl; 1% (w/v) Triton X-100; 0.5% (w/v) sodium deoxycholate; 1% (w/v) SDS) was added. Ten µl of anti-SYNV serum (section 2.13.1) was added to the above mixture and the vials were then incubated at 4°C overnight to allow the antibodies to bind to the antigens. Ten mg of either protein A-sepharose or protein A-agarose (Sigma) was directly added to each vial. Vials were tumbled in a rotary mixer for about 2-3 h at 4°C to allow the protein A-sepharose to bind to the complexes. Protein A-sepharose and bound immunoprecipitated proteins were collected by centrifugation for 4 m in the microcentrifuge (11.600g). The pellets were washed twice with 0.2ml PBSTDS. Immunoprecipitates were prepared for gel electrophoresis by resuspending the wet pellets (protein A-sepharose-antibody-antigen complexes) in 50 µl of boiling buffer (section 2.10.2) and boiling for 2-5 m. Twenty five µl per track were loaded onto polyacrylamide gel (section 2.10.2).

Total *in vitro* synthesised protein were prepared for gel electrophoresis by adding boiling buffer (section 2.10.2) to the *in vitro* translation reactions in a ratio of 2:1. Samples were boiled for 2-5 m and an appropriate quantity to give about 200.000 TCA precipitable c.p.m. was added per track.

2.11.3.3 Fractionation of *In Vitro* Synthesised and Immunoprecipitated Proteins:

The radiolabelled polypeptides were fractionated by SDS-PAGE. After electrophoresis, gels were subjected to fluorography by the procedure of Bonner and Laskey (1974). Proteins were fixed and unreacted methionine was removed by washing the gels twice (30 m each wash or occasionally overnight) in 7% (v/v) acetic acid. Gels were then dehydrated by three washes in about 500ml dimethylsulphoxide (DMSO) (30 m per wash) followed by soaking in 22% (w/v) 2,4 diphenyloxazole (PPO, scintillant grade) in DMSO at 37°C with continuous gentle shaking for 2-2.5 h, (PPO is insoluble in water but very soluble in DMSO). PPO was precipitated within the gels by washing in gently running tap water overnight. Gels were then dried on Whatman 3MM filter paper using a Bio-Rad Model 483 slab gel dryer at 60°C for 90 m. Gels were exposed to pre-flashed Fuji RX X-ray films at -70°C for 24-72 h.

2.12. *IN VIVO* LABELLING OF PROTEIN:

Discs (2 mm diameter) were cut from leaf tissue. A half gram was placed into 50 mm petri dishs (single vent) containing 2ml sterile distilled water and 50µci L-^{35}S methionine (NEN, >1000 ci/mmol^{-1}), vacuum infiltrated for 2-3 m and incubated in a growth chamber for 4-6 h under environmental conditions identical to those used for growing SYNV-infected plants. Samples were drained, quick frozen in -196°C for 10 m and then ground in 0.2ml buffer (62.5mM tris-HCl pH 6.8; 2% SDS) using a small glass rod homogenizer. Extracts were clarified by centrifugation for 2 m in the microcentrifuge

(11.600g). Supernatants were saved and analysed by SDS-PAGE for both total solubilized proteins and immunoprecipitated protein, exactly as described for *in vitro* protein synthesis (section 2.11.3).

2.13 DETECTION OF SONCHUS YELLOW NET VIRUS PROTEIN:

2.13.1 Preparation of Anti-Sonchus Yellow Net Virus Antisera:

Anti-SYNV antisera preparations, from injections of the rabbit to preparation of sera, were kindly carried out by Dr. I.D. Hamilton (Department of Biochemistry, University of Glasgow).

SYNV was purified from *N. edwardsonii* leaf tissues and the concentration of virus protein was determined (sections 2.4.1 & 2.4.3). Purified virus (380µg protein) was emulsified in 50% Freund's complete adjuvant and injected subcutaneously at many sites in a New Zealand white rabbit. Six weeks later the animal was boosted with a similar injection of virus (380µg protein) emulsified in 50% Freund's incomplete adjuvant. Approximately one year later the animal was boosted with virus (250µg protein) in 50% Freund's incomplete adjuvant. After 14 d, blood was taken from the animal, allowed to coagulate, the clot removed, and the whole sera stored in 1ml aliqouts at -20°C.

2.13.2 Pre-Absorption of Anti-SYNV Antiserum:

Despite the care taken during the virus purification, the virus preparations may contain low levels of contaminating plant proteins, and the presence within antisera of antibodies to these proteins may give misleading results. These antibodies to the host

protein were removed from anti-SYNV serum by incubating the latter overnight at 4°C with crude sap from healthy *N. edwardsonii* plants in a ratio of 1:4. Pre-absorbed antiserum was recovered by two 5 m centrifugation at 11.600g in the microcentrifuge. This step removes precipitates formed from the reaction between antibodies to host proteins present in the healthy sap. The supernatant was then stored in 0.5ml aliqouts at -20°C, and each sample was given a brief centrifugation (15-30 s) before use.

Occasionally, healthy plant sap (8-10µl) was soaked up onto small squares (approximately 2.5 x 2.5 mm) of nitrocellulose membrane (Schleicher & Schull, BA 85). These were air dried, stored at 4°C for 15 m to stabilize the binding of the proteins to the membranes and then squeezed into an eppendorf vial containing anti-SYNV antiserum at 4°C. The pre-absorbed anti-SYNV antiserum was recovered, centrifuged for 2 m to remove any detached crude sap and the resultant supernatant was stored as above.

2.13.3 Preparation of Immunoglobulin G (IgG) from Anti-SYNV Serum:

IgG was prepared from anti-SYNV antiserum by salt precipitation and DEAE-cellulose filtration by the method described by Clark *et al.* (1986). All equipment used throughout this procedure was sterilized.

Four ml of anti-SYNV serum was diluted in 16ml sterile AnalaR water. Serum was precipitated by addition of 20ml 36% (w/v) $(NH_4)_2SO_3$, and mixed at room temperature until a flocculent precipitate developed (usually after 45-75 m). The precipitate was pelleted and collected by 10-15 m centrifugation at 3.000 r.p.m. (950g) in the MSE-18 centrifuge at room temperature. The pellet was dissolved in 20ml

sterile AnalaR water and reprecipitated with 20ml 36% $(NH_4)_2SO_3$ as above. The precipitate was dissolved in 4ml half-strength PBS (1:1, PBS:sterile water. PBS is 0.136M NaCl; 9.2mM Na_2HPO_4; 0.87mM KH_2PO_4 and 2.68mM KCl; pH 7.4), transferred to dialysis tubing and dialysed against 4 changes of 500ml of half-strength PBS over a period of 32 h. The recovered IgG preparation had a bluish haze indicating the presence of lipid contaminants.

To remove lipids, the IgG preparation was subjected to DEAE-cellulose chromatography. Five grams of DEAE-cellulose (Sigma) were swollen in 100ml PBS, autoclaved, cooled to room temperature and poured into 10 cm x 1 cm column. The column was equilibrated with half-strength PBS and the effluent monitored spectrophotometrically at 280_{nm} until the absorbance was zero. The IgG preparation (4ml) was carefully applied on to the top of the column, and the protein eluted with half-strength PBS. Fractions of 1ml (25 drops each) were collected and monitored spectophotometrically at 280_{nm}. The first 3-4 fractions containing the protein peak ($A280_{nm}>0.1$) were combined and diluted with half-strength PBS to $A_{280} = 1.4$, equivalent to 1mg/ml.

The final IgG preparation had a water-clear appearance and an A_{280}: A_{252} ratio of 2.54. IgG was stored in 0.5ml aliqouts at -20°C.

IgG, from the same batch of anti-SYNV antiserum, purified by affinity chromatography on a protein-A sepharose column, was a kind gift from Dr. I.D. Hamilton.

2.13.4 Preparation of Protein A-Gold Probe:

Staphylococcus aureus (Covan strain) protein A (Sigma) was complexed with chloroauric acid (about 51% Au, BDH) according to the

procedure of Frens (1973). Prior to the preparation, all glassware was soaked in 0.5M NaOH for 15 m, thoroughly washed with sterile distilled water, dried and then autoclaved.

2.13.4.1 Preparation of Gold Sol:

Chloroauric acid was made up to 4% (w/v) with sterile AnalaR water, and stored at 4°C. A 25µl aliqout of the above stock was diluted to 0.1% by adding sterile AnalaR water, boiled for 2 m, and 0.75ml of 1% (w/v) sodium citrate was added in order to reduce the $HAuCl_2$. The mixture was boiled for another 5 m, until a deep red colour developed. The mixture was allowed to cool to room temperature and the pH adjusted to 7.0 with 0.2M potassium carbonate.

2.13.4.2 Preparation of Protein A Solution:

Protein A was dissolved in 5mM NaCl at a concentration of 0.05mg/ml and stored at -20°C until required.

2.13.4.3 Preparation of Protein A-Gold Complex (PA/G):

The optimal amount of protein A required to stabilize a certain volume of colloidal gold has to be determined by titration. The test involves preparation of a dilution series with small volumes of protein A, addition of a standard amount of gold colloid, addition of a standard amount of salt to destabilize the colloid and then determination of the amount of protein A that is just enough to protect the colloid against salt destabilization (the end point). This destabilization point was marked by a colour change from red to the blue and could be quantitated by an increase in A_{550}.

Dilutions of protein A were made in 0.1ml aliquots of 0.5mM NaCl as shown in table 2-6.

Table 2-6

Protein A(µl) (0.05mg/ml)	100	90	80	70	60	50	40	30	20	10
5mM NaCl (µl)	0	10	20	30	40	50	60	70	80	90

Colloidal gold, pH 7.0 (0.5ml) was added to each dilution. After vortexing and standing for 3 m, 0.1ml of 10% (w/v) NaCl was added. Five to seven minutes later the end point was determined (the lowest dilution giving unchanged colour) either by eye or spectrophotometrically at 550_{nm}. In addition the serial dilutions of protein A complexed to colloidal gold were examined in the electron microscope (Baschong *et al.*, 1985). Five µl of each dilution were individiually placed on formvar-carbon coated 200 mesh copper grids for 5 m. The grids were jet washed with sterile AnalaR water for 10 s, air dried and inspected for aggregates.

The optimal amount of protein A to stabilize the gold sol was taken to be 1.2 times the amount required to reach the above end point (I.M. Roberts, personal communication) and was found to be 144µg protein A per 40ml colloidal gold solution. The protein A-gold complex was made up to 1% (w/v) polyethylene glycol (PEG, MW 6.000) and centrifuged at 11.500 r.p.m. (17.600g) for 1 h at 4°C in the

Sorvall AH629 rotor. The loose pellets were combined and resuspended in 2-2.5ml 0.2mg/ml PEG 6,000 in PBS, giving a deep red solution. The protein A-gold probe was stored at 4°C.

2.13.5 Immunogold Labelling of Cell and Tissue for Electron Microscopy

SYNV-infected and healthy *N. edwardsonii* tissues were fixed in glutaraldehyde, embedded in Spurr resin, sectioned and mounted on nickel grids as previously described (sections 2.9.1.1 & 2.9.1.2). Immunogold labelling of these sections was carried out at room temperature in petri dishes containing sheets of dental wax (Agar Aids). All buffers, solutions and distilled water were filtred through Whatman No. 1 filter paper before use. Grids were handled throughout this procedure with sections face downwards.

For good penetration of the immunogold label into the sections, the latter were etched by floating for 20-30 m on droplets of saturated sodium metaperiodate, washed twice for 5 m with distilled water and the grids refloated on droplets of 0.1N HCl for 10 m. Grids were then washed as above and immunolabelling was carried out using either gold conjugated to goat-anti-rabbit IgG (Janssen Life Science) or gold conjugated to protein A (prepared as previously described in section 2.13.4) according to the procedure of Craig & Goodchild (1982) with minor modefications.

Grids were placed for 30-45 m on the surface of 20-30µl droplets of immunogold labelling buffer (IGL buffer is 20mM tris-HCl; 0.5M NaCl; 0.05% (v/v) Tween 20; 0.1% (w/v) BSA; pH 7.4). Five percent normal goat serum (Scottish Antibody Production Unit) was added to IGL when gold conjugated goat-anti-rabbit IgG was used, but omitted when gold conjugated protein A was used. The primary

incubation was carried out by incubating the grids for 90 m on the surface of 25µl droplets of anti-SYNV IgG (section 2.13.3) diluted to 1:15 with IGL buffer. Unbound IgG was removed by washing the grids 3-5 times in a series of 50µl droplets of IGL buffer for 5 m each. The secondary incubation with gold conjugated to goat-anti-rabbit IgG, or to protein A, was carried out by incubating the grids for 90 m on the surface of a 25µl droplets of gold conjugated goat-anti-rabbit IgG or gold conjugated protein A diluted 1:10-1:20 with IGL buffer. Grids were washed thoroughly in a series of five 50µl droplets of AnalaR water (5 m each), drained onto filter paper and dried at room temperature. Sections were stained with uranyl acetate and lead citrate and examined by electron microscopy as previously described (section 2.9.1.1).

Two controls were carried out with both healthy and infected tissues. These were:

1- Anti-SYNV IgG was omitted and replaced with IGL buffer.

2- Anti-SYNV IgG was replaced by non-immune rabbit serum.

2.13.6 Enzyme-Linked-Immunosorbant Assay (ELISA) for Virus Protein:

2.13.6.1 Preparation of Sap Samples for ELISA:

For preparation of samples from infected tissues. Plants which had been inoculated as described in section 2.3.2 were randomly divided into three batches. Leaves which had been inoculated were marked with a tag. At various times after inoculation, samples of (i) inoculated leaves, (ii) uninoculated leaves, which were fully expanded at time of inoculation (referred to as expanded leaves), (iii) leaves which were less than 1cm long at the time of inoculation (referred to

as unexpanded leaves) and (iv) roots, were taken from one plant out of each batch and sap prepared as follows:

One gram of plant material was homogenized, in a glass rod homogenizer, with 10ml of PBS-TPO (0.136M NaCl; 9.2mM Na_2HPO_4; 0.87mM KH_2PO_4; 2.68mM KCl; 0.05% (v/v) Tween-20; 2% (w/v) polyvinylpyrollidone (PVP) and 0.2% (w/v) BSA; pH 7.2; Clark *et al.*, 1986) and clarified by centrifugation for 2 m in a microcentrigue. All sample preparation steps were carried out at 0 to 4°C and samples were stored at -20°C. One hundred µl was taken from each sample, made up to 2% SDS, heated to 65°C for 15 m and cooled to room temperature. Ten µl of each denatured sample was diluted (1:50 to 1:500) with coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) containing 5% (v/v) normal donkey serum (Scottish Antibody Production Unit).

2.13.6.2 ELISA procedure:

ELISA was carried out according to the method of Lommel *et al.* (1982) as detailed by Clark *et al.* (1986), with minor modifications. Duplicate 200µl dilutions of each denatured sample were bound to the microtitre wells (U-well plate, Sterilin Ltd, Feltham, England) by incubating 75 to 90 m at 35°C. To remove unattached antigen, wells were emptied, filled with washing buffer (0.154m NaCl; 0.05% (v/v) Tween-20; pH 7.4) and left for 1 m. Three further washes were carried out, any the residual liquid was shaken out and the plates were dried by inverting them on paper tissue at room temperature. Preabsorbed anti-SYNV antiserum (section 2.13.2) was diluted 1:75 with PBS-TPO containing 5% (v/v) normal donkey serum, 200µl aliquots were added to each well and the plates were incubated, washed and dried as above. Donkey anti-rabbit IgG conjugated to horseradish peroxidase (Scottish

Antibody Production Unit) was diluted 1:1000 with PBS-TPO containing 5% (v/v) normal donkey serum and 200µl added to each well. Plates were incubated, washed and dried as above.

The plates were developed by adding 150µl o-phenylenediamine (0.5mg/ml) in 25mM sodium acetate pH 5.5, 0.03% (v/v) H_2O_2 "100 volumes" for 5 to 10 m in the dark. The reactions were stopped by adding 50µl of 4M H_2SO_4. The plates were shaken gently for 2 m and the absorbance at 492_{nm} was determined using a Titertek Multiscan MC plate reader.

Known concentrations of SYNV virus protein, as determined by the Coomassie Brilliant Blue binding assay (section 2.4.3), and sap samples from healthy plants were run in parallel with the above samples on each plate.

Controls were performed to confirm the specificity of the reactions between antibody and antigen. These were:

1- Anti SYNV serum was omitted and replaced with PBS-TPO buffer.

2- Anti SYNV serum was replaced by non-immune rabbit serum.

2.13.7 Dot-Immunobinding Assay:

The dot-immunobinding assay (DIBA) (Hawkes *et al.*, 1982) is a modified form of immunoblotting technique of Towbin *et al.* (1979). This simple technique has been described by various names, such as the dot-immunosorbent assay (Dot-ELISA) and the nitrocellulose-immunosorbent assay (NC-ELISA), reported by Pappas *et al.* (1983) and Bode *et al.* (1984) respectively. The principles of DIBA, under any name, are the same as these of ELISA, differing only in that antigen

is bound to a nitrocellulose membrane and that the coloured product of the enzyme reaction is insoluble.

Samples from infected and healthy tissues were prepared by grinding 1g of tissue in 5ml TBS buffer (0.05M tris-HCl, 0.2M NaCl, pH 7.4), and denatured as previously described for ELISA (section 2.13.6.1). A appropriate series of dilutions of the samples was made in TBS buffer. Squares (1 x 1 cm) were drawn on a piece of nitrocellulose membrane (filter) using a pencil and 2µl of each dilution was spotted onto a square. Filters were air dried at room temperature and the binding antigens to the filters stabilized by incubating them 15 to 20 m at 4°C. Non-specific binding sites were blocked with blocking solution (3% (w/v) BSA, made up with TBS, containing 5% (v/v) normal donkey serum). Filters were individually placed into convenient trays containing blocking solution (0.5ml/cm^2) and incubated at room temperature with gentle shaking for 10 to 20 m. Blocking solution was removed by aspiration and the filters reacted with anti-SYNV antiserum. Pre-absorbed serum (section 2.13.2) was diluted 1:500 with blocking solution, 0.5ml of this per cm^2 was added and the filters incubated as above but for 90 m. Unbound antiserum was removed by three washes of 5m each with TBS buffer (2ml/cm^2) with gentle shaking . Filters were soaked with blocking solution as above. Donkey anti-rabbit IgG conjugated with horseradish peroxidase (Scottish Antibody Production Unit) was diluted 1:1000 with blocking solution, 0.5ml of this per cm^2 was added and filters incubated for 90 m as above. Unreacted second antibody was removed by five washes with TBS buffer as above. To detect the antibody-antigen complex, 4-chloro-1-naphthol (97%; Aldrich Chemical Co. Ltd; 3mg/ml in methanol) was diluted 1:4 with TBS containing 0.01-0.03% (v/v) H_2O_2

"100 volumes". This was prepared freshly on each occasion. Filters was incubated with 0.5ml/cm^2 of the above developer. Positive signals appeared as blue spots against the white background of the membrane. When the reactions were complete, filters were washed twice with about 1l of distilled water, dried between 3MM Whatman paper at room temperature and protected from the light until ready for photography.

2.13.8 Immunoblotting of Sonchus Yellow Net Virus Protein:

Protein samples (e.g. sap from healthy and infected *N. edwardsonii* plants, purified SYNV) were prepared and fractionated by polyacrylamide gel elecrtophoresis as described previously (section 2.10.2). Proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell,BA 85) by the contact-diffusion method (Bowen *et al.*, 1980).

A plastic box (10 x 5 x 5 cm) was centrally located inside a larger plastic tray. A glass plate (17 x 15 cm) was placed on top of the box, and a piece of 3MM Whatman paper (35 x 15 cm) was layered over the glass plate, forming a bridge (see fig. 2-1). One l of transfer buffer (25mM tris; 192mM glycine; 20% (v/v) methanol) (Towbin *et al.*, 1979; Burnette, 1981) was poured into the tray and over the bridge.

After running the gel, the stacking gel was removed, and the separating gel was carefully placed on top of the pre-wetted 3MM Whatman paper bridge. A piece of nitrocellulose membrane, cut to a size 1cm longer than the separating gel on each side, was pre-wetted with transfer buffer and carefully layered on the top of the gel. Care was taken to avoid any air bubbles between the 3MM paper and the separating gel, and between the gel and the membrane. Ten dry pieces

of 3MM Whatman paper (the same size as the separating gel) were layered on the top of the membrane, followed by a glass plate (17 x 15 cm) and about a 2 to 2.5 kg weight to ensure a good contact between the gel and the membrane. This was left for 24 to 36 h at room temperature to allow the protein to transfer onto the membrane. The membrane was removed, air dried, and the proteins were fixed to the membrane by 15 m incubation at 4°C (Hawkes *et al.*, 1982). Protein immunodetection was carried out as previously described for dot-immunobinding assay (section 2.13.7).

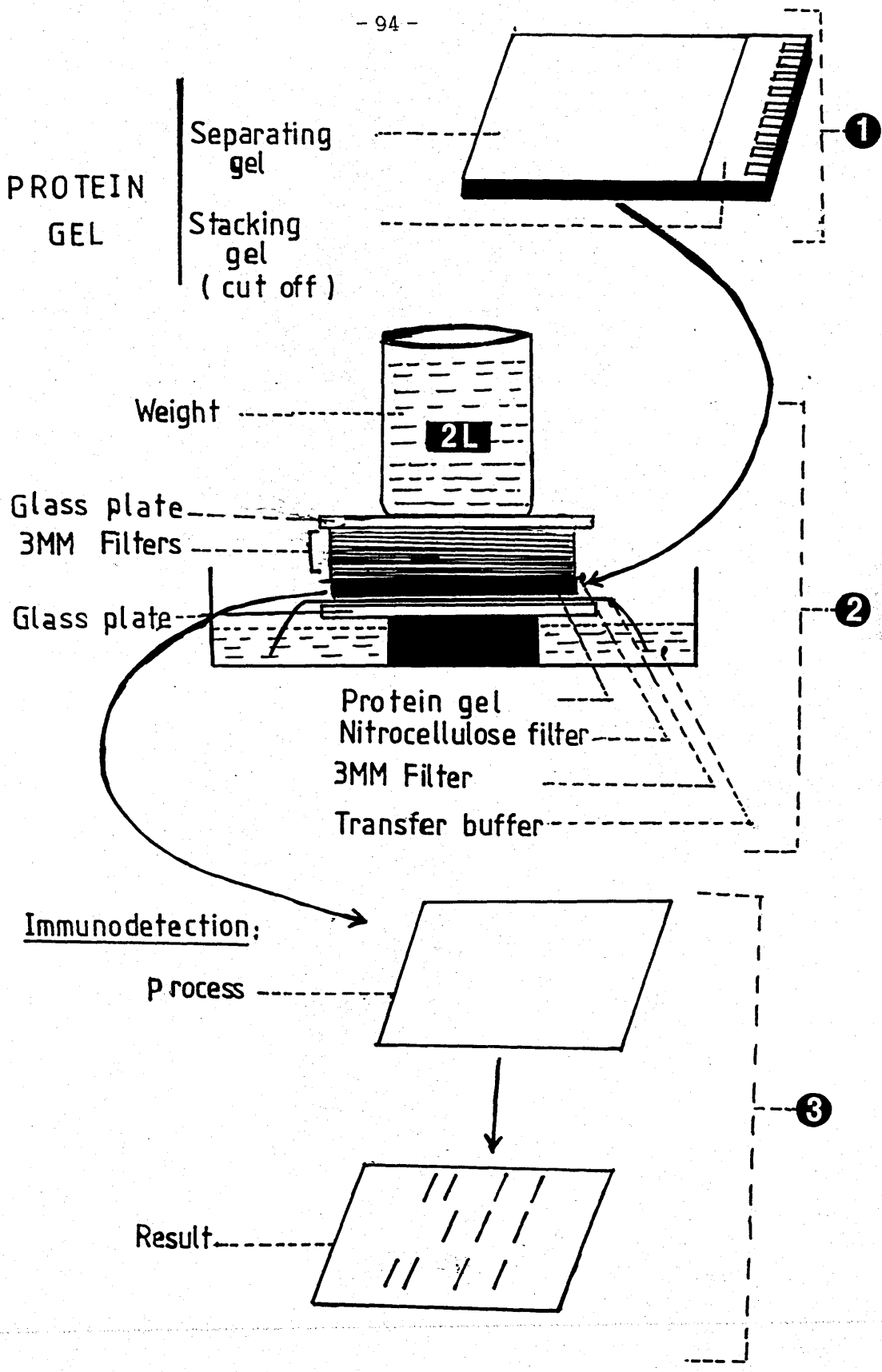


Figure 2-1:

Diagrammatic representation of protein immunoblotting.

2.14 THE DETECTION OF SONCHUS YELLOW NET VIRUS RNA BY DOT-BLOT HYBRIDIZATION:

2.14.1 Preparation of Samples for Dot-Blot Hybridization:

Sap was extracted from healthy and from SYNV-infected *N. edwardsonii* leaf and root tissues at various times after inoculation as previously described (section 2.13.6.1) but using as extraction buffer 0.2M K_2HPO_4, 10mM EDTA, 0.1% (v/v) Triton X-100. Total cellular RNAs from healthy and infected root and leaf tissues were prepared as previously described (section 2.7). RNA from purified SYNV was extracted as previously described (section 2.4.2). Samples were disaggregated by heating at 65°C for 15 m before being applied to nitrocellulose filters.

2.14.2 Preparation and Sap-Spot Hybridization of Filters:

Sap samples were applied to nitrocellulose by the technique of Baulcombe *et al.* 1984. Nitrocellulose membrane (Schliecher & Schull, BA 85) was handled with forceps and gloves as it is very fragile, especially when dry. Nitrocellulose pieces were cut from the roll with a sharp razor blade. Filters were marked into grid squares (1 x 1 cm) with a soft pencil or ball point pen. Six sheets of 3MM Whatman paper were cut to the size of the filter plus 1 extra cm on each side. Three sheets were immersed for 10 m in sterile 20x SSC. At the same time the nitrocellulose filter was immersed in sterile distilled water for 5 m and then transferred to sterile 20x SSC for a further 5 m. The three dry sheets of Whatman paper were placed flat on the bench, the three sheets soaked in 20x SSC were placed on top

and the wet nitrocellulose filter was carefully layered on the top of wet Whatman paper avoiding any trapped air bubbles.

Immediately, 2-5µl of each sample was spotted on the filter using a micropipette. The spots rapidly soaked into the filter which was then carefully removed and air dried at room temperature. Filters were enclosed in folders of Whatman paper and the edges of the folders were stapled. These were baked in a vacuum oven for 4-6 h at 80°C to fix the RNA to the filter (see fig. 2-2a).

Prehybridization and hybridization steps were carried out in heat-sealed plastic bags according to the procedure of Flores *et al.* (1985). Filters were sealed flat in plastic bags along with prehybridization buffer (1ml/10 cm^2 membrane) consisting of:

50%(v/v)	deionized formamide (section 2.2.4)
5x SSC	(0.75M NaCl, 75mM sodium citrate, pH 7.0)
100µg/ml	denatured DNA from herring sperm (Sigma)
0.02%(w/v)	each of BSA, PVP, and ficoll.

Care was taken to avoid trapped air bubbles inside the bags. Filters were prehybridized for 4 to 6 h at 42°C in a water bath.

The prehybridization buffer was replaced by the hybridization buffer, which was the same buffer supplemented with the cDNA probe which had been denatured by boiling for 3-5 m (for the preparation of the probe see section 2.14.3). Sufficient denatured ^{32}P labelled probe was added to a the hybridization buffer to give a concentration of $2x10^6$ c.p.m./ml and 1ml was used per 20 cm^2 filter membrane. Occasionally diluted probe was incubated for 2 h at 45°C with one

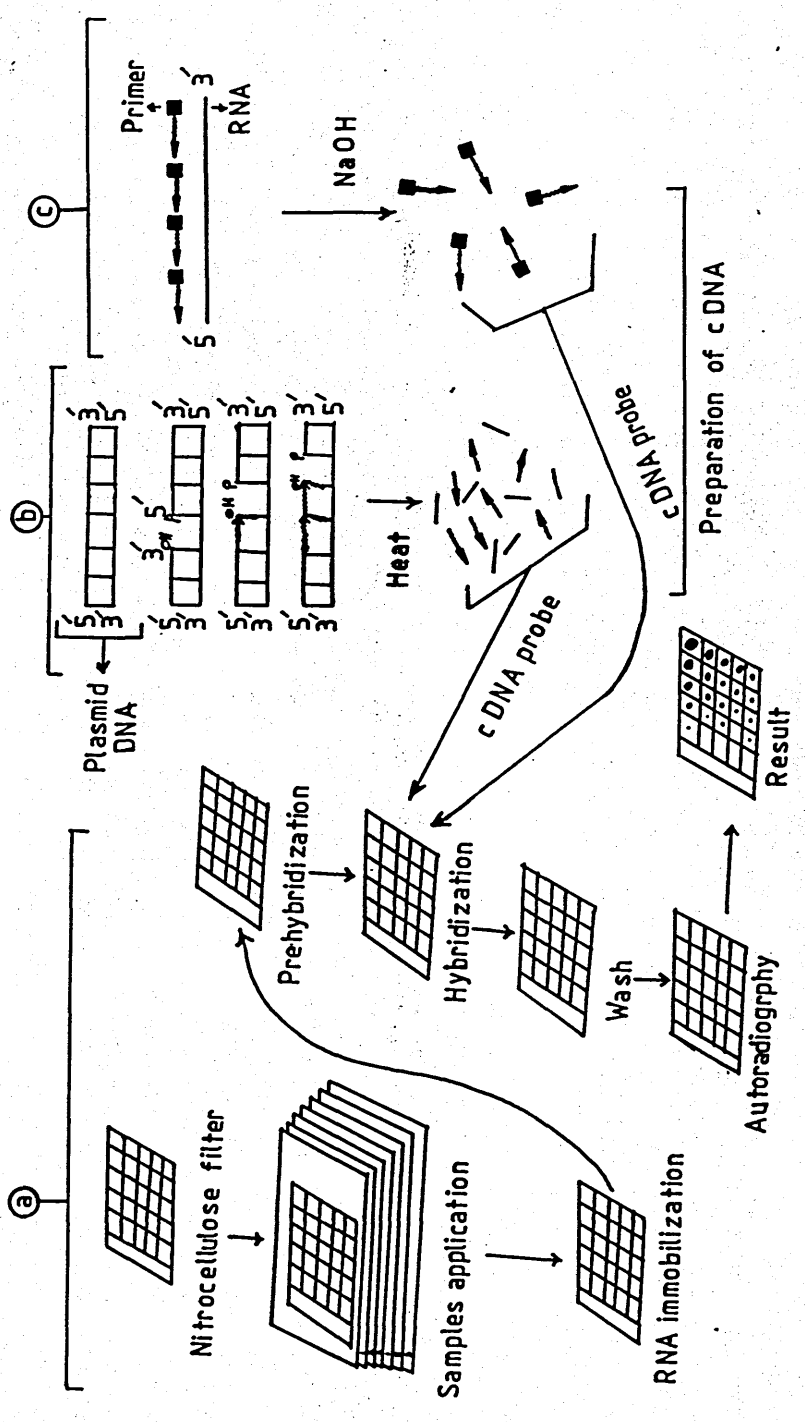


Figure 2-2:

Diagrammatic representation of dot-blot hybridization.

tenth volume of sap from healthy *N. edwardsonii* in order to prehybridize any contaminating cDNA to host RNA. Hybridization was carried out for 20 to 24 h at 42°C.

Filters were taken out of the bags with forceps and washed 4 times (5 m each) at room temperature in 500ml of 2X SSC, 0.1% SDS. Finally, filters were washed twice (30 m each) at 55°C in 500ml of 0.1x SSC, 0.1% SDS. Filters were air dried at room temprature and exposed to pre-flashed Fuji RX X-ray film for 24 to 72 h at -70°C.

2.14.3 Preparation of The Complementary DNA (cDNA) Probe:

2.14.3.1 Labelling Plasmid DNA with ^{32}P by Nick Translation:

Probes were prepared from plasmid DNAs pSYN305, pSYN402 and pSYN502. There cDNA clones contain inserts of 1.7, 0.8, and 0.5Kbp derived from SYNV viral complementary mRNAs sc3, sc4 and sc5 respectively (P.T. Richardson & J.J. Milner, in preparation). pSYN405 DNA was prepared as described (section 2.16), pSYN305 and pSYN502 DNAs were a kind gift from Dr. J.J. Milner.

DNAs were individually labelled with $\alpha[^{32}P]$dCTP to a specific activity of about $8x10^7/\mu g$ dpm by the procedure of Mackey *et al.* (1977). $\alpha[^{32}P]$dCTP (25µci) was dried in a siliconized eppendorf vial under a continuous gentle stream of N_2, placed on ice and the following were added:

0.4µl 0.1mM dCTP

0.5µl 0.4mM each of dATP, dTTP, dGTP

0.75µl 2x Nick translation buffer (0.133M KPO_4; 13.4mM $MgCl_2$ pH 7.5)

0.2µl pancreatic deoxyribonuclease I (50pg/ml) in activation

buffer (10mM tris-HCl, pH 7.5, 5mM $MgCl_2$; 0.1mg/ml BSA).

0.5 µl plasmid DNA (about 120µg/ml).

The above mixture was incubated at room temperature for 1 m, quickly chilled on ice, and 0.5µl of DNA polymerase I (Anglian Biotechnology, 2-4 unit/µl) was added. The mixture was incubated for 8-10 h at 14°C. Fifty µl 0.1x STE buffer (0.018M NaCl; 2mM tris-HCl, pH 7.5, 0.2mM EDTA) and 5µl 0.25M EDTA (pH 8.0) were added, and the DNase and DNA polymerase were denatured by heating the mixture to 65°C for 10 m.

Labelled DNA was precipitated by addition of 2.5 volumes pre-chilled ethanol, 0.1 volumes 7.5M NH_4-acetate, and 8µl transfer RNA (3mg/ml) as carrier, and incubated overnight at -20°C. DNA was collected by centrifugation for 10 m in a microcentrifuge. The pellet was washed twice with 75% ethanol, vacuum-dried and resuspended in 100µl 0.1x STE buffer. In some cases DNA was separated from unreacted dCTP by a single-passage of the probe through a Sephadex G-50 column.

Two µl of labelled DNA were removed and counted in the liquid scintillation spectrometer. The remaining probe was stored at -20°C.

2.14.3.2 Preparation of Complementary DNA Probe to SYNV RNA:

cDNA was synthesised according to Maniatis *et al.* (1982) with some modifications (Flores *et al.*, 1985) (fig. 2-2c).

The reaction mixture (final volume 40µl) was assembled in a siliconized eppendorf vial:

25µci α [^{32}P] dCTP (3,000 Ci/mmol)

2µl 1M tris-HCl pH 8.3 (at 43°C)

5.5µl 1M KCl

4µl 0.1M $MgCl_2$

10µl calf thymus oligonucleotide primers (17.5mg/ml) prepared as described in section 2.15.

1.25µl 0.7M ß-mercaptanethanol

4µl 5mM each of dATP, dGTP, dTTP

2µl 0.5mM dCTP

4µl SYNV RNA (0.55mg/ml; prepared as in section 2.4.2.2)

1µl reverse transcriptase (32 units/µl; Anglian Biotechnology

sterile AnalaR water to a final volume of 40µl.

SYNV RNA and calf thymus oligonucleotide primers were mixed, heated for 1 m at 100°C and quickly chilled in ice before addition to the remaining components. After adding the reverse transcriptase, the reaction mixture was incubated at 37°C for 3 h. The reaction was stopped by the addition of 4µl 0.25M EDTA (pH 8.0) and 3.8µl 1M NaOH and heated for 1 h at 60°C. The reaction mixture was neutralized by adding 25µl 1M tris-HCl (pH 8.0) and 25µl 1M HCl. The cDNA probe was loaded onto a 10 x 1 cm Sephadex G-50 column (equilibrated with 0.1X STE) and eluted with 0.1X STE to separate high-molecular weigh cDNA from unreacted nucleotides. Fractions of 0.5ml were collected and 2µl aliqouts of each fraction counted for radioactivity. Fractions containing the DNA, which was eluted at the exclusion volume of the column, were pooled and stored at -20°C.

2.15 PREPARATION OF CALF THYMUS OLIGONUCLEOTIDES PRIMERS:

A hundred mg of DNA (calf thymus, type I, sodium salt, Sigma No. D-1501) was dissolved in 20ml of 5mM tris-HCl pH 7.5 and 5ml of

10mM $MgCl_2$ and digested by adding 0.2ml of 5mg/ml DNase I (Sigma, DN-25) for 45 m at 37°C. One mg of proteinase K (BRL) was added and the solution was then incubated at 37°C for a further 45 m. After the addition of 0.25ml of 4M NaCl, the final solution was extracted with phenol/chloroform and centrifuged at 8.000 r.p.m. (6.580g) for 10 m in the MSE-18 centrifuge. The upper aqueous phase was saved and the phenol/chloroform phase was re-extracted with 5ml of buffer A (5mM tris-HCl; pH 8.0, 0.1M NaCl; 1mM EDTA). The two aqueous phases were combined and re-extracted with 10ml of chloroform. The upper aqueous phase was saved and stored at 4°C.

A column of DEAE sephadex was prepared. One gram of DEAE-sephadex A-25-120 (Sigma) was suspended in 20ml of buffer A and swollen by autoclaving. The cooled slurry was poured into a siliconized column (10 cm x 1 cm), and equilibirated at room temperature with 300ml of buffer A using a peristaltic pump at 20ml/h. The digested calf thymus DNA was then carefully loaded onto the surface of the equilibrated column, which was washed thoroughly with buffer A until the A_{260} of the eluate had fallen to 0.1-0.2 (usually 500 to 700ml of buffer A over a period of 12 to 18 h). Bound calf thymus oligonucleotides of about 6-8 b in length were then eluted with buffer B (5mM tris-HCl, pH 8.0; 0.3M NaCl; 1mM EDTA). Fractions (10ml) were collected in 15ml sterile glass tubes. The A_{260} of each fraction was measured. Fractions which had an A_{260} mor than 0.3 were combined and precipitated with 2.5 volumes of ethanol at -20°C overnight. The precipitate was collected by centrifugation for 20 m at 15.000 r.p.m. (23.120g) at 4°C in the MSE 18 centrifuge. Pellets were washed twice with 75% ethanol, dried under gentle vacuum, resuspended in 1ml sterile AnalaR water and stored in 200µl aliquots

at -20°C. DNA concentration was determined spectrophotometrically assuming that a 1mg/ml solution has an A_{260} of 20.

2.16 PREPARATION OF PLASMID DNA:

DNA was prepared by the alkaline lysis method of Birnboim and Doly (1979), with minor modifications (Maniatis *et al.*, 1982).

2.16.1 Growth of Bacteria:

E. coli containing a recombinant plasmid with viral complementary sequences, were grown overnight, with shaking, in sterile 15ml glass test tubes containing 10ml of sterile L-broth medium (tryptone 10g/l, yeast extract 5g/l, NaCl 5g/l, pH 8.0) plus ampicillin (50mg/l). The inocula were transferred to sterile flasks, each containing 500ml sterile L-broth and ampicillin at the above concentration. Flasks were shaken at 37°C and the growth of the bacteria monitored spectrophotometrically at 660_{nm} using L-broth containing ampicillin as blank. When the optical density reached 0.6-0.8, chloramphenicol was added at 150mg/l inhibiting further replication of chromosomal DNA but allowing continued replication of the plasmid DNA. Flasks were shaken overnight, and the bacteria harvested by centrifugation at 8.000 r.p.m. (6.580g) for 10 m in the MSE 18 centrifuge.

2.16.2 Preparation of the Plasmid:

Pellets were resuspended in 18ml lysis solution (35mM tris-HCl, pH 8.0, 10mM EDTA; 50mM glucose), a further 2ml lysis solution containing 200mg lysozyme was added and left on ice for 30 m.

Forty ml of alkaline-SDS (0.2M NaOH; 1% (w/v) SDS) was added and the mixture stirred thoroughly on ice for 15 m. Thirty ml of 3M potassium acetate (pH 4.8) was added with continued stirring on ice for a further 15 m. Chromosomal DNA was removed by 5 m centrifugation at 8.000 r.p.m. (6.580g) in the MSE-18 centrifuge and the supernatant was collected by filtration through two layers of Kleneex tissue.

DNA was precipitated by adding 0.6 volumes of pre-chilled isopropanol and leaving at -20°C for 1 h. Precipitates were pelleted by centrifugation for 5 m at 8.000 r.p.m. (6.580g). Pellets were dried by inverting the centrifuge tubes over Kleneex tissue for 10 m and resuspended in 7ml of 0.1x STE buffer (0.018M NaCl, 0.2mM EDTA, 2mM tris-HCl, pH 7.5). CsCl to a final concentration of 1.157g/ml and ethidium bromide to a final concentration of 3mg/ml were added. The final solution was adjusted to a refractive index of 1.3970, pipetted into two 6ml polyallomer centrifuge tubes (13 x 66 mm). Tubes were topped up to their shoulders with sterile liquid paraffin, and centrifuged at 45.000 r.p.m. (151.000g) for 18 h at 20°C in the Sorvall TV 865 vertical rotor. By the end of centrifugation two fluorescent bands were visible in each tube under UV light. The upper (thinner) band contained both the chromosomal and linearized or relaxed plasmid DNA whereas, the lower (thicker) band contained the closed circular plasmid DNA.

Bands were collected through the side of the tube using a specially constructed 10ml syringe, made by bending the tip of the needle to about 90 degree. Ethidium bromide was removed by shaking the solution containing the plasmid bands with an equal volume of isopropanol which had been equilibirated with saturated CsCl. The upper phase, containing most of the ethidium bromide, was piptted off

and the procedure repeated seven times. The lower phase containing the plasmid DNA was dialyzed overnight against three changes of 1l of 0.1x STE buffer at 4°C.

To remove any residual protein, the plasmid DNA solution was incubated for 30 m at 37°C with proteinase K at a final concentration of 50μg/ml, followed by a phenol/chloroform extraction (section 2.4.2.1). Plasmid DNA was precipitated by an overnight incubation with 2.5 volumes pre-chilled ethanol and 0.1 volumes 3M potassium acetate (pH 4.8), collected by 10 m centrifugation, washed twice with 75% ethanol, vacuum dried and resuspended in 100μl of 0.1x STE buffer. The concentration of plasmid DNA was determined spectrophotometrically assuming that a 1mg/ml solution has an A_{260} of 20, and the DNA was stored at 4°C.

CHAPTER 3

SONCHUS YELLOW NET VIRUS; ITS MORPHOLOGY, MOVEMENT AND INTRACELLULAR LOCATION WITHIN INFECTED *NICOTIANA EDWARDSONII* PLANTS

3.1 INTRODUCTION:

Symptoms induced by plant rhabdoviruses have been discussed in a number of reviews (Francki, 1973; Francki & Randles, 1980; Francki *et al.*, 1981; Jackson *et al.*, 1987). The symptoms, in general, are either chlorotic or necrotic local lesions or systemic vein clearing and leaf cupping or curling. However, since symptomology is of limited use for virus diagnosis and identification, no major studies of symptom expression have been made of this virus group.

There has been no simple and generally applicable procedure yielding adequate amounts of pure virus. A number of different purification procedures have been discussed (Francki, 1973; Jackson *et al.*, 1987). The yields of purified plant rhabdoviruses, are very low compared to the most other plant viruses (Matthews, 1981; Jackson *et al.*, 1987).

In order to understand, and perhaps eventually to control, pathological effects, we need to know the way in which viruses spread systemically through plants and the way virus levels vary within the tissues of infected plants. Samuels (1934) was among the first to use local-lesion infectivity assays to follow the spread of TMV from the initial site of inoculation, in mechanically infected tomatoes. Since then there have been a large number of such studies. In most cases, virus spreads from the initial site of inoculation within as little as 36 h . Translocation of a number of viruses appears to involve the phloem (for review, see Matthews, 1981). In the case of LNYV, infectivity can be found associated with the xylem sap (Francki & Randles, 1970). Özel (1973) has demonstrated, by electron microscopy, the presence of virus particles in the roots of *S.*

oleraceus 48 h after aphid-mediated inoculation of leaves with SYVV. He suggests that the initial movement of virus is into the roots and involves the phloem. Multiplication takes place and subsequent movement from roots to leaves involves the xylem.

In the case of several plant rhabdoviruses, virus levels, as determined by infectivity assays, show an initial increase within various tissues, reach a maximum, and then decline (Jackson & Christie, 1977; Francki & Randles, 1970). Such assays, however, are only semi-quantitative and are considerably less sensitive than techniques such as enzyme-linked-immunosorbant assay (ELISA).

The main aim of the work described in this chapter is to establish the time at which the virus leaves the mechanically inoculated leaves of *N. edwardsonii* plants, and to follow the virus movement and its intracellular location in the infected cells of these plants. ELISA, dot-immunobinding assay, dot-blot hybridization and electron microscopy have been used to establish the concentrations of virus protein and RNA, and particle location.

3.2 RESULTS:

3.2.1 Symptoms on *N. Edwardsonii* Plants:

Local lesions were occasionally observed on inoculated leaves about 4-5 d after inoculation. Two to three days later systemic symptoms (vein-clearing and leaf-cupping) were quite distinct in unexpanded leaves, and on the buds when present. The time of onset of symptoms was quite consistent in plants maintained in the growth chamber as described in sections 2.3.1 & 2.3.2, but symptom expression

was delayed 4-6 d in plants which had been maintained in the greenhouse. This perhaps reflects the physiological state of these plants. Systemic symptoms appeared in the buds of plants infected at the flowering stage before appearing on unexpanded leaves. Expanded leaves usually showed less severe symptoms, although these were more distinct if the tops of the plants and subsequently the buds were removed 6-7 d after inoculation. Typical SYNV symptoms on *N. edwardsonii* leaves are shown in figure 3-1. Plant recovery was noticeable 25-30 d after inoculation. Systemic symptoms disappeared gradually from the tip of the leaf towards the stem, and new leaves were asymptomatic. Infected plants appeared similar to healthy 60-70 d and on after inoculation. Recovered and healthy plants are shown in figure 3-2. Virus effects in these plants are reported in chapter 5.

3.2.2 Purification of SYNV:

3.2.2.1 Yield of Purified Virus:

Twenty seven batches of virus were purified from leaf tissues harvested from infected *N. edwardsonii* plants 10 d after inoculation as described in section 2.4.1. The yield of virus was estimated by measuring the virus protein using the CBB-binding assay as described in section 2.3.4, and ranged between 240 and 750 µg/100g tissue. The final step in the purification of SYNV involves sucrose gradient centrifugation. The absorbance profile of one such gradient is shown in figure 3-3. The yield of the virus purified from unexpanded leaves of *N. edwardsonii* plants which were growm, inoculated at age 4-5 weeks, and maintained in the greenhouse were about 240 to 370 µg/100g tissue. The highest yields of virus (500 to 750 µg/100g tissue) were

Figure 3-1:
(a) Healthy and (b) SYNV infected *N. edwardsonii* plants 10 d after inoculation.

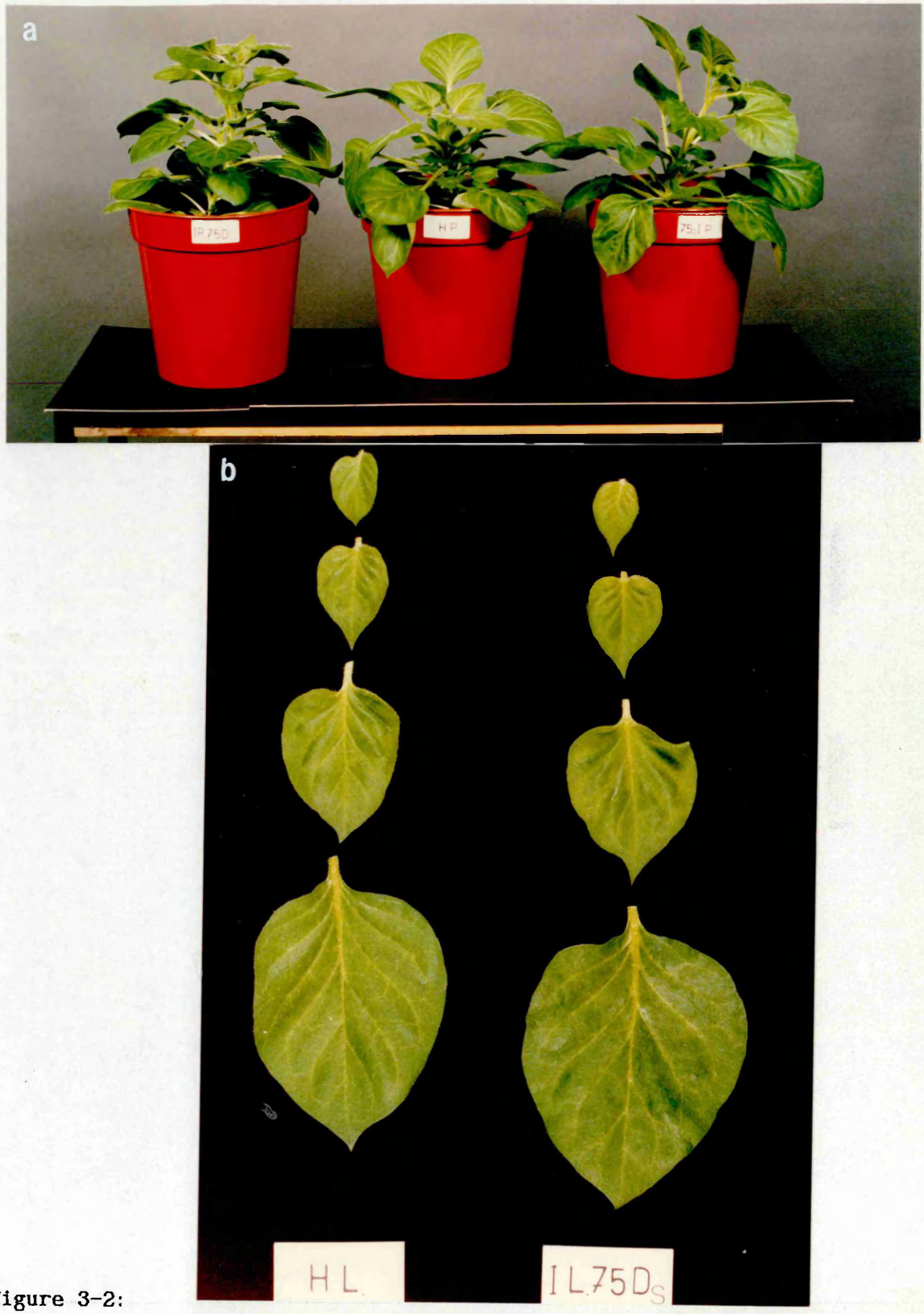


Figure 3-2:
Healthy and chronically infected *N. edwardsonii* 75 d after inoculation. (a) Plants and (b) leaves. Note the identical appearance between samples.

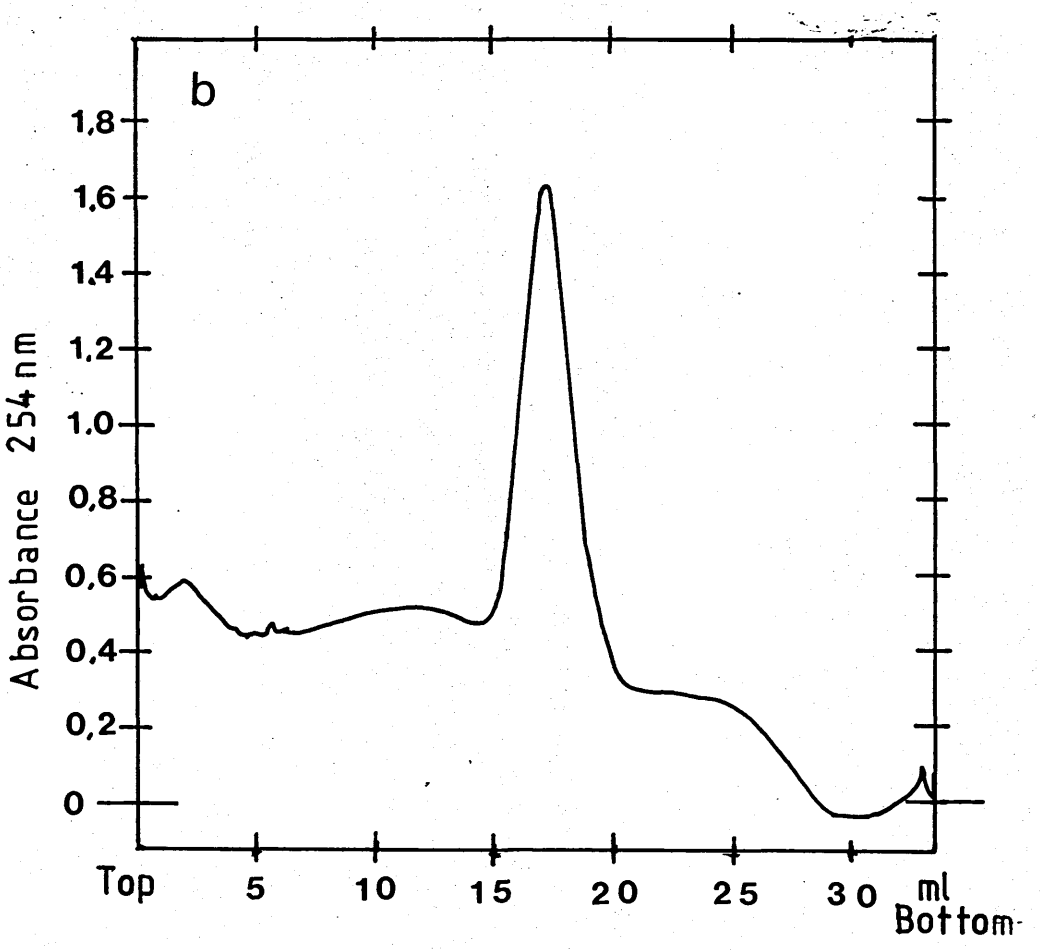


Figure 3-3:
Photometric scan of a 5-30% rate zonal sucrose gradient of a purification of SYNV. Leaves were from plants (10 d after inoculation) which had been grown in the greenhouse out of direct light and inoculated and maintained in the growth chamber.

obtained when unexpanded leaves and buds were taken from *N. edwardsonii* plants which were grown in the greenhouse out of direct light, inoculated at the beginning of flowering stage (about 7-8 weeks) and maintained in the growth chamber.

3.2.2.2 Estimation of the Virus Losses During the Purification-Steps:

The losses of viral protein at each stage of the purification procedure were monitored by ELISA (fig. 3-4). Reduction in the virus yield was negligible in the first step of the purification procedure (grinding the material and muslin filtration) when grinding was carried out for 30-45 s under chilled conditions. Losses were greater (2-3%) when heat was generated during the grinding step as a result of 2-3 m grinding time. Low speed centrifugation (2500-3000 r.p.m) of the brei and the filtration of the supernatant reduced the yield of the virus about 1.8-2.5%. The yield of the virus obtained after centrifuging the supernatant in discontinuous sucrose gradients was reduced about 4-5%. In this step, loss of the virus was sometimes greater when the interface between 60% and 30% sucrose layers (green band) was disrupted. Most of the loss of yield occurred during filtration through 0.6-0.7cm thick celite pads. Seventy to eighty percent of the virus was lost at this stage when celite pads thicker than 2.0cm were used, and no virus particles were detected spectrophotometrically following sucrose gradient centrifugation. No significant reductions in yield occurred when celite pads thinner than 0.3cm were used, but these gave green virus filtrates contaminated with host cell components that subsequently co-sedimented with SYNV in the sucrose gradients. Pelletings, sucrose gradients

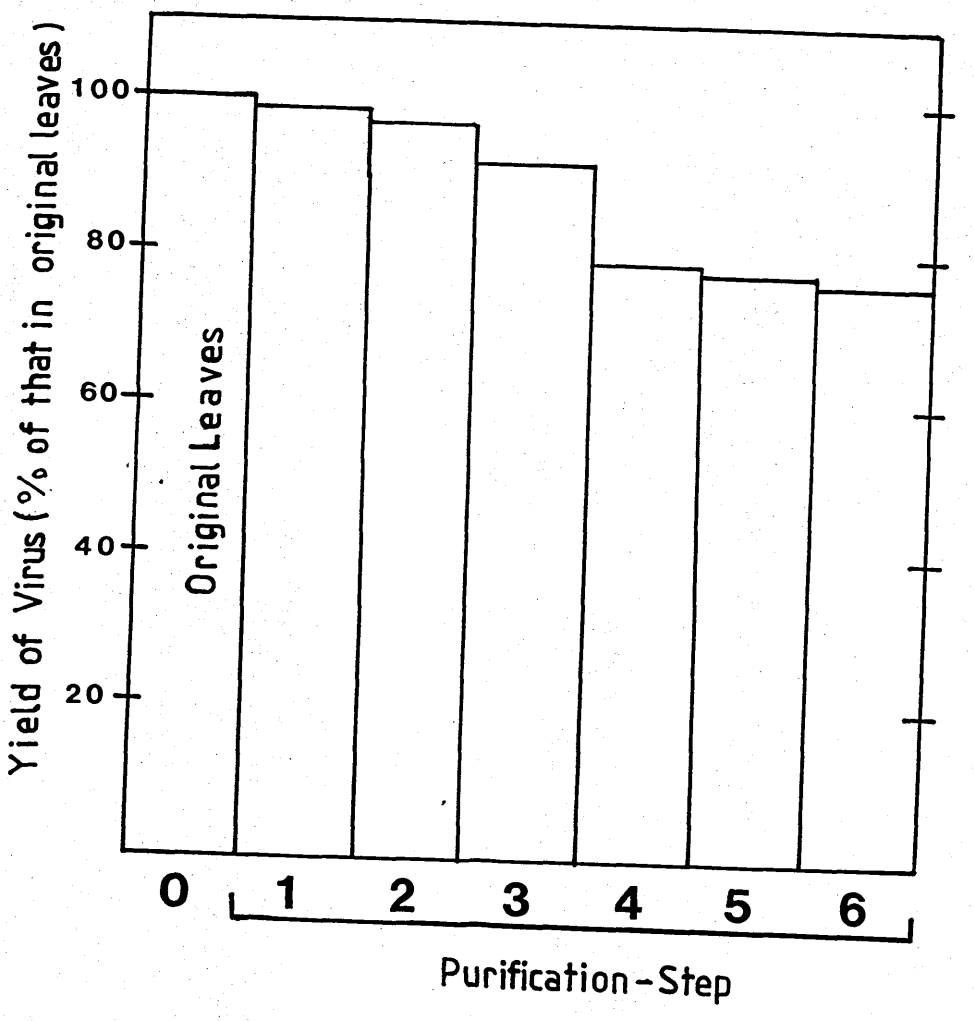


Figure 3-4:
Determination, by ELISA, of the losses of virus yield during purification. Steps from 1 to 6 are the solutions obtained after (1) grinding and muslin filtration, (2) low speed centrifugation and Kleenex-paper filtration, (3) centrifugation of discontinuous sucrose gradients, (4) celite-pad filtration, (5) pelleting and resuspending of the virus and (6) final recovery of the virus from sucrose gradients.

centrifugations and final recovery of the virus reduced yield by about 2.0-2.5%.

3.2.3. The Morphology of SYNV:

Electron microscopy of ultrathin sections of SYNV- infected *N. edwardsonii* revealed distinct bacilliform particles. Both mature enveloped virions and unenveloped nucleocapsids were observed; the presence or absence of the virus envelope could be clearly distinguished in sections (fig. 3-5a,b,c). Particles in cross section showed a series of concentric rings corresponding to the nucleocapsid and envelope with its projections. The lucent central part, perhaps reflects the helical organisation of the nucleocapsid in the intact particle, (fig. 3-5c). The dimensions of the mature virions were 216 ± 3nm x 74 ± 3nm and those of nucleocapsids were 200 ± 3nm x 51 ± 2nm (length x diameter; average of 70 particles ± standard deviation).

Bullet-shaped particles were observed when leaf dips of infected *N. edwardsonii* were stained with phosphotungstate (fig. 3-6b). However, when uranyl acetate was substituted, bacilliform particles were observed (fig. 3-6a). Bacilliform particles were observed using either phosphotungstate (fig. 3-6d) or uranyl acetate (fig. 3-6c) when the decoration procedure was used. Unwound nucleocapsids were also observed when the decoration procedure was used (fig. 3-6e).

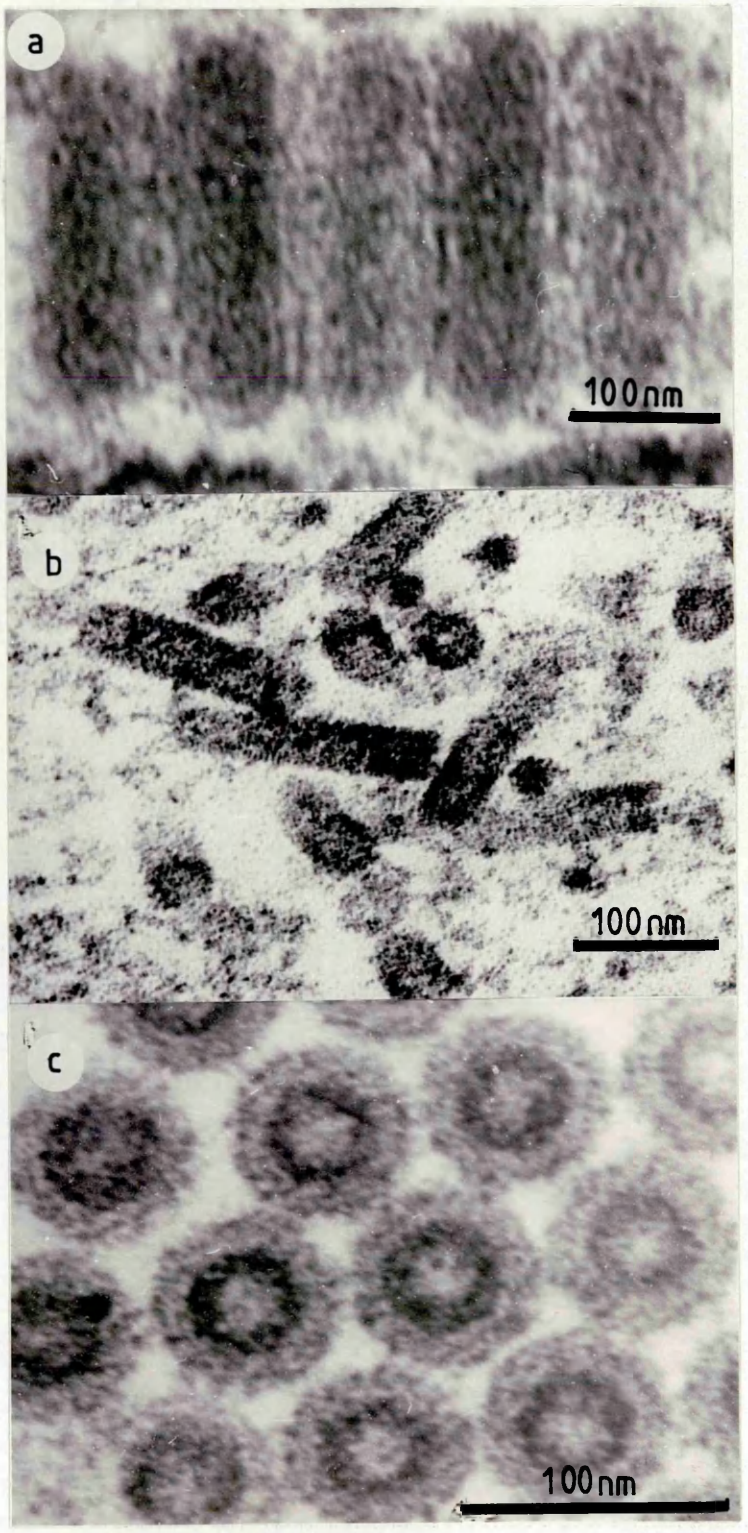


Figure 3-5:

Electron micrographs in thin sections of SYNV in an infected *N. edwardsonii* leaf 10 d after inoculation. (a) Mature virions, (b) nucleocapsids, (c) detail of virus particle in cross-section.

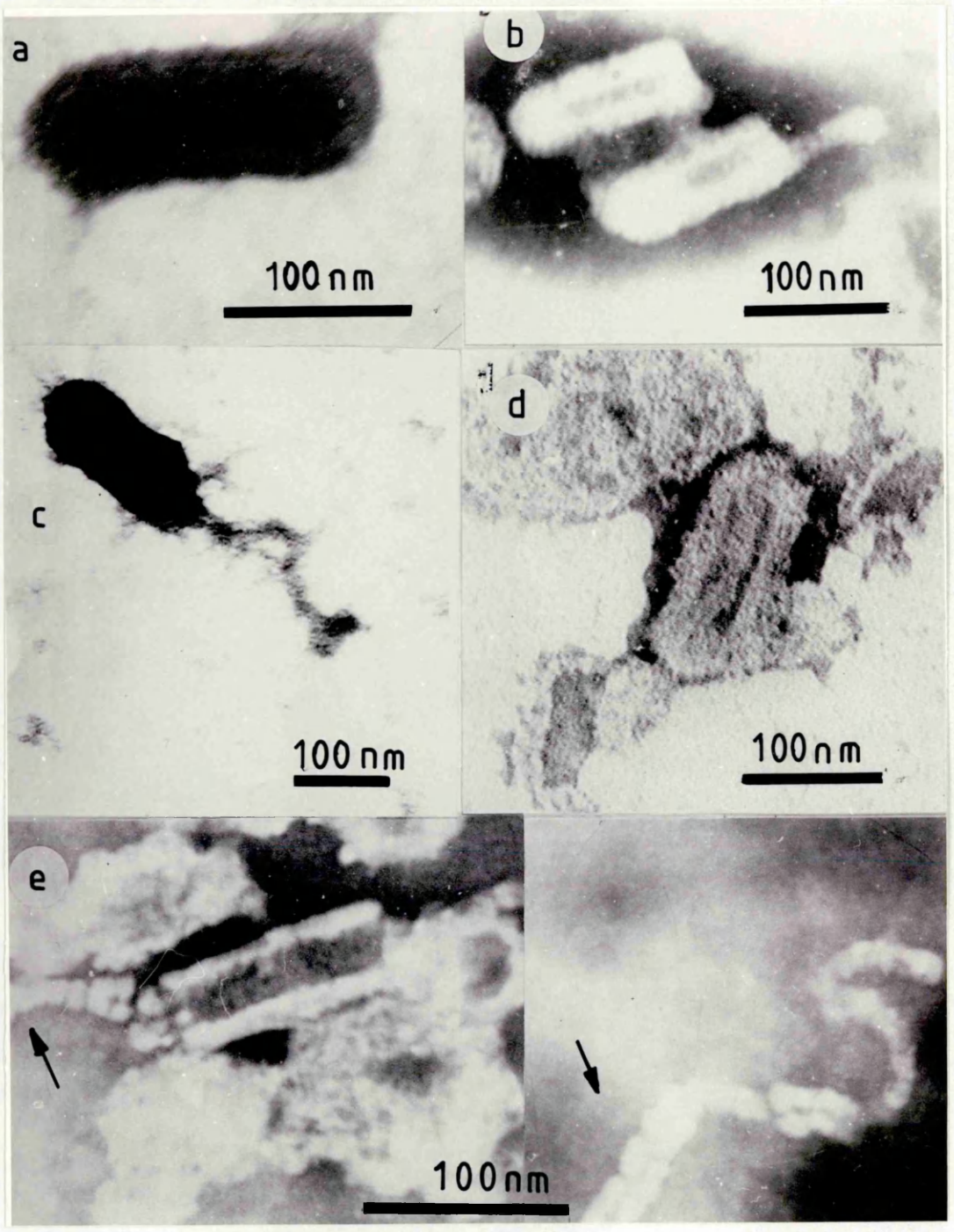


Figure 3-6:
Electron micrographs of SYNV in a leaf-dip preparation of SYNV-infected *N. edwardsonii* negatively stained with (a) uranyl acetate (b) phosphotungstate (c) uranyl acetate, after being incubated with anti-SYNV serum (d & e) phosphotungstate, after being incubated with anti-SYNV serum.

3.2.4 Determination of the Time at Which the Virus Moves Out of the Inoculated Leaves:

The minimum time for the virus to move from the mechanically inoculated leaves to the other parts of the plant was investigated. *N. edwardsonii* plants were mechanically inoculated with SYNV. The three inoculated leaves on each plant were marked with a tag, the plants were divided into three batches and maintained in the greenhouse. All these inoculated leaves were removed from one plant out of each batch at various times after inoculation (2 h, 4 h, 8 h, 12 h, 24 h, 36 h, 2 d, 3 d and 10 d). Healthy plants were used as a control. Plants were scored for evidence of systemic infection by development of symptoms, ELISA and western immunoblot assays in order to determine the minimum time taken for the virus to move from inoculated leaves into the other parts. The results are shown in table 3-1. Removal of inoculated leaves up to 12 h after inoculation prevented systemic spead of the virus. In two out of three batches of plants systemic symptoms developed when inoculated leaves were removed 24 h after inoculation and in the third batch after 36 h.

To confirm that expression of symptoms was indicative of systemic virus infection, plants, from which the inoculated leaves had been removed as described above, were tested 10 d after inoculation for systemic spread of virus using two serological tests, ELISA and western immunoblotting. Sap extracted from healthy and from infected (10 d after inoculation) leaf tissues was used as negative and positive controls and purified virus was used as a standard. The results are shown in figure 3-7a,b. No significant differences between the healthy plants and inoculated ones from which leaves had been removed 2 h, 4 h, 8 h and 12 h after inoculation, were detected

by ELISA (fig. 3-7a). Also, no positive reactions were detected when these were scored by the immunoblotting detection assay (fig. 3-7b). Those plants which had the inoculated leaves removed 24 h or longer after inoculation gave a high A_{492} in the ELISA test (fig. 3-7a) and in the western blot the four major structural viral proteins were detectable. These results confirm that symptoms are due to systemic infection. Virus thus appears to move out of the inoculated leaves between 12 and 24 h after mechanical inoculation.

Table 3-1 Determination of the time at which the SYNV leaves inoculated *N. edwardsonii* leaves.

Time after inoculation	Sample		
	Batch 1	Batch 2	Batch 3
2 h	-	-	-
4 h	-	-	-
8 h	-	-	-
12 h	-	-	-
24 h	+	+	-
36 h	+	+	+
2 d	+	+	+
3 d	+	+	+

- : Plants with no systemic symptoms.

+ : Plants with systemic symptoms.

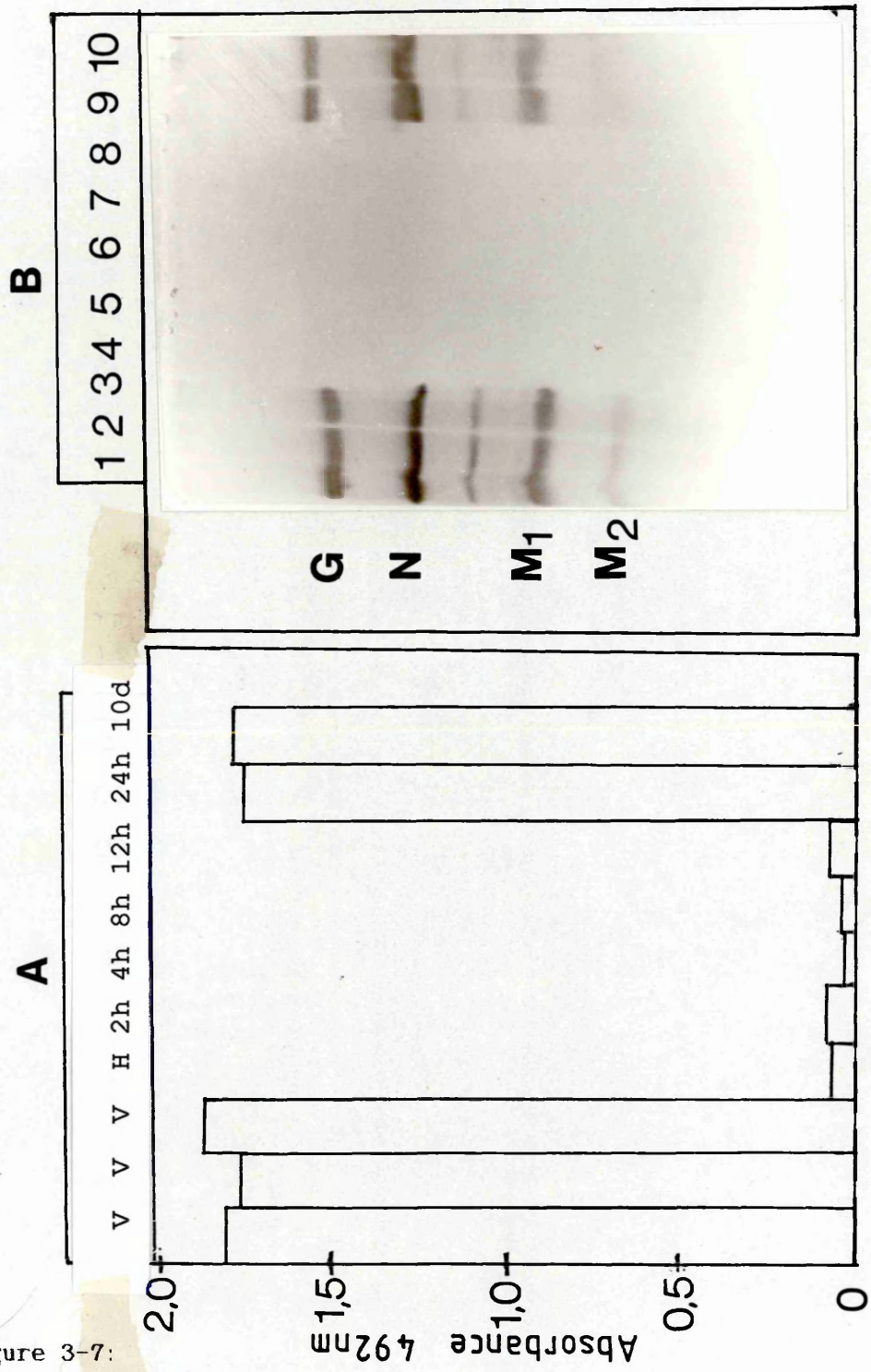


Figure 3-7:
Determination of the time at which the virus moves from infected leaf into the other plant parts by (A) ELISA and (B) immunoblotting. Samples are, (1 to 3) purified SYNV, (4) healthy control and (5 to 10 respectively) non-inoculated leaves removed 10 d after inoculation from plants in which inoculated leaves had been removed at 2 h, 4 h, 8 h, 12 h, 24 h, 10 d.

3.2.5 Temporal changes in SYNV-concentration in Infected *N. Edwardsonii* Plants:

The concentration and location of SYNV in infected tissues were determined at various times after inoculation. Dot-immunobinding and ELISA assays were used to measure the viral protein concentrations, and the dot-blot hybridization assay was used to estimate the viral RNA concentration. The location of virus particles was determined by using electron microscopy.

3.2.5.1 Determination of Virus Protein by Dot-Immunobinding Assay:

The specificity and the sensitivity of the assay was first checked using sap extracts of healthy and infected (10 d after inoculation) leaf tissues. Duplicate nitrocellulose filters were prepared from a series of tenfold dilutions (10^{-1} to 10^{-6}) of these extracts and from known concentrations of purified SYNV. Non-preabsorbed anti-SYNV serum gave positive reactions with infected leaves to a dilution of 10^{-6}, but also reacted to a dilution of 10^{-4} to healthy sap (fig. 3-8a). However, when anti-SYNV serum was preabsorbed or when "used" anti-SYNV serum was used, healthy sap reacted only weakly to a dilution of 10^{-1} whereas infected leaves still reacted to 10^{-6} (fig. 3-8b). Comparable non-specific binding to highly concentrated healthy sap has been reported by Hibi and Saito (1985), Hsu (1984), Brada and Roth (1984) and Bode *et al.* (1984) using a similar assay.

Levels of virus protein were estimated by dot-immunobinding assay. A series of fourfold (1:50 to 1:3200) dilutions of crude sap was prepared from leaf tissues of healthy and infected (2, 4, 6, 8, 9, 10, 12, and 20 d after inoculation) plants and spotted onto

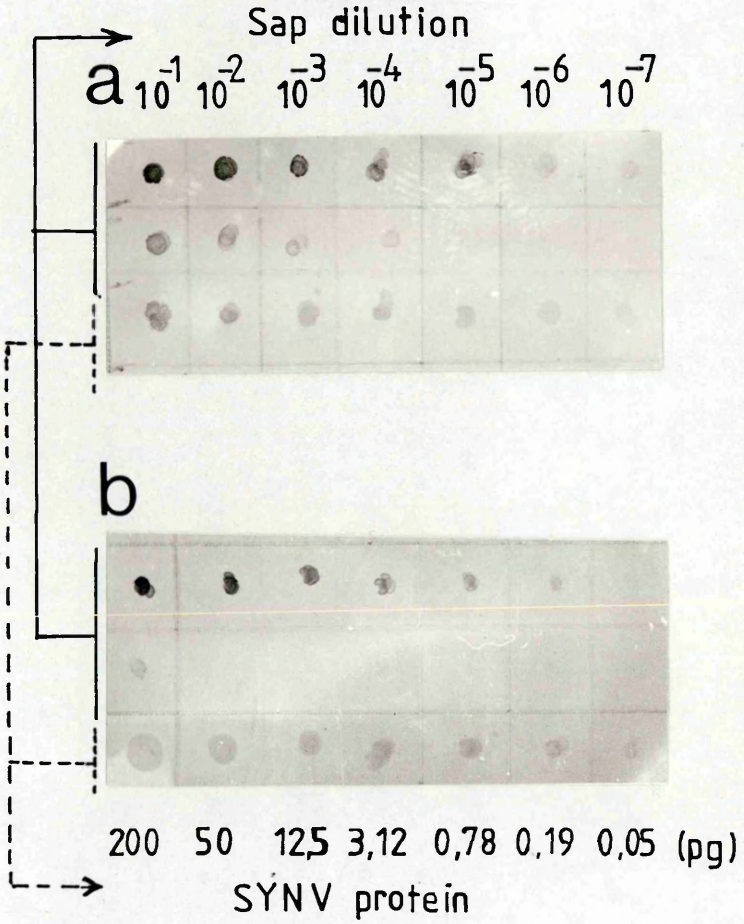


Figure 3-8:

Determination of SYNV protein by dot-immunobinding assay. (a) and (b) sap samples from (top row) infected *N. edwardsonii* leaves 10 d after inoculation, (middle row) control leaves from a healthy plant. Spots from left to right are a series of ten fold dilutions. The bottom row contains purified SYNV at the indicated concentrations. (a) was incubated with crude anti-SYNV serum. (b) was incubated with pre-absorbed anti-SYNV serum.

nitrocellulose. Known concentrations of purified SYNV were used as a standard. Virus protein was detectable in leaves 2 d after inoculation, and the amount increased rapidly from day 6 reaching a maximum 10 d after inoculation and gradually falling thereafter (fig. 3-9).

3.2.5.2 Measurment of Virus Concentration by ELISA Assay:

ELISA was used to quantify the concentration of virus protein in tissues at various times after inoculation.

3.2.5.2.1 Determination of ELISA Working Conditions:

As the aim of the experiment was to quantify the viral protein during the infection course, it was necessary to check the antibodies raised against the virus protein for specificity, and to optimise the conditions of the experiment so as to maximise the detection of very small quantities of viral protein. In order to make sure that anti-SYNV antiserum reacted specifically to all SYNV structural proteins, purified SYNV (100, 20 and 4 ng viral protein) was disrupted with SDS and the protein fractionated by SDS-PAGE on 10% polyacrylamide gels and blotted onto a nitrocellulose membrane. Two identical blots were prepared, one was incubated with anti-SYNV antiserum diluted to 1:200, the other was incubated with non-immune rabbit serum (normal rabbit serum) diluted to 1:200. The results (fig. 3-10) showed that the antiserum reacted specifically to the SYNV G, N, M1 and M2-proteins when 100ng viral protein per track was fractionated, but reaction to M1 and M2 proteins was not detectable when 20ng or 4ng of viral protein per track were fractionated. Presumably these proteins are

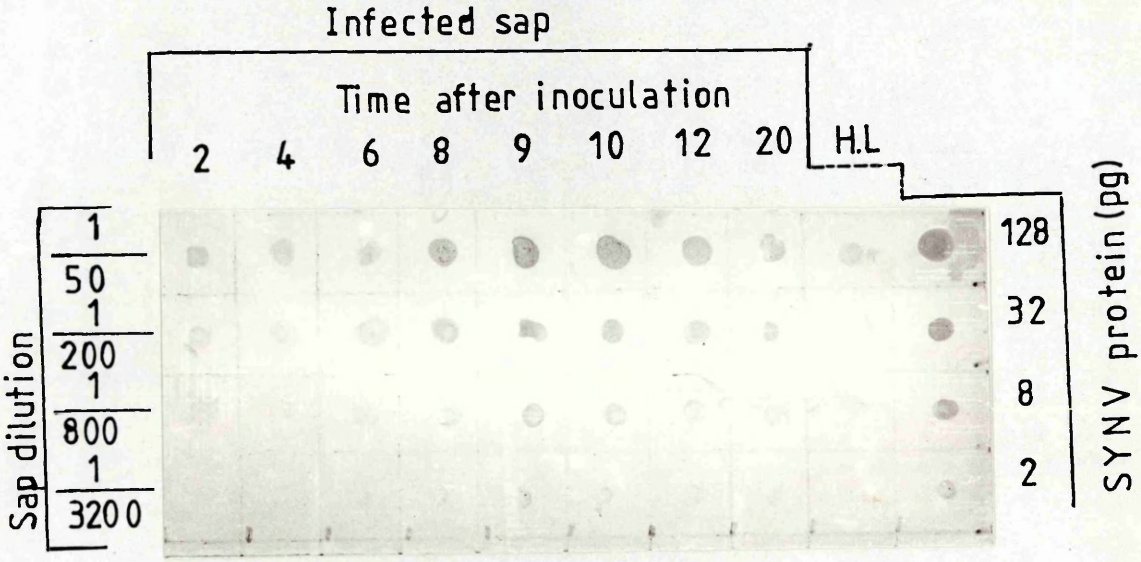


Figure 3-9:

Determination of SYNV protein by dot-immunobinding assay. Sap samples from infected plants obtained at intervals from 2 days to 20 days after inoculation and from healthy plants (H.L). Spots from top to bottom, are a series of indicated dilutions. The right hand column contains purified SYNV at the concentrations indicated.

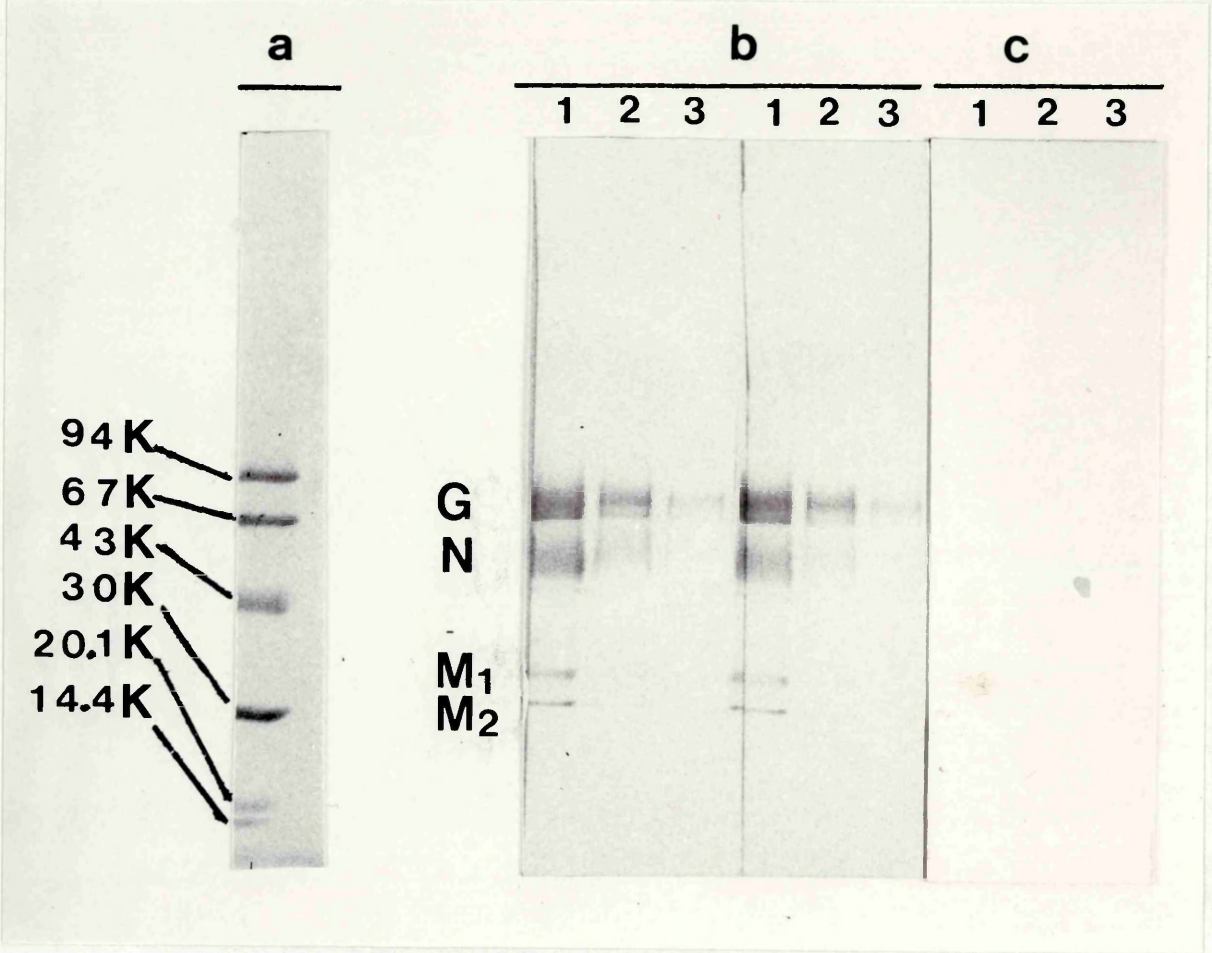


Figure 3-10:

Polyacrylamide gel electrophoresis of SYNV protein blotted onto nitrocellulose membrane. (a) Molecular weight markers stained with amido black. Sizes in KD are as indicated. (b) and (c) Purified SYNV. Lanes 1, 2 and 3 contain 100, 20 and 4 ng SYNV-protein. (b) Was incubated with anti-SYNV and (c) was incubated with non-immune serum (normal rabbit serum).

poorer immunogens than G and N proteins. No reaction was detectable in the blot which was incubated with non-immune serum.

The titre of anti-SYNV serum giving the most sensitive detection of antigen was determined. Five different dilutions of antiserum (1:5, 1:25, 1:75, 1:125 and 1:250) were tested against known concentrations of virus protein at a series threefold dilutions (0.48µg down to 0.1pg). The results are shown in figure 3-11. A dilution of 1:75 was used in subsequent ELISA assay since the linear part of the curve corresponds to the range 10pg to 2ng virus protein.

In order to confirm the specificity and the sensitivity of the antiserum at a dilution of 1:75, duplicate dilutions of SDS-disrupted SYNV preparation diluted with coating buffer (twofold 1:100 to 1:51200) were applied to a microtitre plate, one of each pair was incubated with antiserum diluted to 1:75, the other was incubated with non-immune serum at the same dilution. In the same plate, duplicate dilutions of coating buffer diluted as above but with water, were applied, and this was incubated with antiserum at dilution of 1:75. ELISA was carried out as previously described (section, 2.13.6.2). The results are shown in figure 3-12. These demonstrate the specificity of the antiserum at dilution 1:75, since no positive reactions were detectable either when antiserum was incubated with coating buffer or when viral antigen was incubated with non-immune serum.

Since the determination of the viral protein induced during the virus infection is carried out in crude sap pressed from infected tissues and since these sap preparations contain predominantly host protein, it was essential to challange the antiserum against crude sap extracted from infected and from healthy

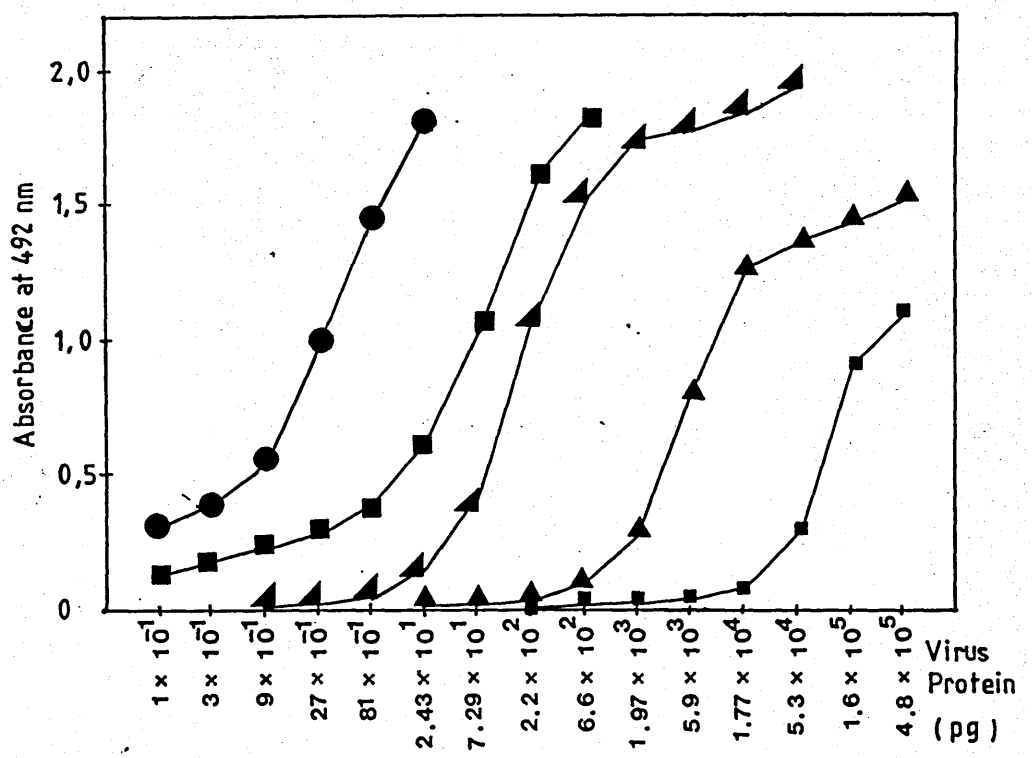


Figure 3-11:

ELISA of purified SYNV at the indicated concentrations. Anti-SYNV serum was diluted with PBS-TPO to (●) 1:5, (■) 1:25, (◢) 1:75, (▲) 1:125 and (■) 1:250.

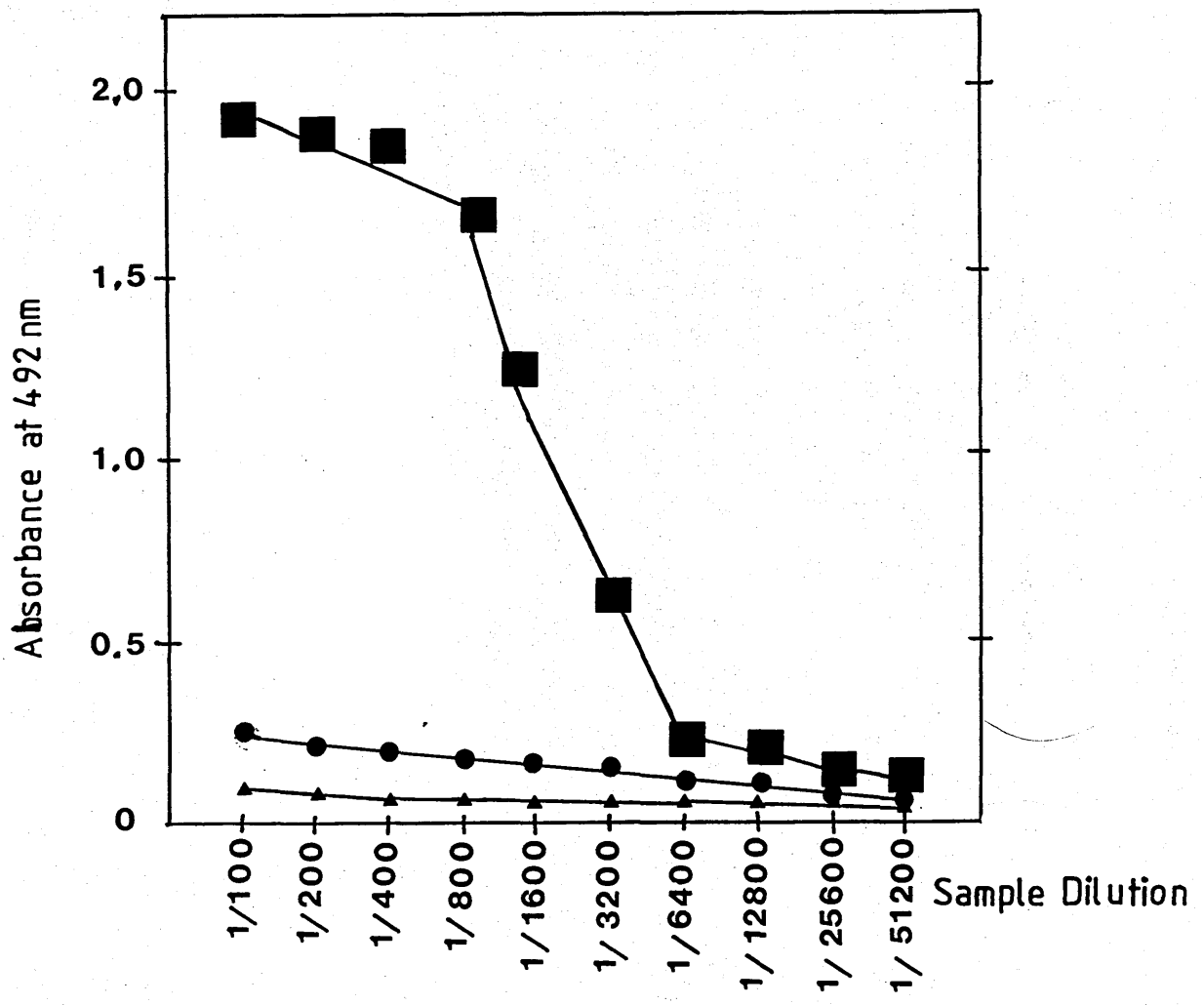


Figure 3-12:

ELISA of (■ & ●) purified SYNV and (▲) coating buffer. Purified SYNV was diluted with coating buffer and coating buffer was diluted with water to the indicated dilutions. (▲) and (■) were incubated with anti-SYNV serum (1:75); (●) was incubated with normal rabbit serum (1:75).

tissue. Duplicate dilutions (twofold, 1:5 to 1:5120) were prepared from sap extracted from healthy and infected (10 d after inoculation) leaf tissue. Dilutions were applied to a microtitre plate, incubated with antiserum at a dilution of 1:75 and the procedure carried out as before. The results are shown in figure 3-13a. Infected sap gave a strong positive reaction with A_{492} being greater than 1.5 down to a dilution of 1:640. The antiserum also reacted with healthy sap although the titre was approximately 64 fold less than with infected sap. This reaction with healthy sap may result from the presence of antibodies to host protein and/or cross reaction between anti-SYNV idiotypes and host proteins. Several procedures were tested for the ability to eliminate or reduce this cross-reactivity. These included, pre-absorbing the antiserum by mixing it with crude sap from healthy tissue (section, 2.13.2), liberating the viral protein from particulate matter by using SDS at various concentrations, and heat-denaturing the proteins to make them more available to the antiserum. Addition of SDS to the brei at concentration of 0.5-3%, followed by heating to 65°C for 10 m increased the A_{492} obtained with any given sample by a factor of up to threefold. The maximal effect was obtained with 2% SDS. The addition of SDS had a further beneficial effect of greatly increasing reproducibility when the same tissue sample was independently tested several times. Consequently, all samples were treated with 2% SDS as described in materials and methods (section, 2.13.6.1). Pre-absorption of antiserum with healthy sap (as described in section, 2.13.2) almost completely eliminated reaction with healthy sap (fig. 3-13).

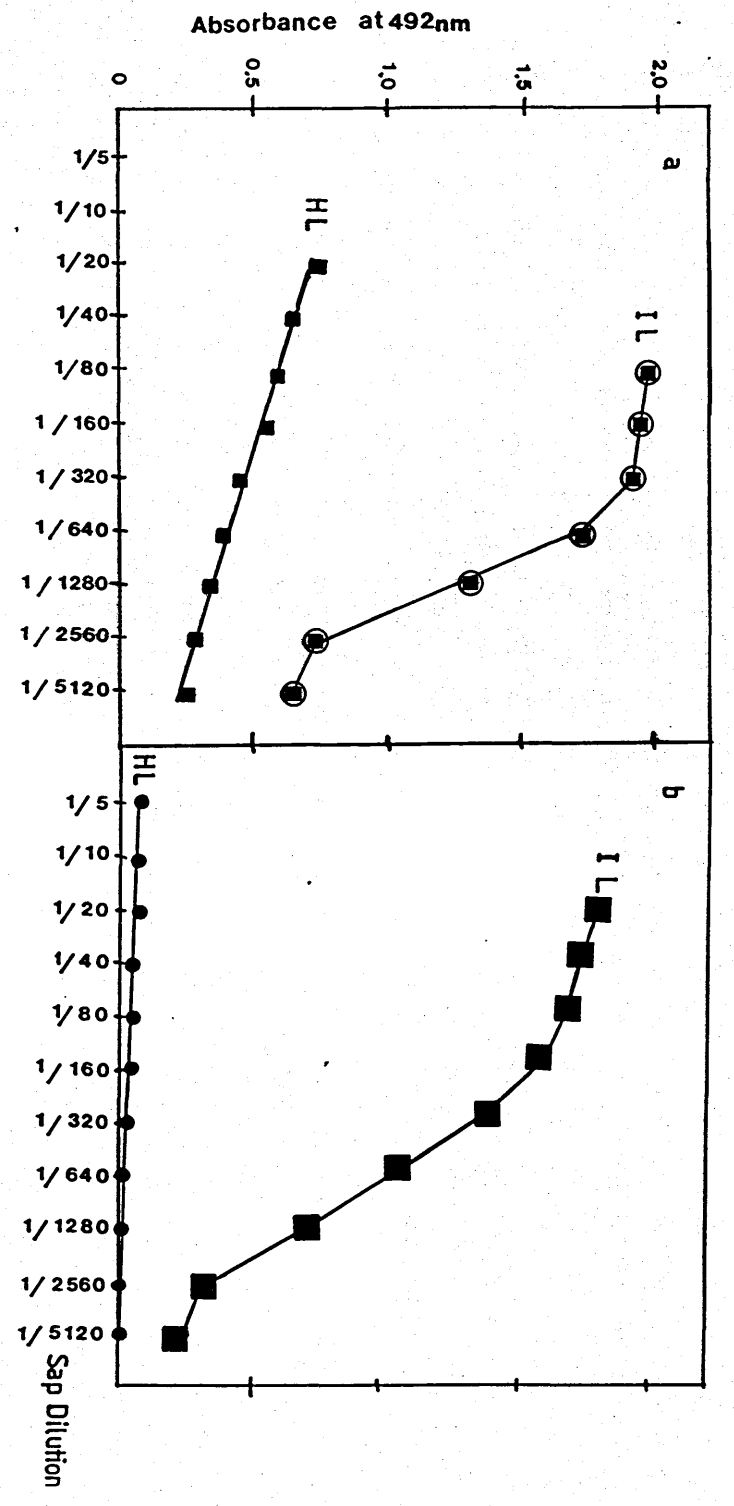


Figure 3-13:

ELISA of (■ & ◉) infected plants 10 d after inoculation and (● & ■) healthy controls. Samples were diluted with coating buffer to the indicated dilutions. (a) was incubated with crude anti-SYNV serum. (b) was incubated with pre-absorbed anti-SYNV serum.

3.2.5.2.2 Determination of SYNV Concentration in Infected Tissue:

The concentration of virus proteins in sap samples was determined by ELISA. Each plate included a standard comprising suitable dilutions of purified virus at known concentrations. Tissue samples were taken from inoculated, expanded and unexpanded (at the time of inoculation) leaves and roots at 12 h intervals from 0 to 4 d after inoculation, at 24 h intervals from 4 to 6 d after inoculation, at 48 h intervals from 6 to 12 d after inoculation and at 5 d intervals from 15 d and after. Suitable dilutions of each sample were assayed for virus antigen by ELISA. For each time point, samples were taken from three independent plants and duplicates of each sample were tested. Sap from healthy plants was used as a control. Figure 3-14 demonstrates a typical standard curve showing the reaction between known concentrations of purified SYNV and the pre-absorbed anti-SYNV antiserum at a dilution of 1:75. Each point on the curve is the average of six separate experiments ± standard deviation. Virus antigen within non-inoculated leaves and roots was detected at a low but reproducible level of approximately 0.1µg of virus protein per g of tissue 24 h after inoculation. This quantity of virus gave an absorbance, using buffer as a blank, which was approximately three times that of sap from healthy plants. Virus antigen was detectable in inoculated leaves immediately after inoculation but its concentration declined to near zero within 24 h, perhaps because it was inoculum which had adhered to leaves and that washed off or degraded within 24 h of inoculation. The concentration of virus in expanded leaves changed little between 24 h and 4 d after inoculation. Virus levels in roots increased only marginally from 0.12 to 0.14µg/g over the same period.

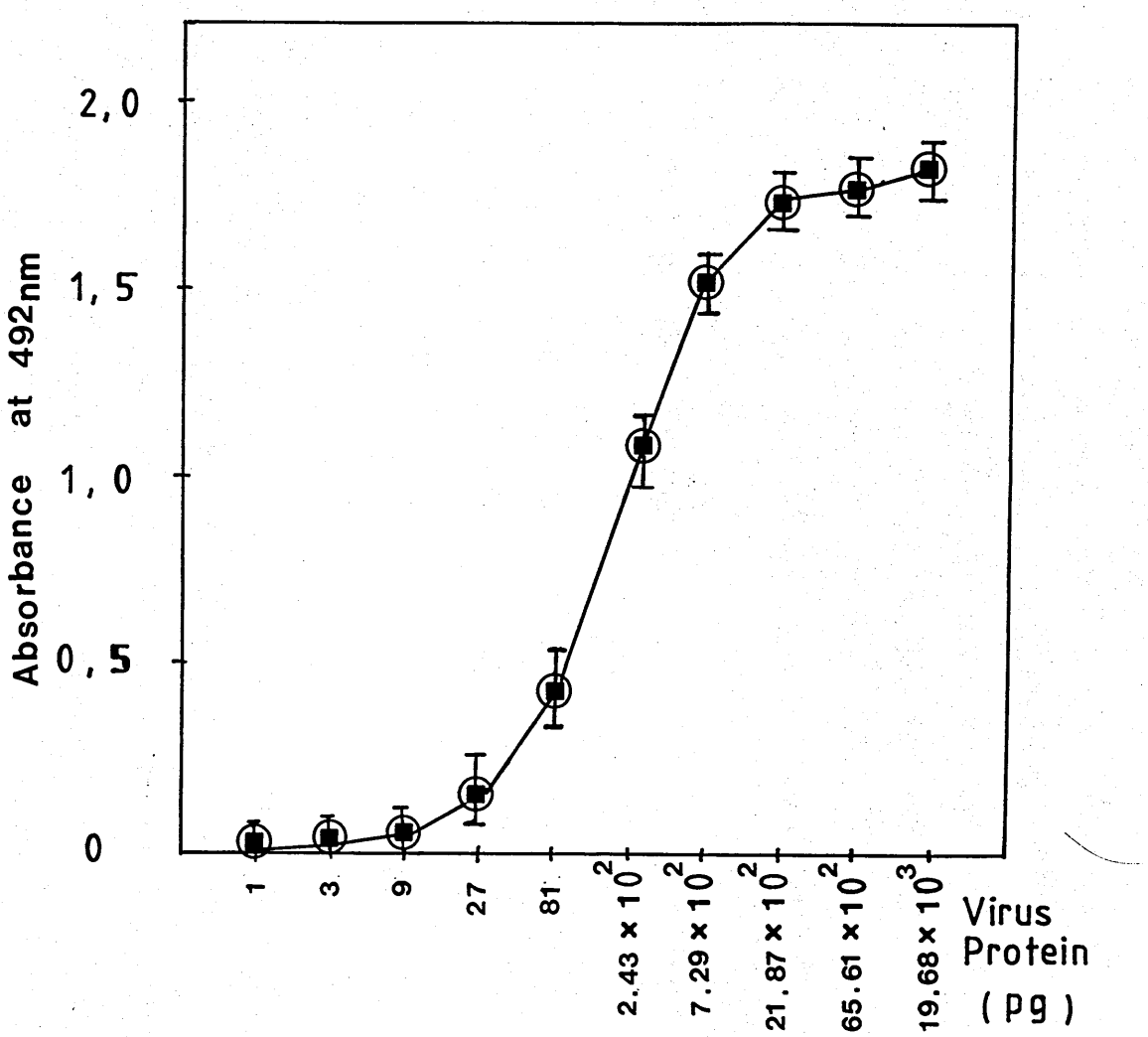


Figure 3-14:
ELISA of known concentrations of purified SYNV and pre-absorbed anti-SYNV serum at dilution 1:75. The error bars indicate the standard deviations.

All tissues showed increasing levels of virus antigen after 4 d with the most dramatic increases occurring between 6 and 10 d after inoculation. Levels of virus within the same tissues from different plants were remarkably similar as reflected by the small standard deviations, which were usually less than 3% (see table 3-2 & fig. 3-15). This indicates that levels of virus replication show little plant to plant variation provided that plants are matched for growth conditions and physiological state. Virus antigen reached a maximum in all tissues except expanded leaves (uninoculated) after 10 d. In the experiment shown in figure 3-15, maximal levels of virus antigen were as shown in table 3-2.

Table 3-2 Maximum levels of virus antigen detected by ELISA.

Tissue	Virus antigen µg/g tissue	Time after inoculation (d)
Inoculated leaves	2.0 ± 0.07	10
Expanded leaves	5.7 ± 0.20	12-20
Unexpanded leave	7.3 ± 0.02	10
Roots	2.8 ± 0.20	10

The concentration of the virus in several experiments differed slightly. Maximum levels for expanded leaves were in the range 4.0 to 7.3µg/g, for unexpanded leaves were in the range of 6.4 to 9.8 µg/g, for uninoculated leaves were in the range 1.7 to 3.4µg/g and for roots

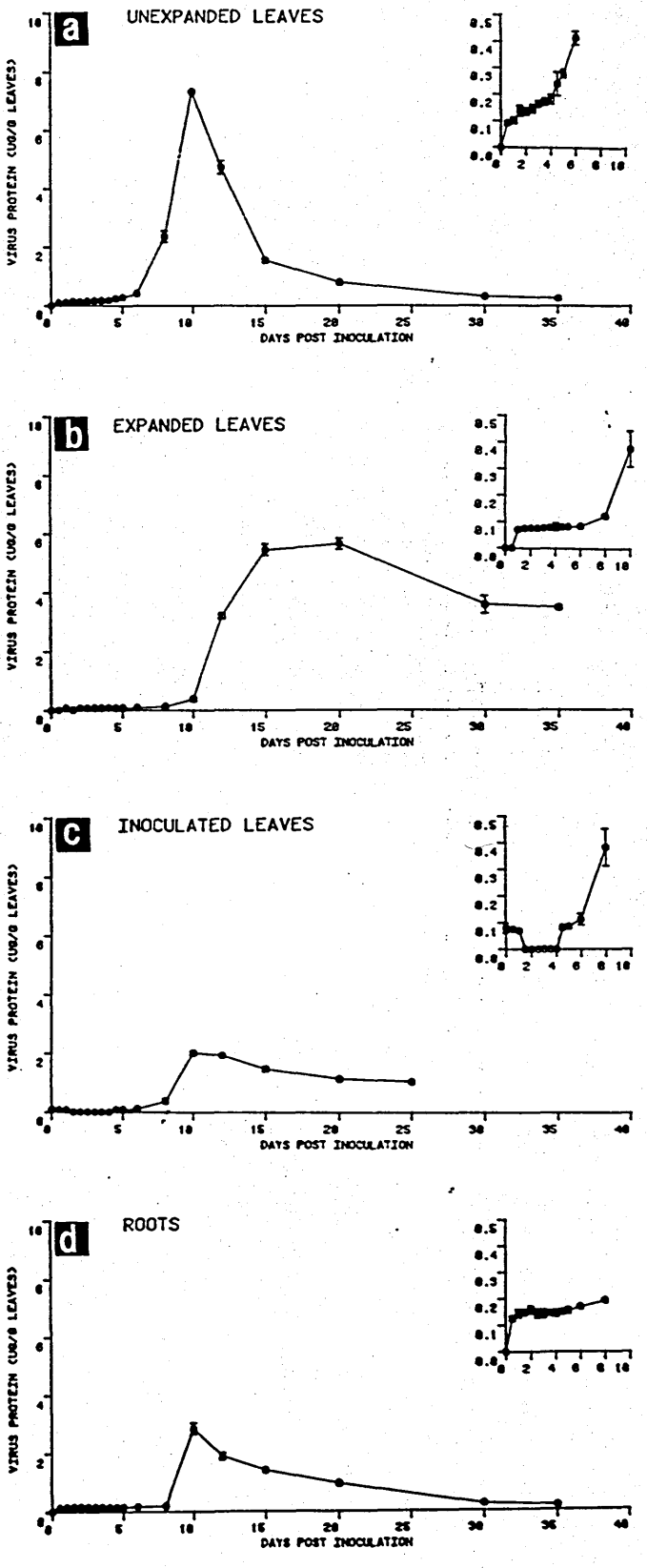


Figure 3-15:
Concentration of virus protein in tissues determined by ELISA. (a) Unexpanded leaves. (b) Expanded leaves. (c) Inoculated leaves. (d) Roots. The insets show the same data for the first 6 days on a larger scale. The error bars indicate the standard deviations.

were in the range 2.2 to 4.3μg/g. The relative levels of virus antigen in the different tissues remained nearly the same. These differences in the maximum viral levels presumably reflect differences in the physiological state of the plants at inoculation. Levels of virus antigen declined rapidly in all tissues (except expanded leaves) between 12 and 20 d after inoculation and declined more slowly thereafter. In expanded leaves virus levels remained constant between 12 and 20 d after inoculation but declined thereafter. The levels of antigen in leaves closely followed the appearance and disappearance of symptoms (vein clearing and cupping of the leaves).

In other experiments, petals and terminal leaves (unexpanded leaves showed symptoms of mottling and vein clearing) of flowering plants were tested by ELISA for virus protein. Petals and terminal leaves were taken from these plants at 30 d and 50 d after inoculation. Sap was extracted and samples were prepared for assay as previously described (section 2.13.6.1). The results from three independent batches of three plants each are shown in table 3-3.

Levels of virus protein within the same tissue from different plants varied only slightly as shown by the relatively small standard deviations. This might reflect small plant to plant variations in virus replication and movement even in plants grown under the same conditions.

Levels of virus protein in terminal leaves and petals after 50 d had declined to about 1/3 the level detected after 30 d.

Table 3-3 Determination of virus protein in petals and in terminal leaves of infected *N. edwardsonii* plants.

Tissue	Maximal levels of virus antigen (µg/g tissue ± SD)	
	30 days	50 days
Petals	5.04 ± 0.98	1.48 ± 0.45
Terminal leaves	5.68 ± 0.70	1.92 ± 0.33

3.2.5.3 Determination of the Changes in Viral RNA Concentration:

Concentrations of viral RNA within unexpanded leaves and roots were estimated by dot-blot hybridization. A series of tenfold dilutions of crude sap was spotted onto nitrocellulose. Sap from uninfected plants was used as a control. Known concentrations of viral RNA were used as a standard. Samples probed with cDNA prepared by reverse transcription of purified viral RNA reacted strongly with crude sap from healthy plants (fig. 3-16a). Total RNA was prepared from sap from healthy and infected plants, separated by electrophoresis on 1% agarose-formaldehyde gel, Northern blotted onto a Biodyne nylon membrane and hybridized with the same cDNA probe. In the lane containing RNA from infected plants, the cDNA probe hybridized strongly to a band of approximately 13 Kb representing the viral RNA and also to bands with the expected mobilities of ribosomal

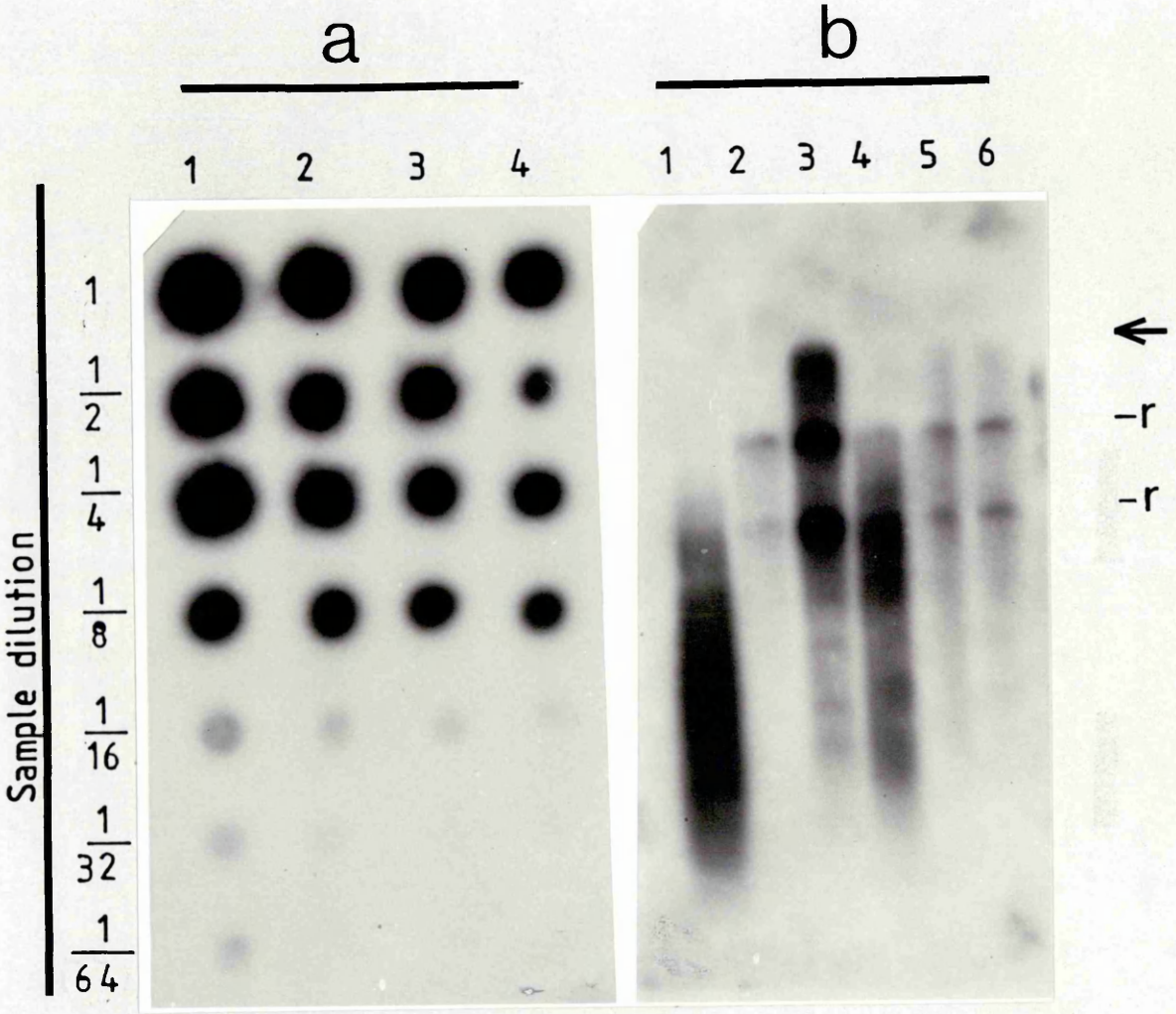


Figure 3-16:

(a) Detection of viral RNA by dot-blot hybridization. (1) purified SYNV RNA, 0.5µg was applied in the first spot. Sap samples from infected (2) leaves and (3) roots 10 d after inoculation. (4) Sap sample from healthy leaves. (b) Northern blot hybridization of purified RNA from (1) healthy leaves; infected leaves (2) 5 d, (3) 10 d, (4) 15 d after inoculation and (5,6) infected roots 10 d after inoculation. Two µg RNA was applied per lane. Detected SYNV RNA is arrowed ribosomal RNAs are indicated by (r). (a) & (b) were probed with cDNA prepared to SYNV RNA.

RNA (rRNA) in both healthy and infected lanes (fig. 3-16b), indicating the presence of cDNAs to rRNA in the probe. This presumably resulted from contamination of the viral RNA template with rRNA. Pre-hybridization of cDNA with RNA or sap from uninfected plants reduced but did not eliminate the cross-hybridization. In order to eliminate this problem, cloned probes were substituted for the reverse transcribed cDNA. A mixture of plasmid DNAs pSYN302, pSYN402 and pSYN503 (see section, 2.14.3.1), labelled by nick translation, reacted only intermittently with undiluted healthy sap but not at all to dilutions of 10^{-1} or more (see below and figs. 3-19 & 3-20). A mixture of these three plasmid DNAs was subsequently used as the hybridization probe.

Viral RNA was detected in both unexpanded leaves and roots 5 d after inoculation and reached a maximum 10 d after inoculation. Levels of hybridizable RNA then fell gradually and were barely detectable 30 d after inoculation in the case of unexpanded leaves (fig. 3-17a) and between 16-20 d after inoculation in the case of roots (fig. 3-18a). No viral RNA was detectable in roots from day 20 and after. In the experiments shown in figures 3-17a & 3-18a the uninfected sap gave a strong positive signal when undiluted, but not at higher dilutions. Hybridization to undiluted sap was weaker in other experiments (see figs. 3-17b & 3-18b). Comparable non-specific binding to undiluted sap samples has been reported by Owens and Diener (1981) and probably results from non-specific binding of the probe to components other than RNA. By comparing a series of threefold dilutions of sap from leaves 10 d after inoculation with dilutions of purified viral RNA (fig. 3-17a,b) the hybridizable RNA (genomic and

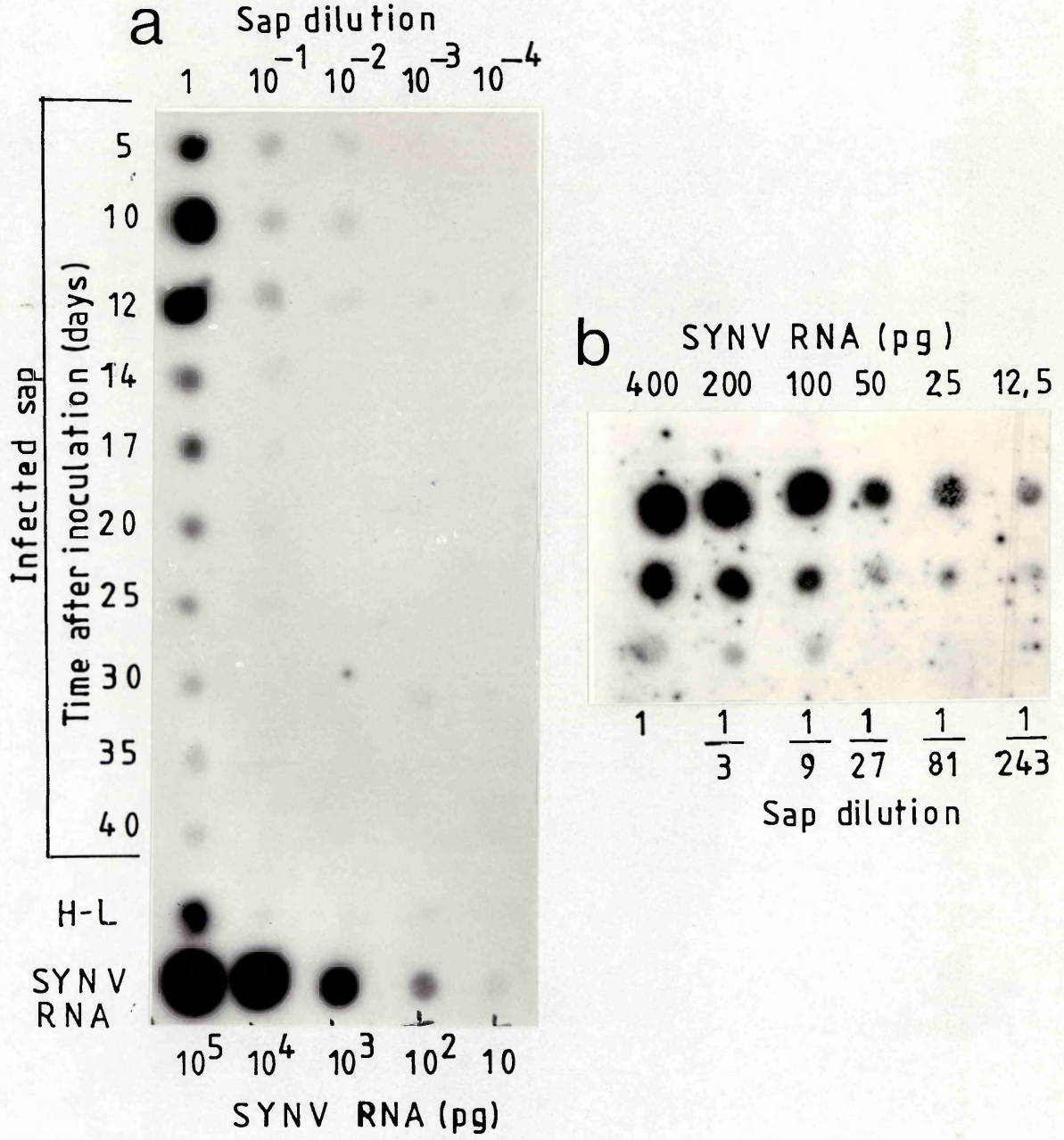


Figure 3-17:

Detection of viral RNA by dot-blot hybridization. (a) Sap samples from infected leaves obtained at intervals from 5 days to 40 days after inoculation and from healthy leaves (H-L). Spots, from left to right, are a series of ten-fold dilutions. The bottom row of spots contains purified SYNV RNA at the indicated concentrations. (b) Sap samples from (bottom row) healthy leaves, and (middle row) ten day infected leaves. Spots, from left to right, are a series of three-fold dilutions. The top row contains purified SYNV RNA at the indicated concentrations.

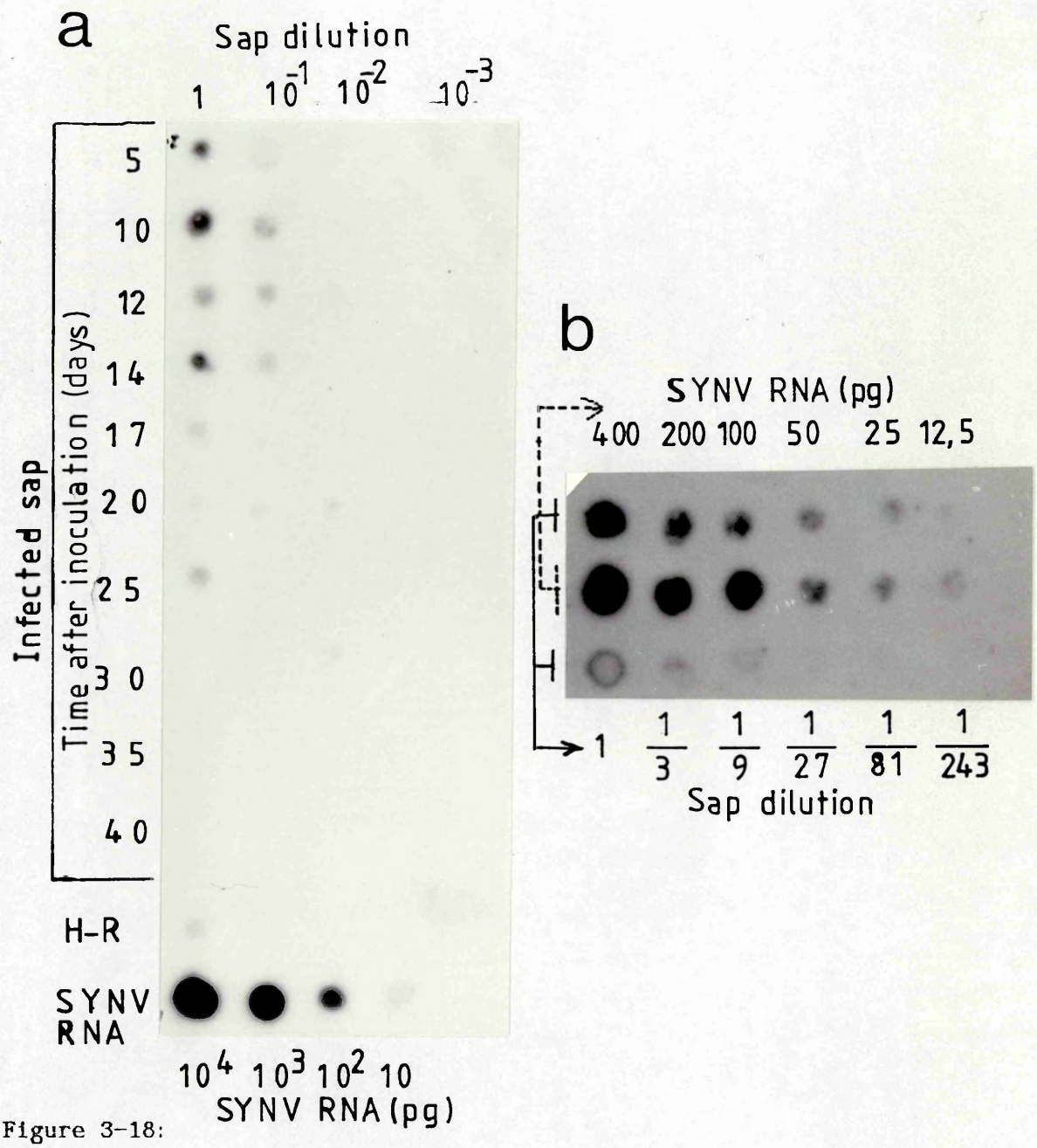


Figure 3-18:

Detection of viral RNA by dot-blot hybridization. (a) Sap samples from infected roots obtained at intervals from 5 days to 40 days after inoculation and from healthy roots (H-R). Spots, from left to right, are a series of ten-fold dilutions. The bottom row of spots contains purified SYNV RNA at the indicated concentrations. (b) Sap samples from (bottom row) healthy roots, and (upper row) ten day infected roots. Spots, from left to right, are a series of three-fold dilutions. The middle row contains purified SYNV RNA at the concentration of (from left to right) 400, 200, 100, 50, 25, 12.5 pg/spot, respectively.

mRNA) was estimated to be 40ng/g tissue. Similarly the concentration of RNA in roots was estimated to be 18ng/g tissue (fig. 3-18a,b).

3.2.5.4 Determination of the Movement and Intracellular Location of SYNV in Infected Tissue by Electron Microscopy:

The results presented in sections 3.2.4, 3.2.5.2.2 & 3.2.5.3 suggest a rapid spread of virus to the roots and subsequently throughout the plants. In order to determine whether this rapid increase in the level of virus antigen resulted from an increase in the amount of virus per cell or from an increase in the number of infected cells, ultrathin sections from a variety of tissues from infected plants were examined by electron microscopy.

Epidermal and mesophyll cells from unexpanded leaves, 5 d after inoculation contained numerous virus particles. Mature virus particles were observed in membrane-bound inclusions within the nucleus and within the perinuclear space, with a few scattered in the cytoplasm (fig. 3-19a). . Nucleocapsids, associated with viroplasms, were observed within the nuclei (fig. 3-19b). Fifteen percent of 180 cells examined in 6 separate grids contained detectable virus particles. Root cells from the same plants also contained viral particles within the nucleus and perinuclear space and also a few particles clustered in the cytoplasm (fig. 3-20). No virus particles were seen in over 200 cells from expanded leaves examined at this stage of infection.

Eight days after inoculation, about 65% of over 200 cells from unexpanded and expanded leaves contained virus particles. Large clusters, containing mature virions, were observed in the perinuclear space as well as in the nucleus (fig. 3-21). Virions could be clearly

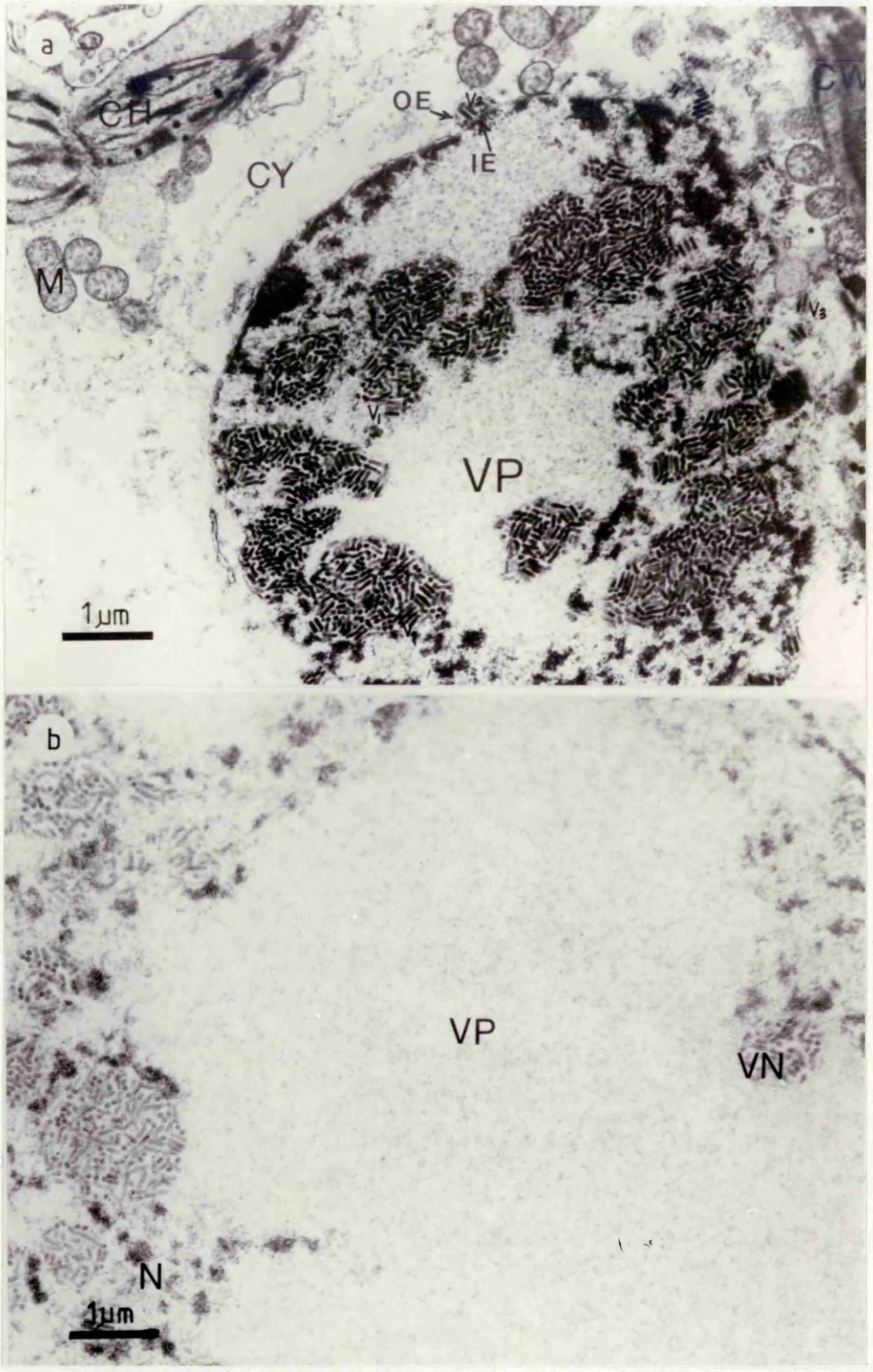


Figure 3-19:

Electron micrographs of the distribution of viral particles in unexpanded leaves 5 days after inoculation. (a) Mature particles (indicated by V1, V2 & V3 respectively) within the nucleus (N), perinuclear space and cytoplasm (CY). (b) Nucleocapsids (VN) associated with viroplasm (VP) within a nucleus. The inner and outer nuclear envelopes are indicated by IE and OE, cell wall by CW, chloroplasts by CH and mitochondria by M.

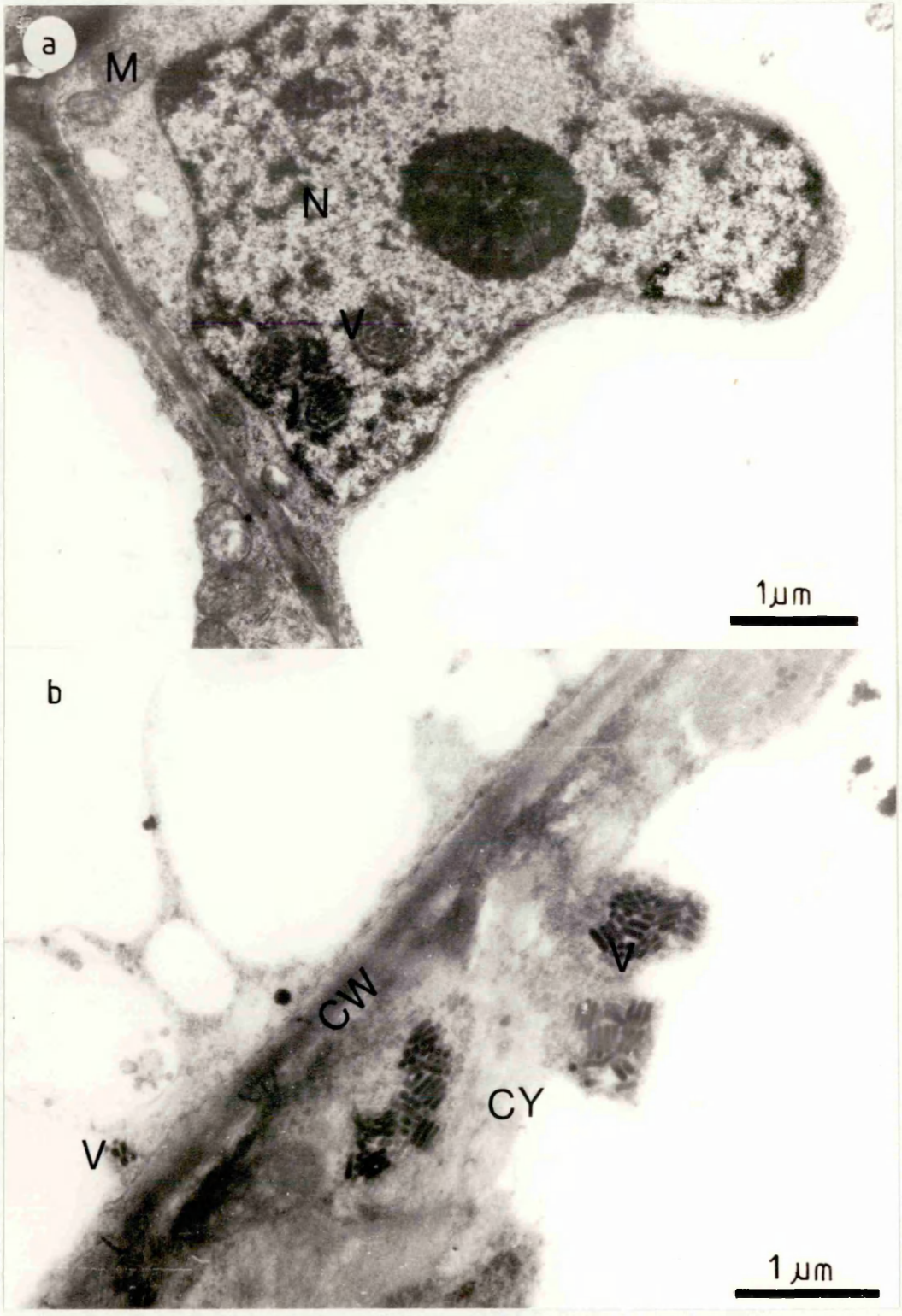


Figure 3-20:

Electron micrographs of viral particles within (a) the nucleus and (b) cytoplasm of root cells from plants 5 d after inoculation. Abbreviations are as in page X

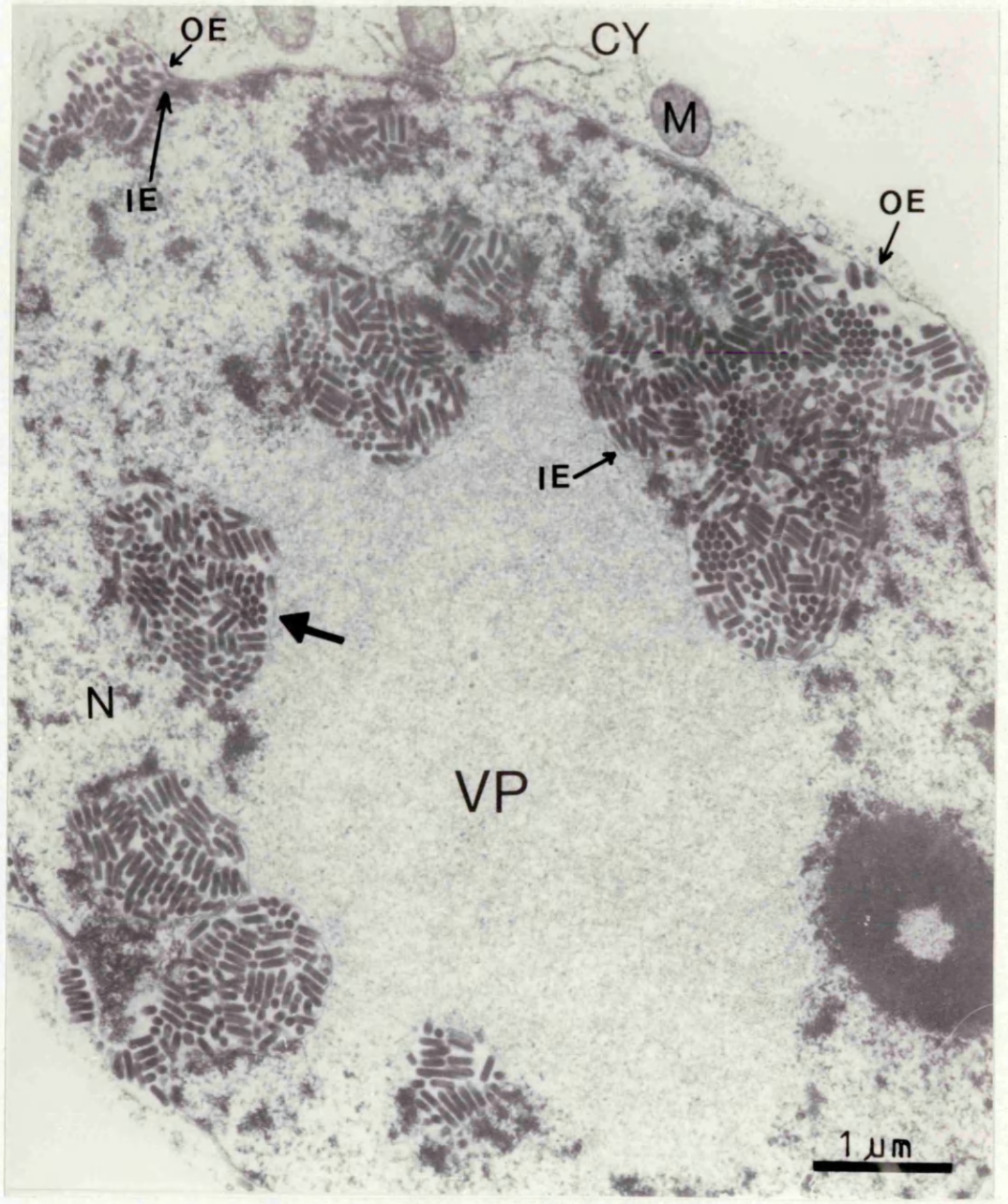


Figure 3-21:

Electron micrographs of infected cells from an unexpanded leaf 8 d after inoculation. Clusters of virus particles are located in the perinuclear space and in the nucleus. The inner and outer nuclear envelopes are indicated by IE and OE. The membrane-bound inclusion, within the nucleus, is indicated by the thick arrow. Other abbreviations are as in page X

seen budding from the nucleus through the inner nuclear envelope into the perinuclear space (fig. 3-22). A few virions were scattered within the cytoplasm, often associated with rough endoplasmic reticulum (fig. 3-23a,b,c). Nucleocapsids were present, scattered within some nuclei as well as associated with granular matrix within densely staining viroplasms (fig. 3-24). Cells of both primary and secondary roots contained mature virions, the majority were observed in the cytoplasm and the minority in the perinuclear space (fig. 3-25a,b).

Ten days after inoculation, sections from all tissues showed the highest proportions of cells which contained virus particles. The location and numbers of particles were similar to these in sections from plants 5 and 8 d after inoculation. In some cells the perinuclear space was extended and intruded both into the cytoplasm and nucleus (fig. 3-26a,b). In both unexpanded and expanded leaves, virus particles were seen in about 90% of over 1000 mesophyll, palisade and epidermal cells examined over a period of two and a half years in a large number of independent sections. Cells of both primary and secondary roots contained mature virions in the cytoplasm, as well as large clusters within the perinuclear space. Some of the virions within the cytoplasm of both roots and leaf cells were in small groups bounded by a membrane. Nucleocapsids associated with nuclear viroplasms were also observed. A significant decrease in the amount of granular matrix was also evident after 10 d (fig. 3-27a,b). The time at which the concentration of virus antigen, as determined by dot-immunobinding assay and ELISA and viral RNA as determined by dot-blot hybridization were greatest, thus coincided with the time at which the highest proportion of cells contained virus particles.

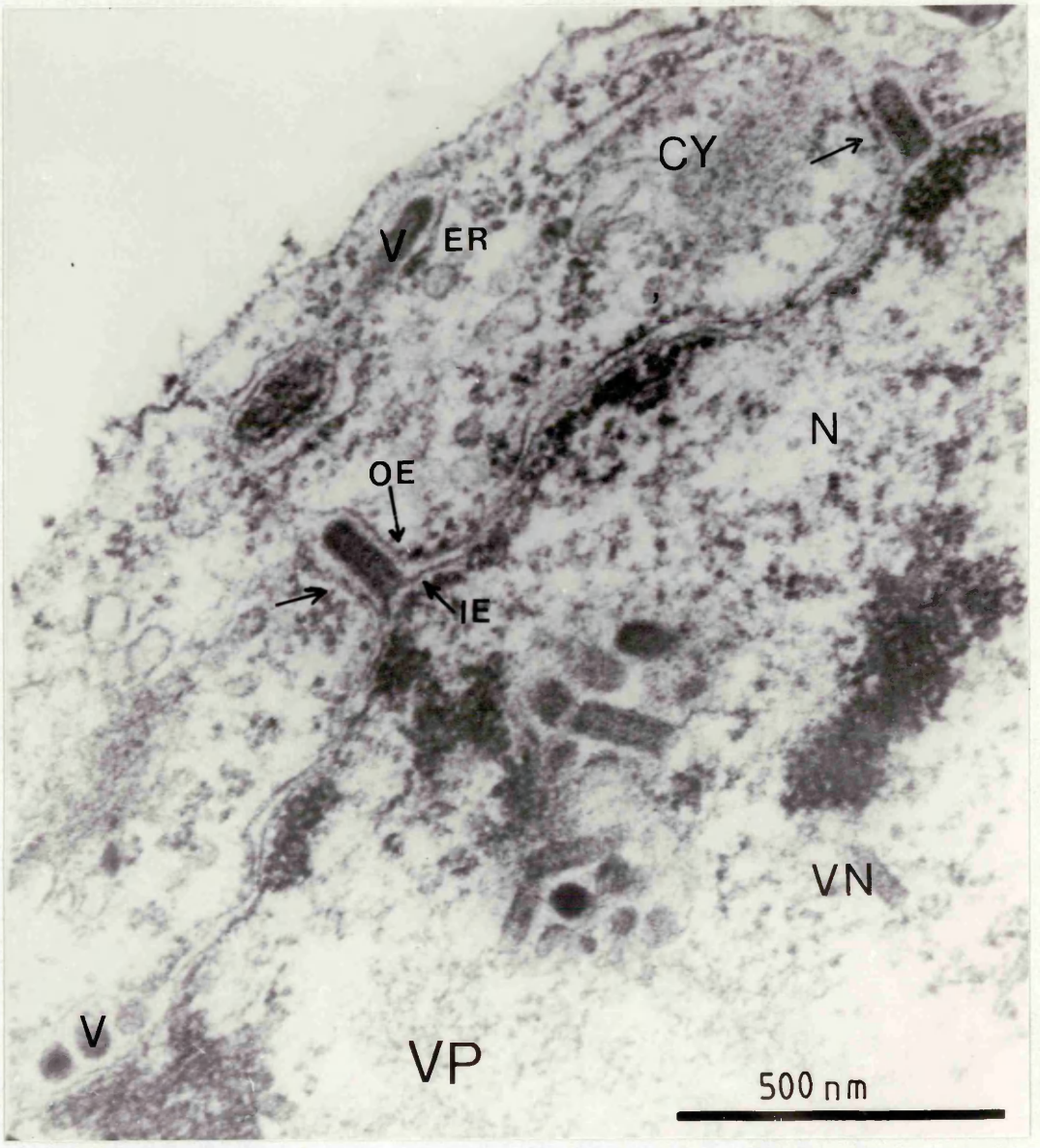


Figure 3-22:

Virus particles within a cell from an unexpanded leaf 8 d after inoculation. Arrows show virus budding from the nucleus through the inner nuclear envelope. Rough endoplasmic reticulum associated with virions is indicated by ER. Other abbreviations are as in page X

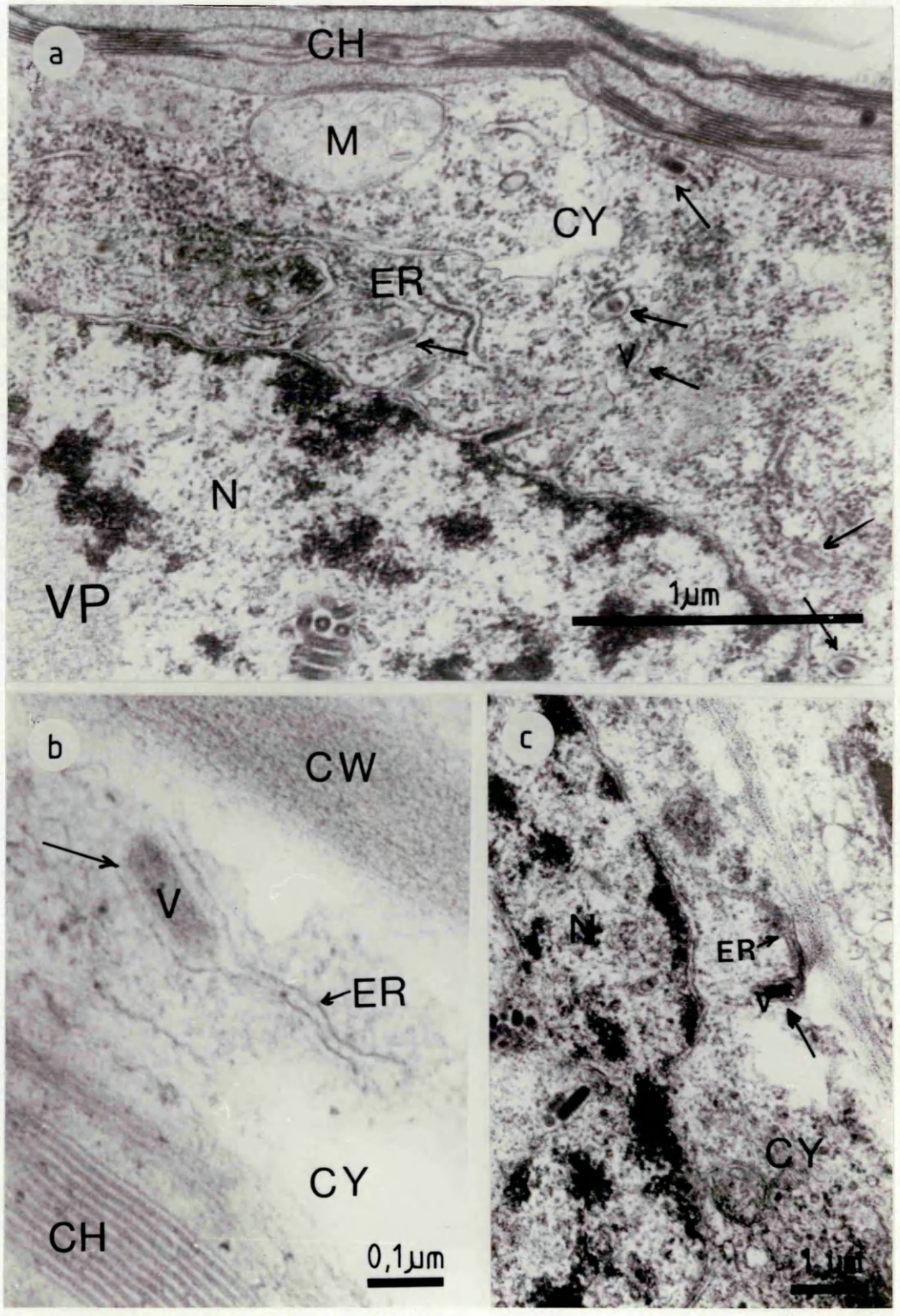


Figure 3-23:

Electron micrographs showing virus particles associated with endoplasmic reticulum (ER) within cells from unexpanded leaves 8 d after inoculation. (a) The developed network of ER; (b) and (c) virus particles vesiculating through ER which is extended to accommodate the virus. Other abbreviations are as in page X

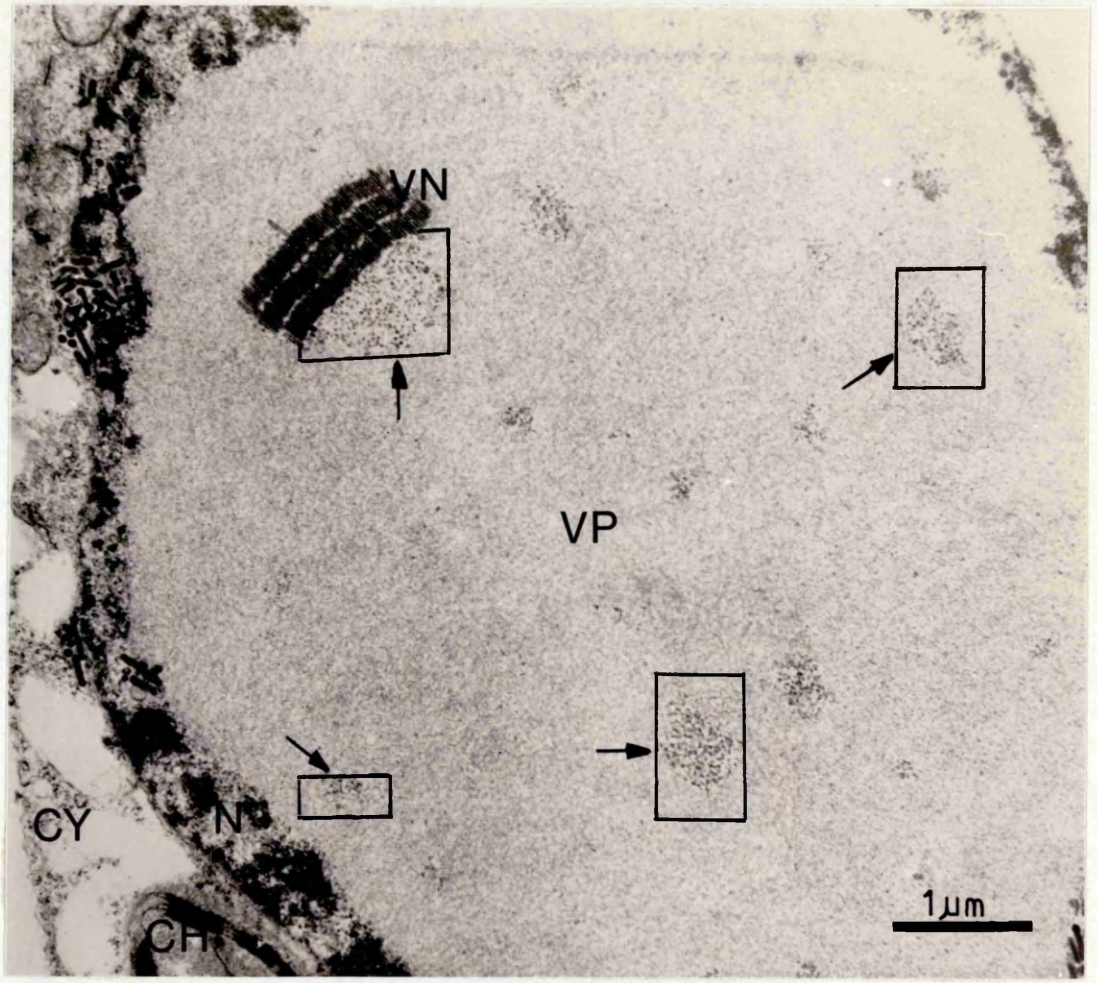


Figure 3-24:
Rows of nucleocapsids (VN) associated with granular matrix in a large viroplasm within a cell from an unexpanded leaf 8 d after inoculation. Examples of granular matrix are boxed and arrowed. Other abbreviations are as in page X

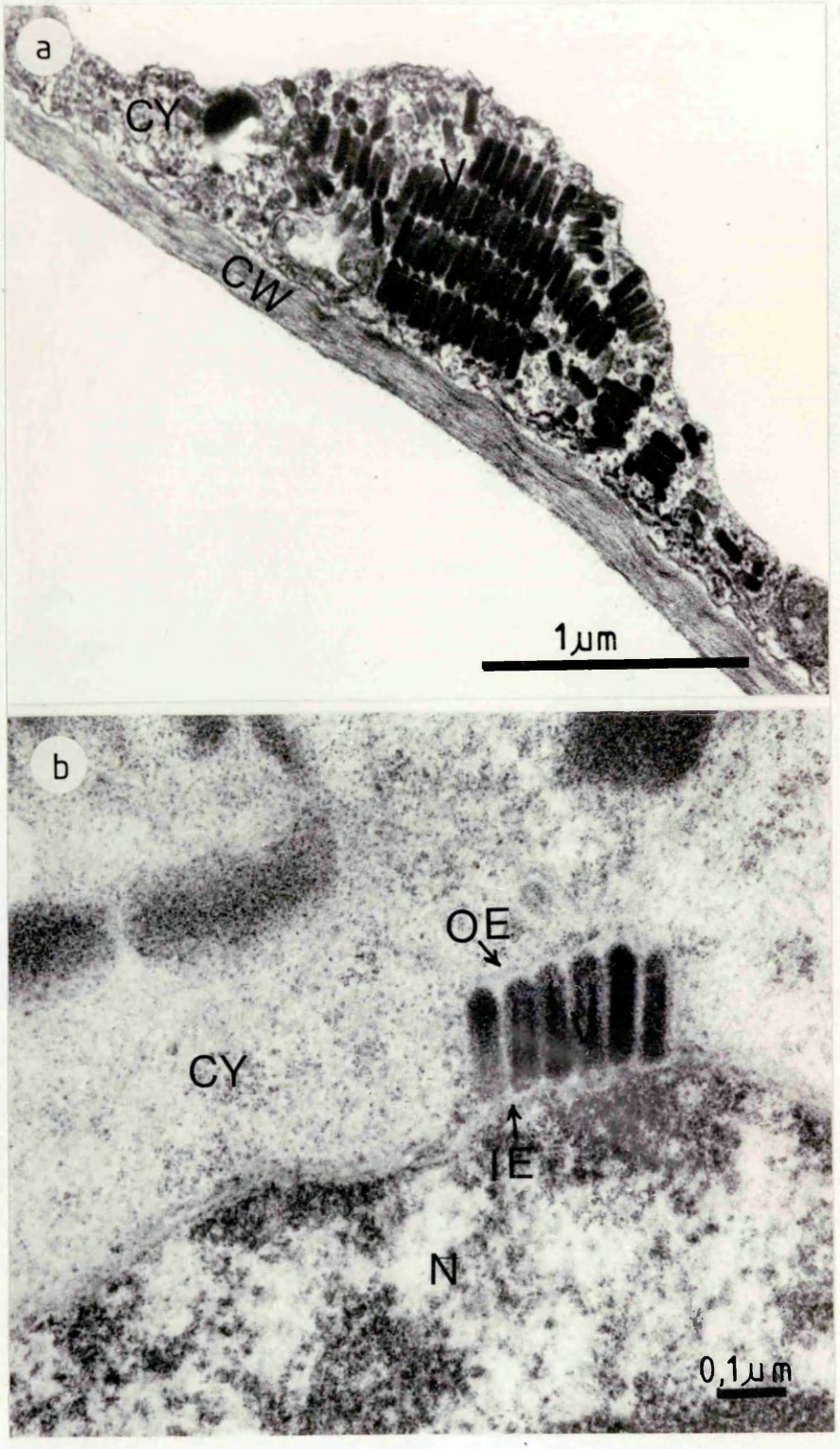


Figure 3-25:

Electron micrographs showing viral particles within roots from plants 8 days after inoculation. (a) viral particles within the cytoplasm. (b) viral particles within the perinuclear space. Other abbreviations are as in page X

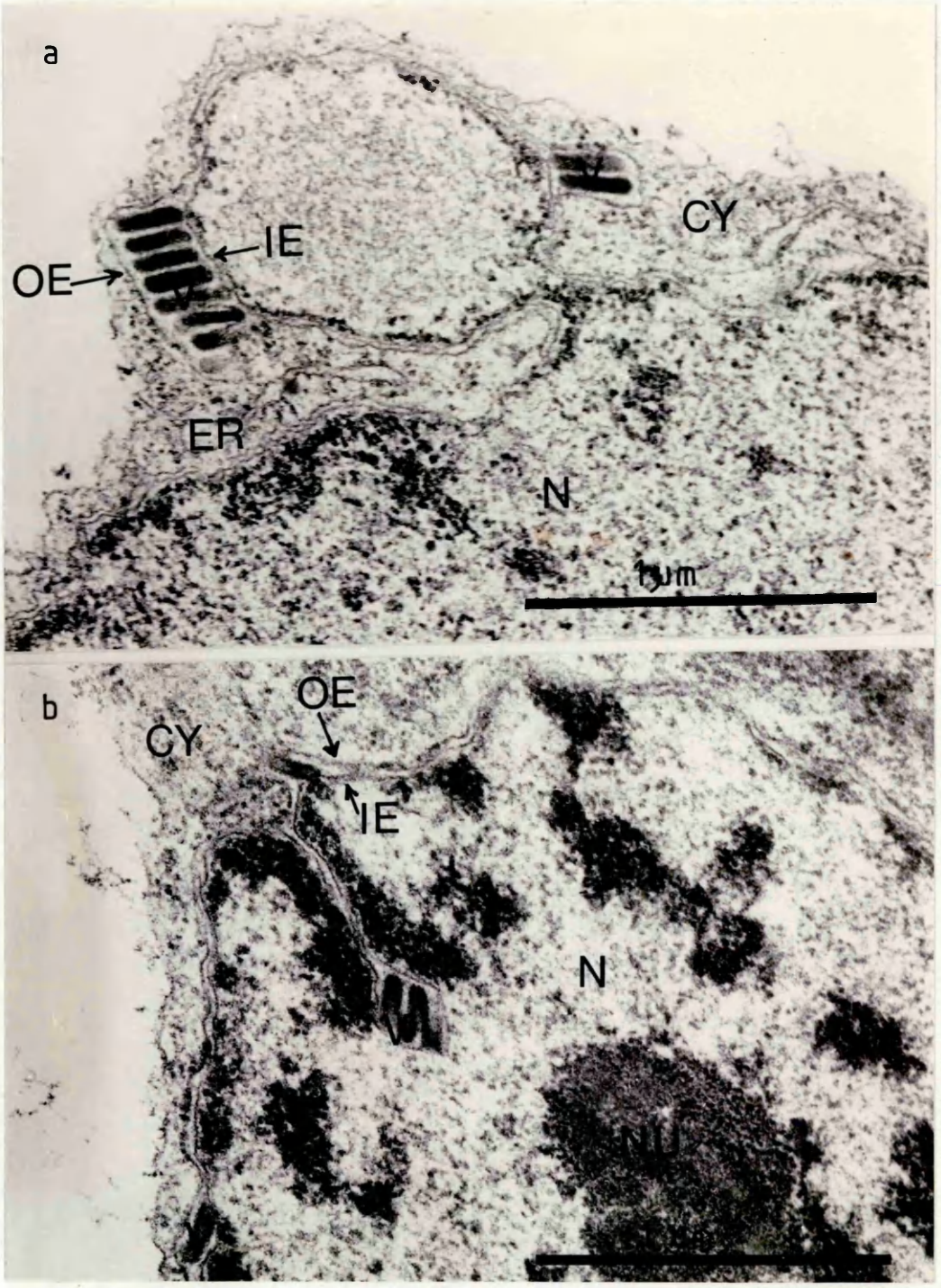


Figure 3-26:

Electron micrographs showing the perinuclear space extended and swollen into (a) the nucleus and (b) the cytoplasm; (a) was sectioned from an infected leaf 15 d after inoculation; (b) was sectioned from an infected leaf 6 d after inoculation. The inner and outer nuclear envelopes are indicated by IE and OE. Other abbreviations are as in page X

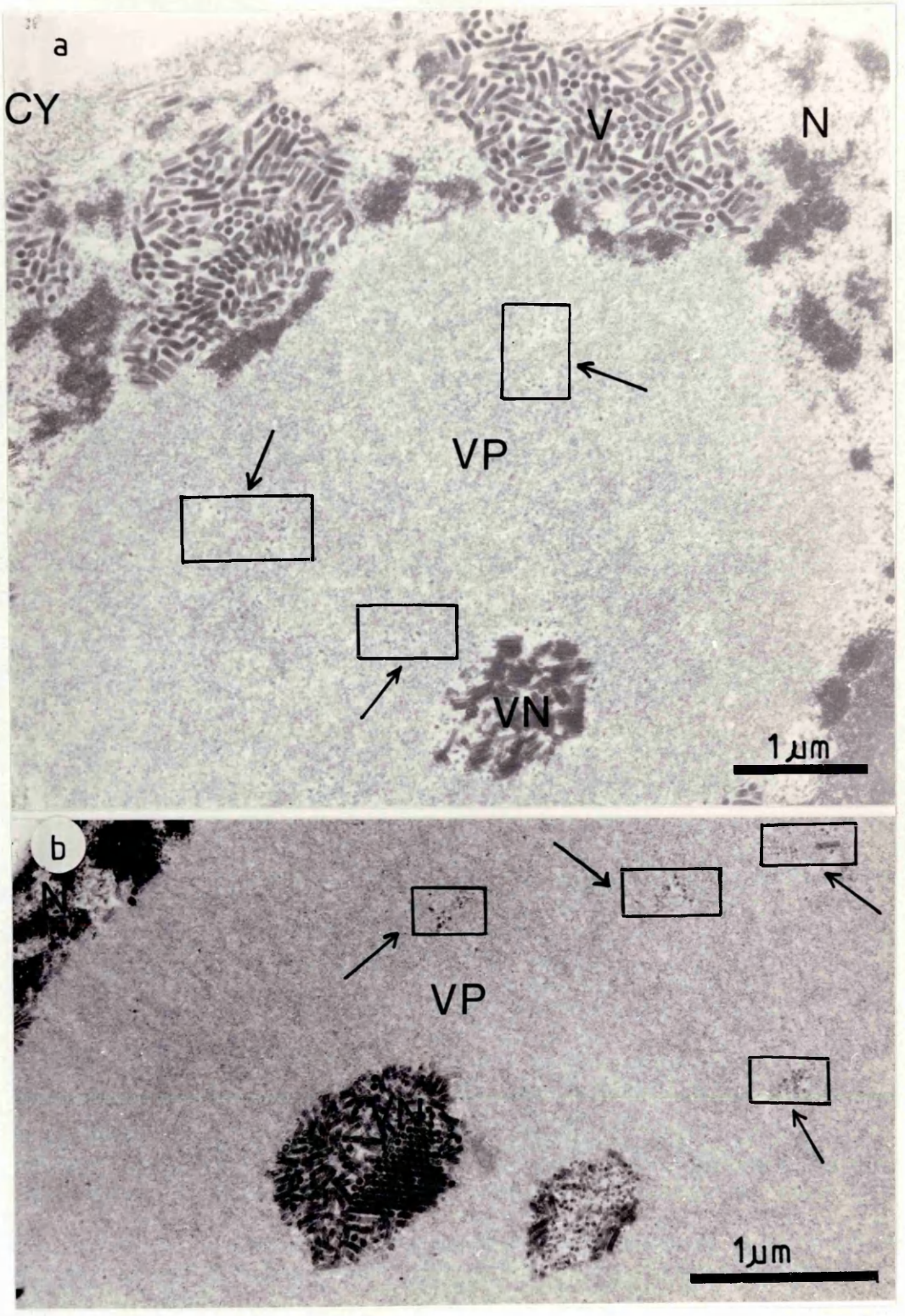


Figure 3-27:

Electron micrographs showing clusters of nucleocapsids (VN) associated with granular matrix in a large viroplasm within cells from unexpanded leaves 10 d after inoculation. Samples of granular matrix are boxed and arrowed (c.f. figure 3-24). Abbreviations are as in page X

Phloem companion and phloem parenchyma cells from plants 8 to 19 d after inoculation consistently contained large numbers of virus particles (fig. 3-28a,b) and scattered virus particles were present in the phloem sieve elements. Virus particles were also observed within the xylem (fig. 3-29a) and xylem parenchyma (fig. 3-29b) cells.

The number of virus particles observed within cells and the proportion of the cells examined which contained virus fell dramatically between 10 and 20 d after inoculation; after 20 d, virus particles could be observed in less than 10% of cells from either unexpanded leaves or roots. When leaf and root material from plants 15 and 20 d after inoculation was examined, most of the virus had disappeared from the nucleus and perinuclear space. Scattered particles were observed in the cytoplasm, sometimes in small membrane-bound inclusions (fig. 3-30). Cells from plants infected for longer than 20 d only rarely contained virus, nearly always within the cytoplasm.

Terminal (unexpanded) leaves of flowering plants were examined 30 d after inoculation. These leaves showed symptoms of mottling and vein clearing. Sections showed up to about 90% cells containing virions, with large numbers of viral inclusions within the perinuclear space and scattered through the cytoplasm. Various parts of the flowers (petals, stamens, anthers and filament) also contained virus particles within nuclei and cytoplasm, the highest proportions of virus observed in the petal tissue (fig. 3-31a). The number of virions observed decreased when corresponding sections from plants 50 d after inoculation were examined, the great majority of virions in these sections were observed in the cytoplasm. No virus particles

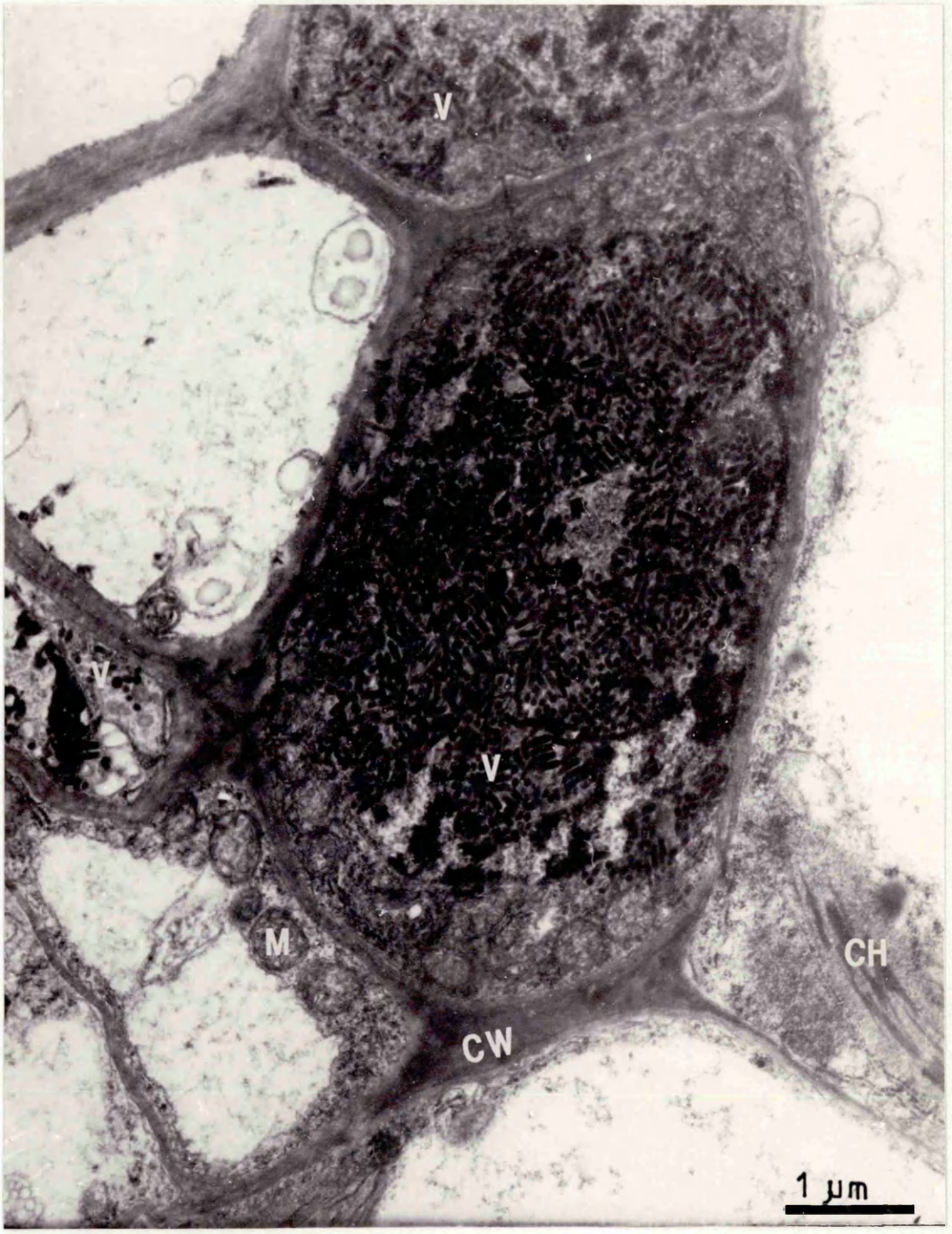


Figure 3-28:
Electron micrograph of phloem companion and phloem parenchyma cells showing large numbers of virus particles within the nuclei. Virus-like particles within the sieve-element are indicated by V. Tissue was taken from leaf 10 d after inoculation. Other abbreviations are as in page X

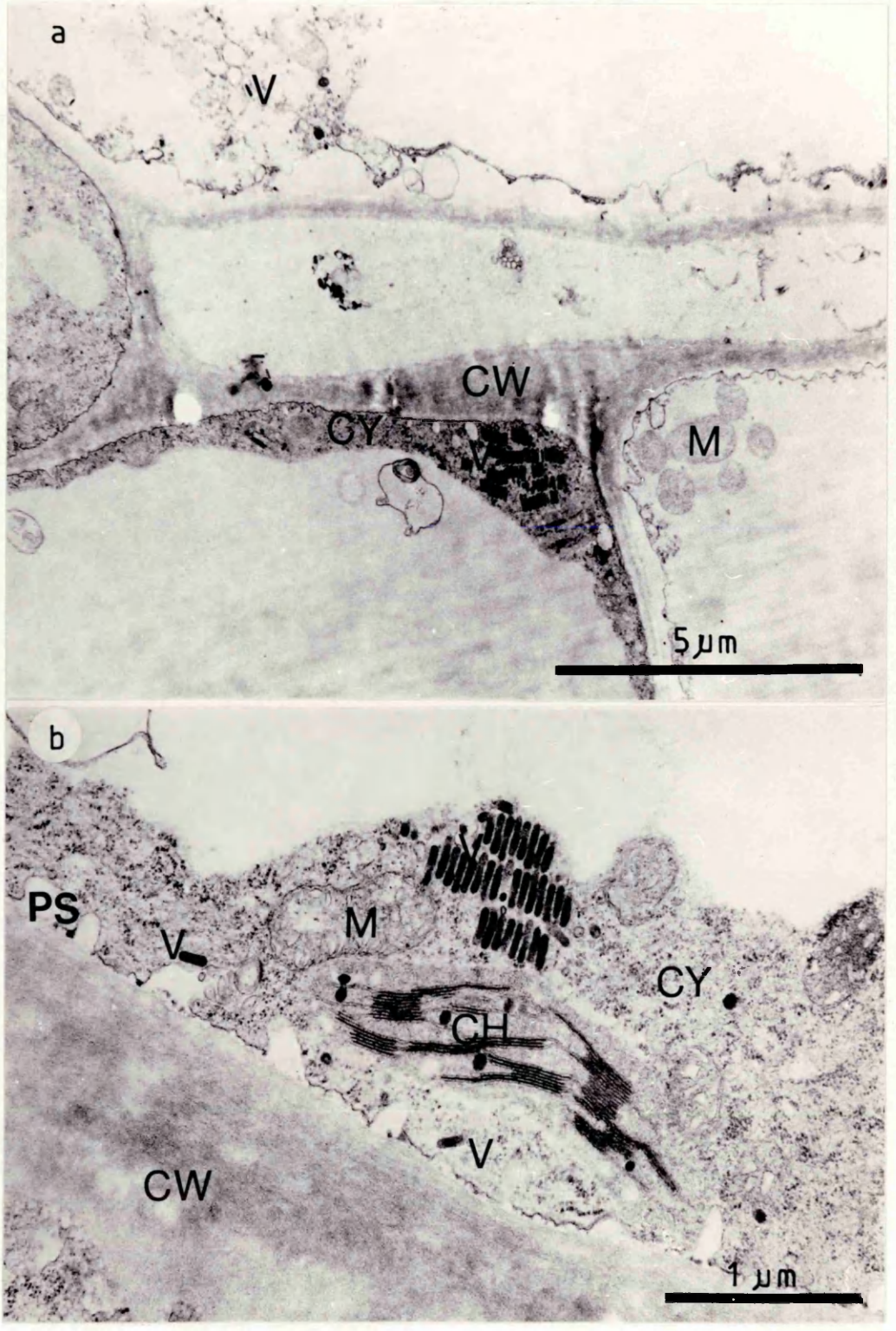


Figure 3-29:

Electron micrographs showing virus particles within (a) xylem and (b) xylem parenchyma cells. Both micrographs are of leaf tissue 10 d after inoculation. Other abbreviations are as in page X

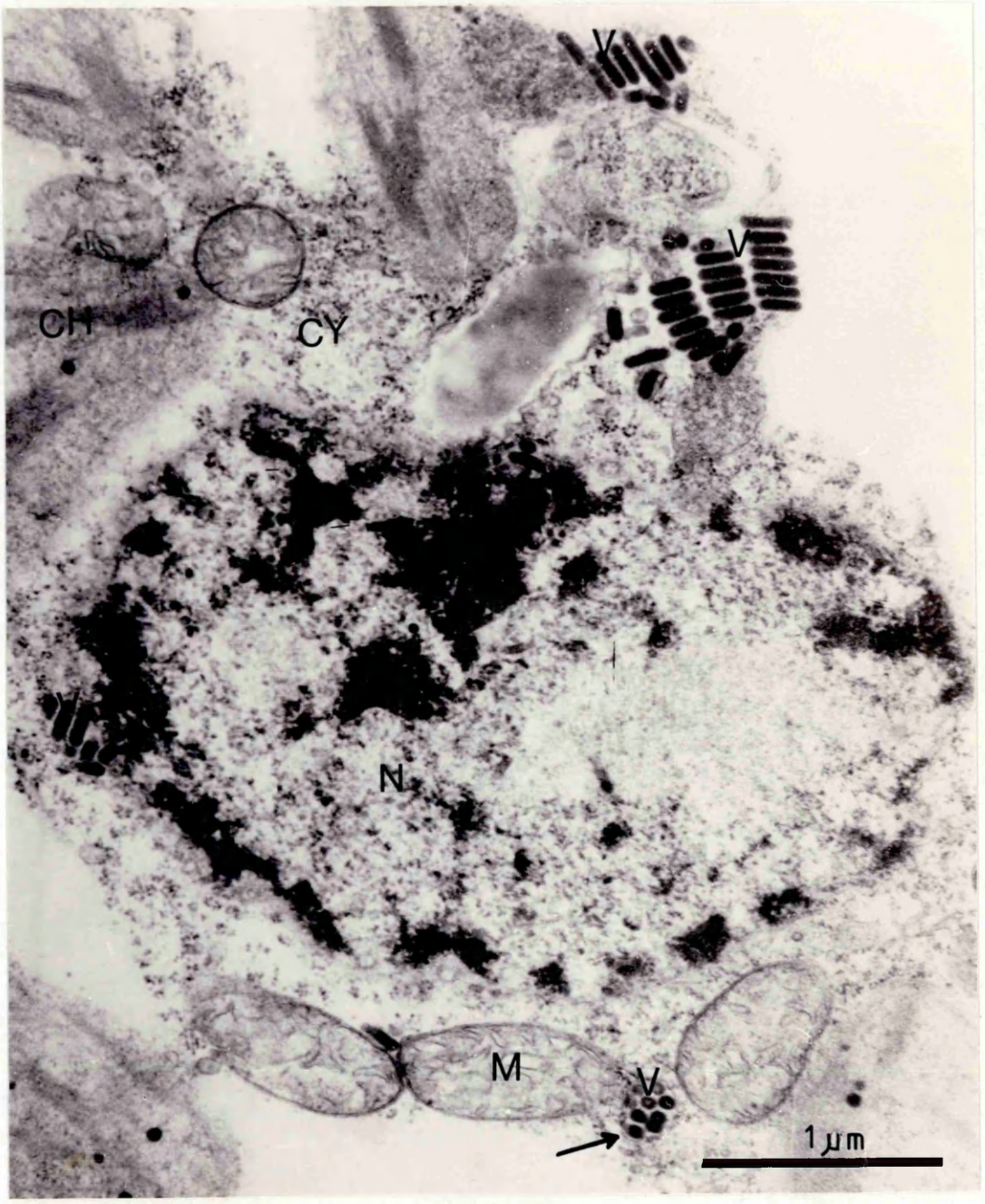


Figure 3-30:

Virus particles within cells of unexpanded leaves from plants 20 d after inoculation. Virus particles within the cytoplasm are indicated by V. A cytoplasmic membrane-bound viral inclusion is arrowed. Other abbreviations are as in page X

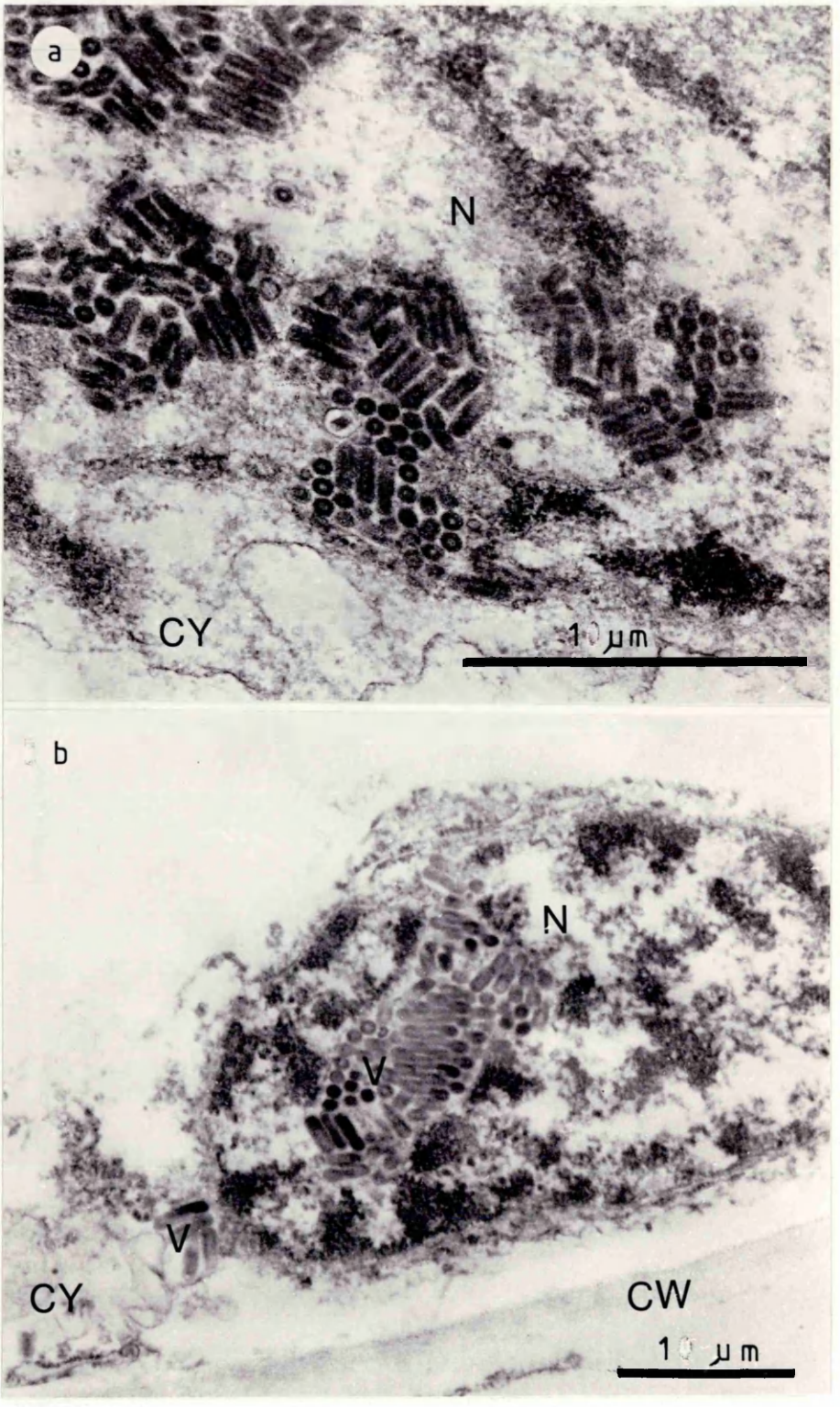


Figure 3-31:

Electron micrographs showing virus particles in flowers of infected plants 30 d after inoculation. Virus particles located within (a) the nucleus of petals and (b) the nucleus and the cytoplasm of filament tissues. Abbreviations are as in page x

were observed in pistil (stigma, ovary and style) either 30 or 50 d after inoculation.

3.3 CONCLUSIONS:

The results presented in this chapter describe the morphology, purification and movement of SYNV in mechanically infected *N. edwardsonii*. The morphology of SYNV particles in negatively stained preparations was dependent on the stain, but authentic bacilliform particles were observed when these preparations were decorated with anti-SYNV serum prior to the staining step.

The spread of SYNV through *N. edwardsonii* has been assayed using ELISA, dot-blot hybridization and electron microscopy. ELISA assays indicated that virus antigen is first detectable in inoculated plants by 12-24 h after inoculation. This result is confirmed by the observation that removal of inoculated leaves up to 12 h after inoculation prevented systemic spread, but removal after 24 h generally failed to do so. All the assays demonstrated the rapid systemic movement and replication of SYNV within the plant with levels in various tissues reaching a peak at 10 d after inoculation. The results also demonstrate the rapid recovery involving reduction in the levels of viral proteins, the levels of viral RNA, the number of infected cells and the number of virions per cell. This later reduction was accompaned by the movement of the virions from the nuclei into the cytoplasm.

Yields of virus during purification were estimated by comparing yields of purified virus with levels in tissues 10 d after inoculation, and also by measuring losses during each stage of

purification. Losses were quite low; the final yield of purified virus evidently reflects the amount of virus within tissues.

CHAPTER 4

CYTOPATHOLOGICAL CHANGES AND IMMUNOCYTOCHEMISTRY OF INFECTED *NICOTIANA EDWARDSONII* CELLS.

4.1 INTRODUCTION:

The interaction between a plant virus and its host may result in visible or other detectable abnormalities in plants, which are recognizable as symptoms. Symptoms may appear on infected plants several days to several weeks after inoculation (section 1.6.1). Virus infection is also likely to be associated with internal effects which can be recognized by light and electron microscopy, such as cytological and ultrastructural changes, and virus-induced inclusion bodies.

Although the site of multiplication and location of several plant rhabdoviruses are known (for review see e.g. Francki *et al.*, 1981; Jackson *et al.*, 1987). Information about the effects of plant rhabdoviruses on the ultrastructure of their host cells is limited. It seems that the study of LNYV in infected *N. glutinosa* (Wolanski, 1969, PhD thesis) is the only complete one, using electron microscopy, ever mentioned in the literature. Also, with the exception of the identification of viral structural proteins in the nucleoplasm of PYDV-infected *N. rustica* cells (Lin *et al.*, 1987) no immunocytochemical studies seem to have been done on plant rhabdovirus-infected plant cells.

The main aim of the work described in this chapter was to investigate, using the electron microscopy, the effects of SYNV on the ultrastructure of mechanically inoculated *N. edwardsonii* at various stages of infection. The immunogold labelling technique was also used to investigate the location of viral proteins in infected cells.

4.2 RESULTS:

4.2.1 Ultrastructural Changes in the Nucleus:

Five days after mechanical inoculation of *N. edwardsonii* with SYNV, nuclei of infected leaf cells started to show obvious abnormalities when these cells were examined by electron microscopy. Large electron-dense viroplasm-like regions developed in the nuclei of some infected cells (section 3.2,5.4 & fig. 3-19b). The chromatin was less abundant, its distribution was altered, being commonly located adjacent to aggregates of nucleocapsids at the periphery of these nuclei. A slight swelling in the perinuclear space was also evident, perhaps because of the virus budding process. The appearance of the nucleoli was unchanged. Other infected nuclei had not developed viroplasm regions and the chromatin distribution was not affected. Nuclei often showed cytoplasmic invaginations (fig. 4-1a,b). These invaginations very often contained cytoplasmic components, such as ER, ribosomes, mitochondria, Golgi bodies and microbodies (fig. 4-1a). The perinuclear space in such invaginations was expanded and contained virus particles (fig. 4-1b). When thin sections of infected leaves 5 d after inoculation, were immunogold labelled using anti-SYNV antibodies, particles reacted very extensively to the granular and fibrillar viroplasm regions, but not to the chromatin (fig. 4-2a,b), indicating the presence of large amounts of free viral proteins within the viroplasm. Tissue sections failed to bind more than the occasional scattered gold particle when they were labelled with non-immune serum (normal rabbit serum) or when healthy controls were labelled with anti-SYNV antiserum. Similar failure of gold particles to bind to healthy controls, or of infected tissue to react with non-immune serum was observed in all subsequent immunogold labelling experiments.

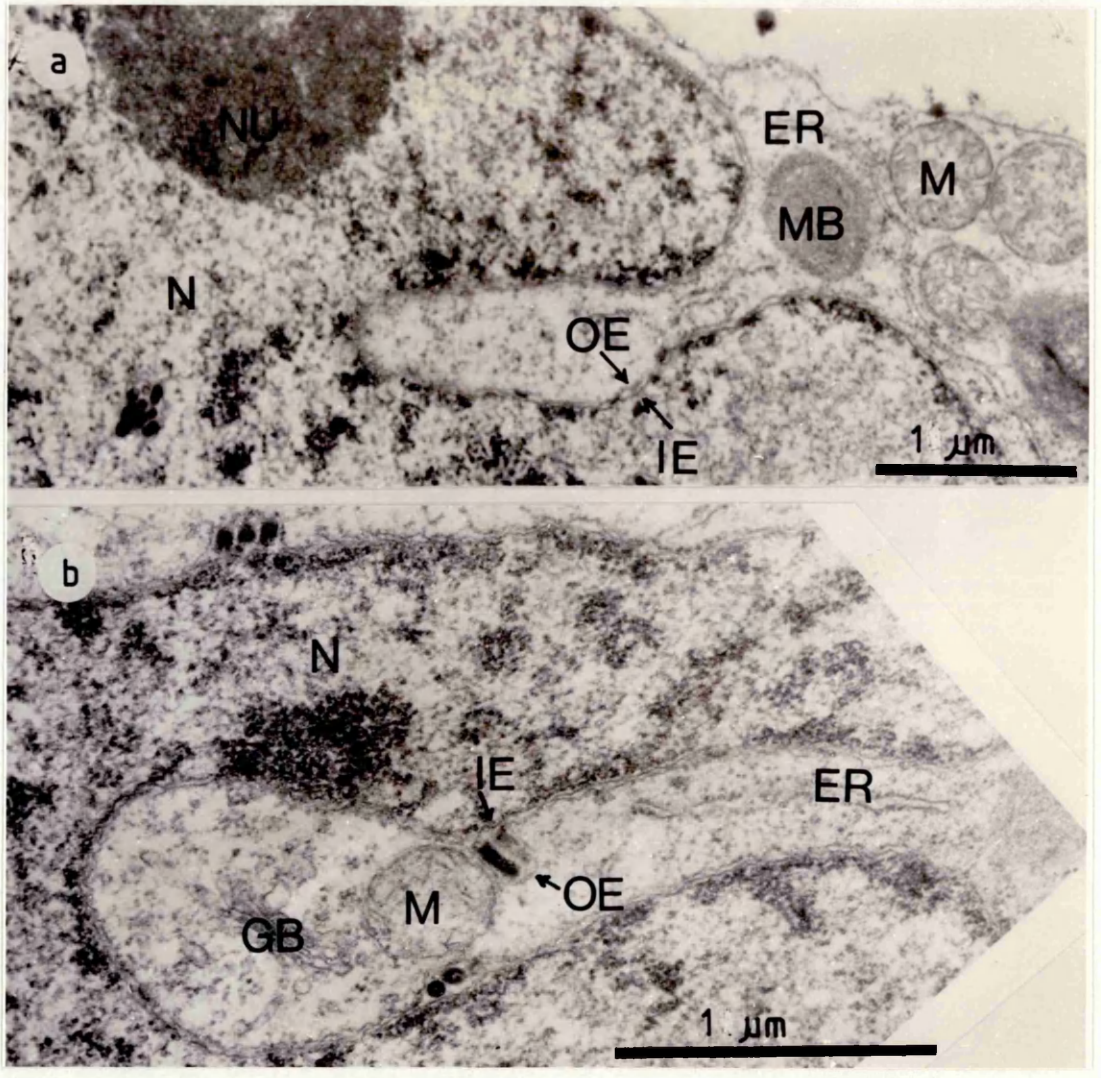


Figure 4-1:
Electron micrographs of infected *N. edwardsonii* cells showing cytoplasmic invaginations into the nuclei. (a) 5 d and (b) 7 d after inoculation. Abbreviations are as in page x

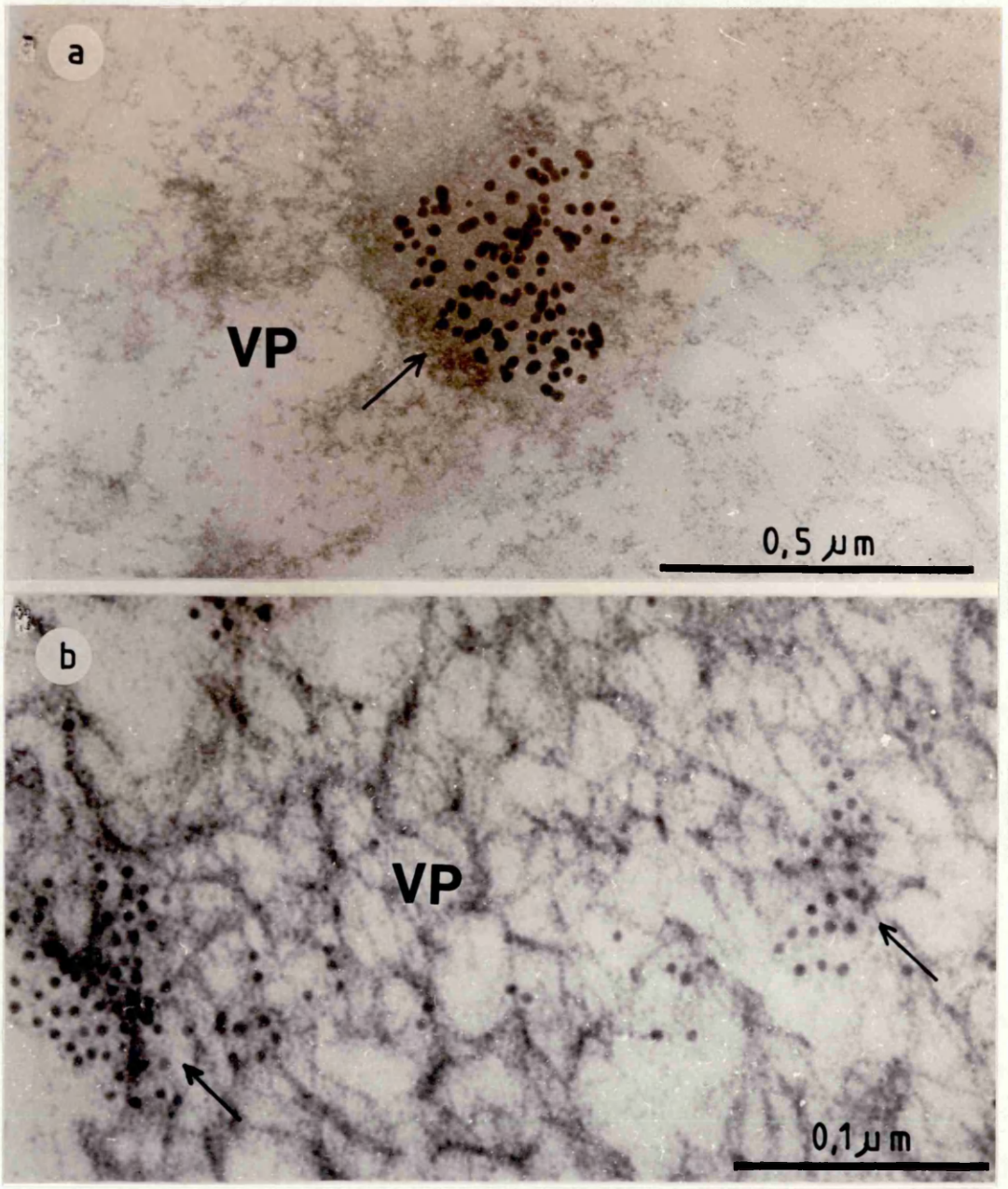


Figure 4-2:

Electron micrographs of infected leaf cells of *N. edwardsonii* 5 d after inoculation labelled with the immunogold probe, showing viroplasms (VP) within nuclei at two magnifications. Clusters of gold particles are arrowed.

By 8-12 d after inoculation, most of the cells were infected and the nuclei had developed viroplasms. These viroplasms contained considerable amounts of granular material aggregated in a number of patches which were associated with nucleocapsids. Large numbers of virus particles were observed within membrane-bound inclusion bodies at the periphery of these nuclei, almost completely embedded in the chromatin area (see section 3.2.5.4 & fig. 3-21). Various degrees of cytoplasmic invagination into the nuclei were also observed. These invaginations were similar to those observed in the tissue examined 5 d after inoculation, but areas of expanded perinuclear space were larger.

At this stage of infection, changes in the nucleoli were also apparent. Swollen nucleoli, bearing up to 4-6 lucent areas were observed. The degree of swelling of these nucleoli seemed to be correlated to the number of such areas (fig. 4-3a,b). The number of lucent areas per nucleolus did not vary with time after inoculation. Immunogold labelling of infected leaves 8-12 d after inoculation revealed a considerable number of clusters of gold particles reacting to the viroplasm regions (fig. 4-4 & 4-5). The gold particles reacted most strongly to these regions of the viroplasm which contained the most dense granular matrix. Gold particles reacted neither to the chromatin nor to the nucleoli nor to any part of the patches within the nucleoli.

Channels of 180-310 nm in width but varying considerably in length were regularly observed running from the chromatin area at the outer edge of the viroplasm into the interior of the viroplasm. These channels contained membrane-bound tubes. Figure 4-6a,b shows typical results. Two membrane-bound tubes of 65 nm width, were observed

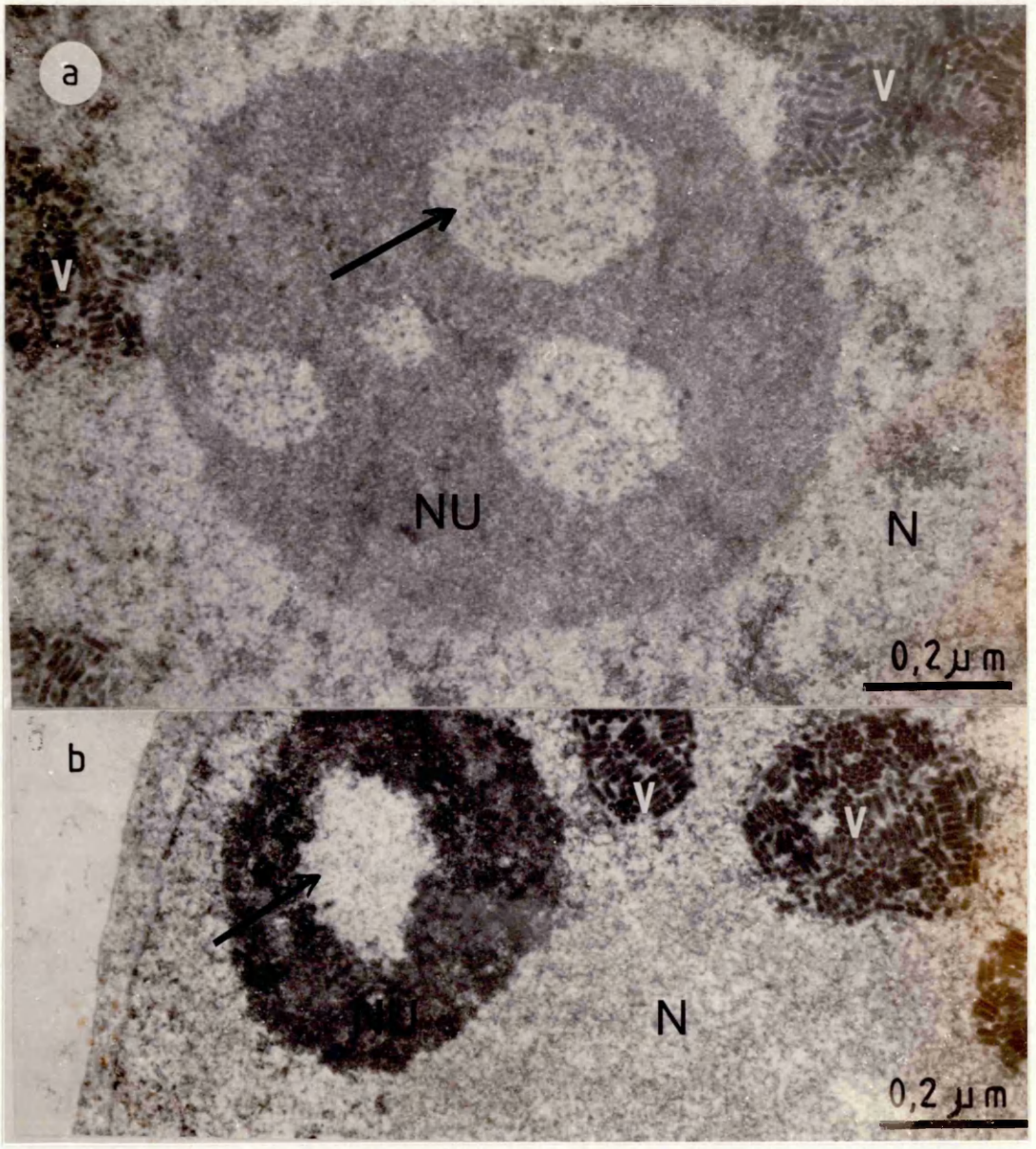


Figure 4-3:
Electron micrographs of infected *N. edwardsonii* showing the hypertrophy of the nucleoli in cells from plants (a) 8 d and (b) 12 d after inoculation. Lucent area is arrowed. Abbreviations are as in page X

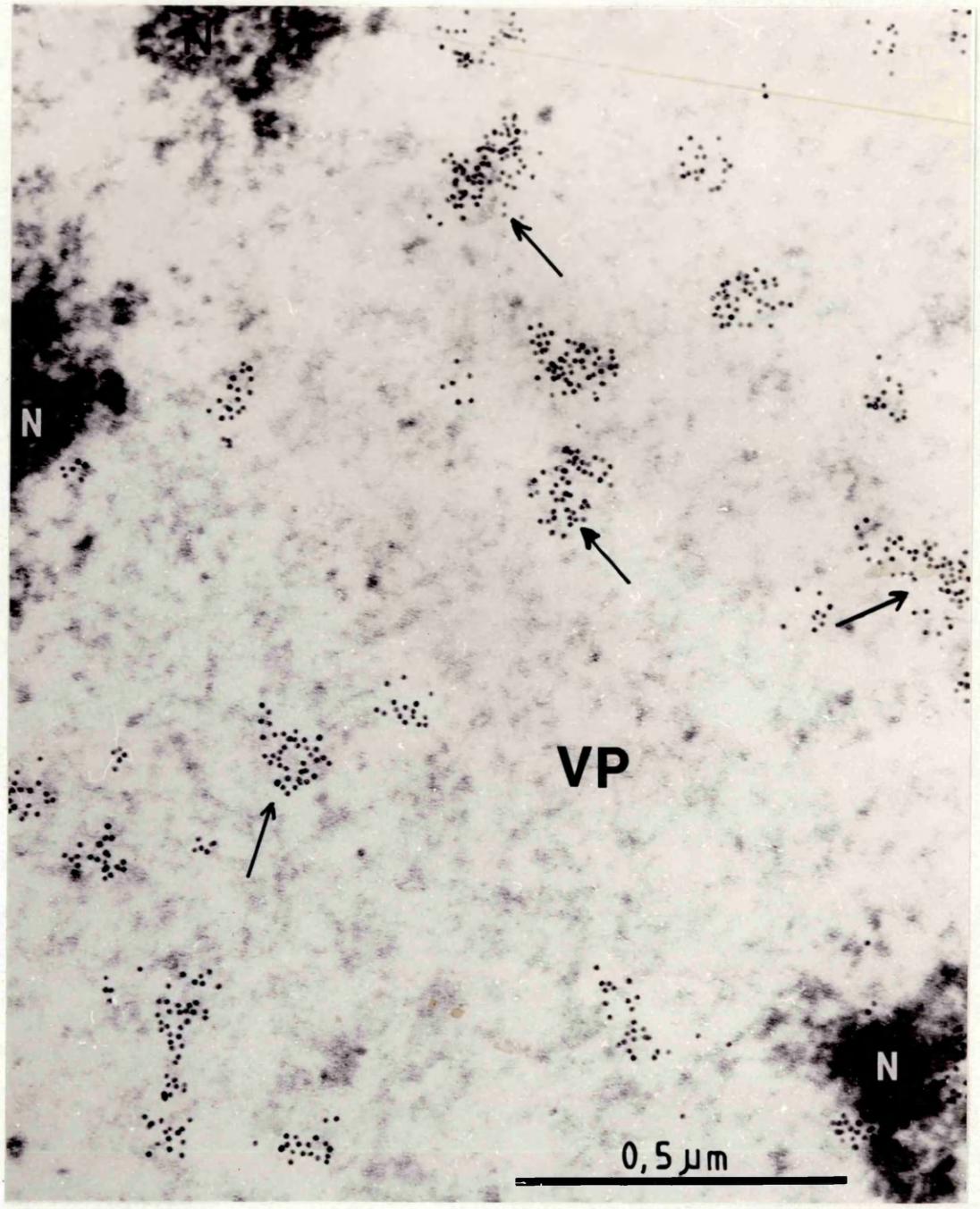


Figure 4-4:

Electron micrograph of immunogold-labelled section of infected *N. edwardsonii* cell 8 d after inoculation showing clusters of gold particles binding to to the nuclear viroplasm. Note the distribution of the gold particles (arrows). c.f. the distribution of the granular matrix in a similar section which has not been immunogold stained (fig. 3-24). Abbreviations are as in page X

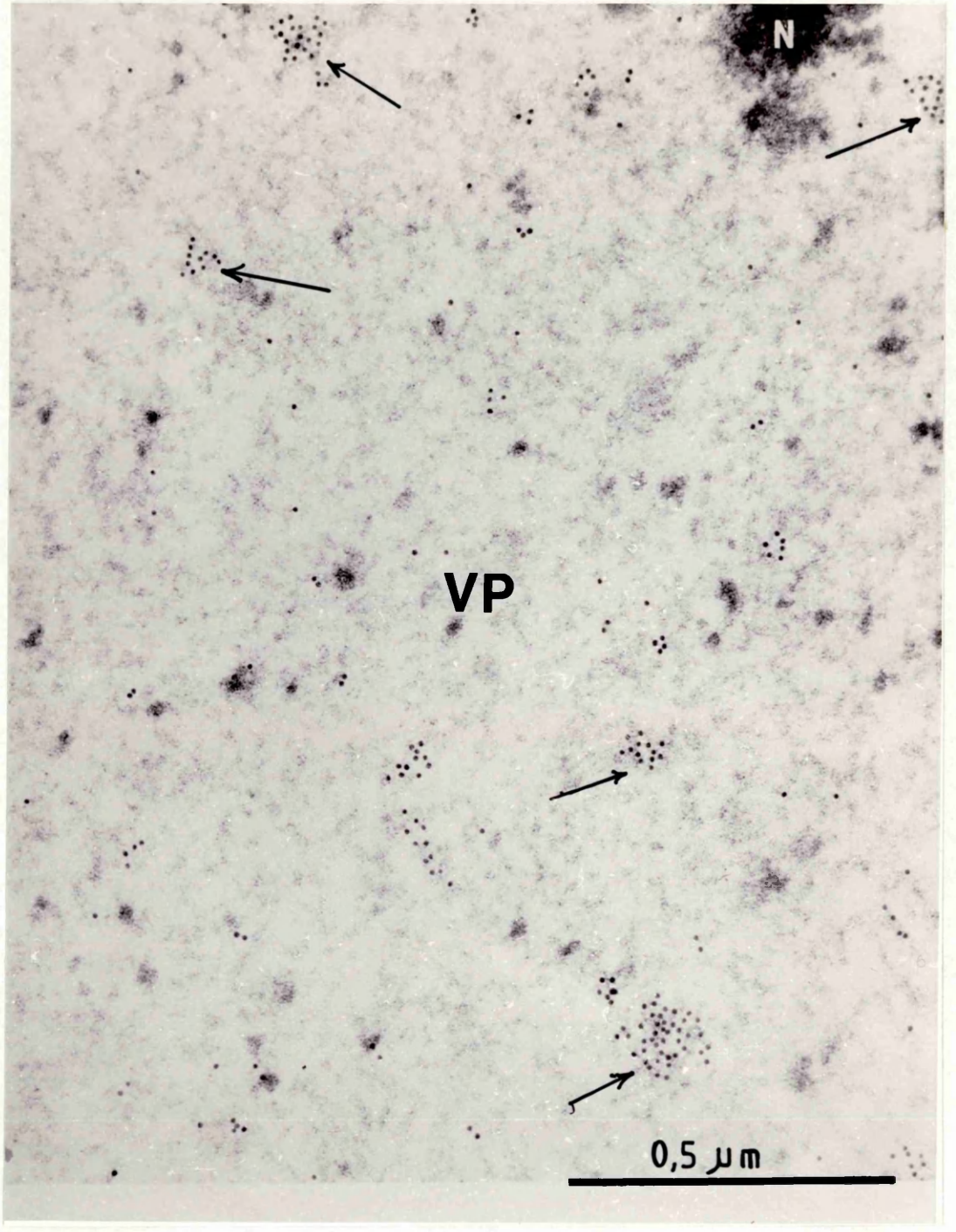


Figure 4-5:

Electron micrographs of an immunogold-stained section of an infected *N.edwardsonii* cell 12 d after inoculation showing clusters of gold particles binding to the nuclear viroplasm. Abbreviations are as in page X

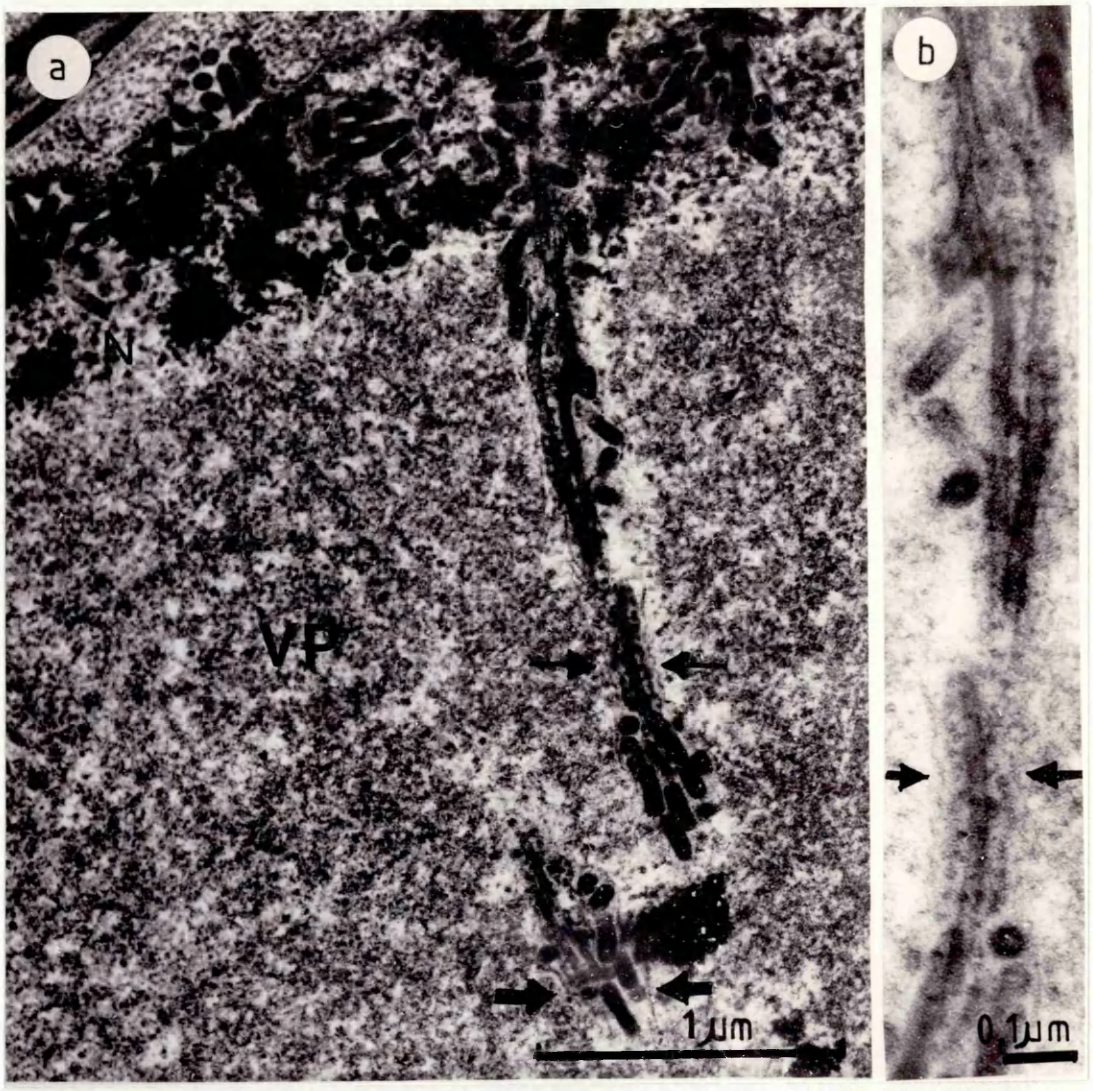


Figure 4-6:

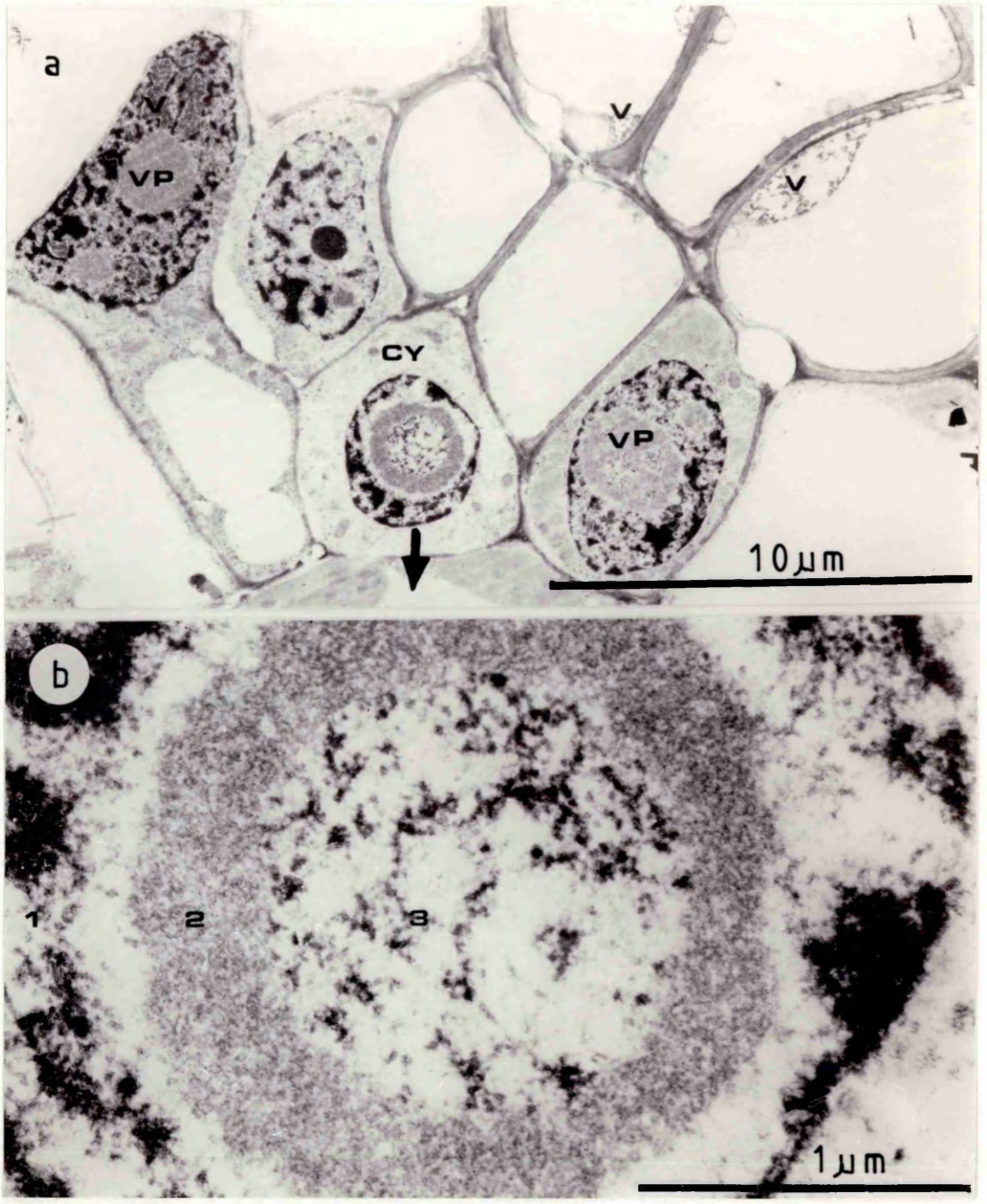
Electron micrographs of an infected *N. edwardsonii* cell 8 d after inoculation showing (a) a channel within the nucleus running from the chromatin area into the viroplasm area. Arrows indicate the sides of the channel. (b) The same channel shown at higher magnification. Note the nucleocapsids within the channel and associated with the outside. Abbreviations are as in page X

running inside the channel, nucleocapsids were arranged lengthwise within these tubes.

Nuclei in cells of the vascular bundle, 10-15 d after inoculation, were enlarged and occupied up to 1/3 to 1/2 of the cell volume. Three distinguishable areas were observed in some nuclei (fig. 4-7a,b). The outer region contained clumps of chromatin scattered very close to the inner envelope, the middle region contained fibrillar material and the inner region contained a mixture of clumps of chromatin and fibrillar material. Other diseased nuclei in the vascular bundle appeared to have altered distributions of chromatin, which was very often aggregated in clumps forming a distinguishable region (fig. 4-7a). In immunogold labelling experiments, no gold particles reacted to any of these regions although they reacted to viroplasms within such cells.

Fibrillar ring-like bodies, resembling these seen in *Phaseolus vulgaris* infected with bean golden mosaic virus (BGMV) (Kim *et al.*, 1978), were occasionally observed in the nuclei of infected cells 10 d after inoculation (fig. 4-8a,b). These fibrillar rings were located in the chromatin regions, close to the inner nuclear envelope.

By 25-30 d after inoculation, few nucleocapsids or mature virions were present in within nuclei (see section 3.2.4.5 & fig. 3-30). Despite the apparent absence of mature virions or nucleocapsids about 35-40 d after inoculation, invaginations of the nuclei were even more convoluted than those observed earlier in infection (figs. 4-9 & 4-10). Such convoluted nuclei were clearly distinguishable from those of healthy cells and were absolutely characteristic of chronically infected plants from 30 d to at least 150 d after inoculation. Occasionally, empty membrane-bound inclusion bodies were observed



Figrue 4-7:

Electron micrographs of infected *N. edwardsonii* cells in vascular system tissue 12 d after inoculation. Note the hypertrophy of the nuclei in (a) which occupy up to 1/3 to 1/2 of the cell volume. (b) Higher magnification of nucleus indicated in (a). Note the three distinguished areas (1, 2 & 3 in micrograph b). Abbreviations are as in page X

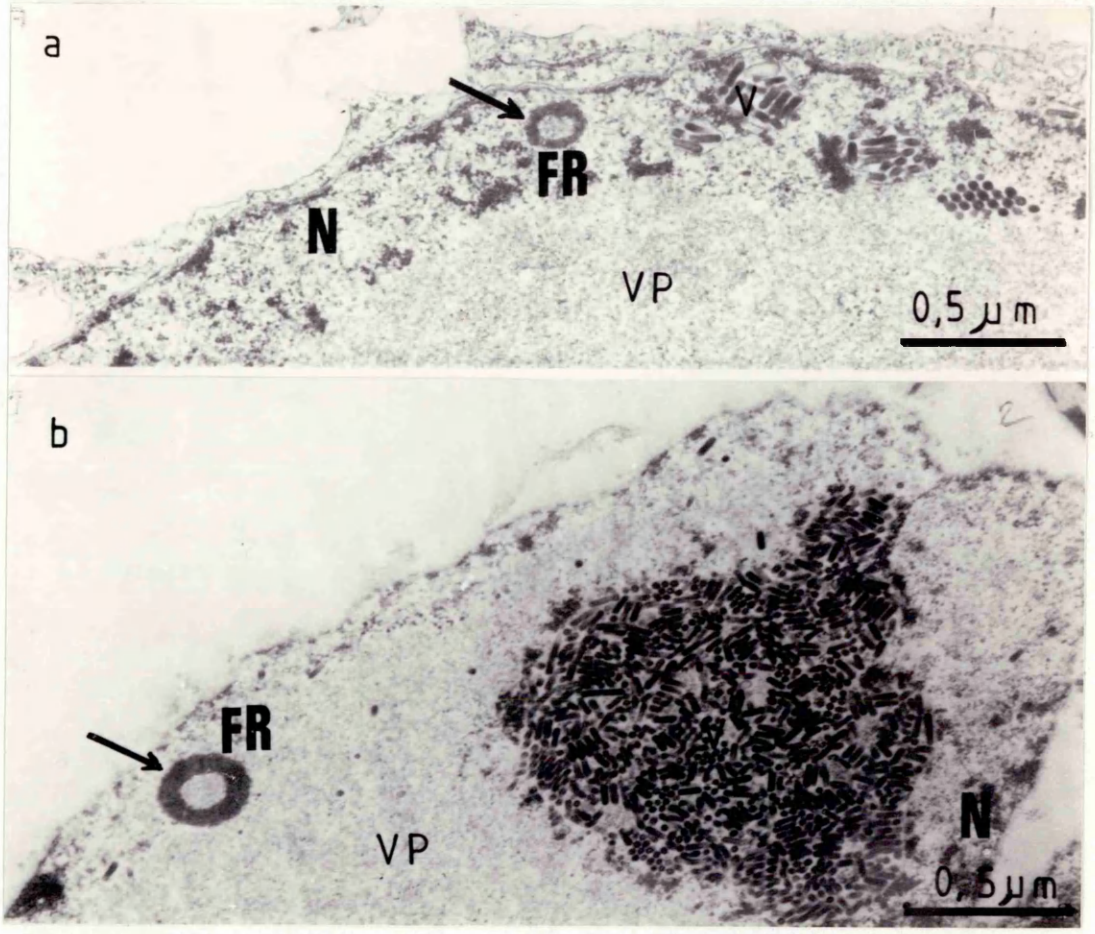


Figure 4-8:
Electron micrographs of infected *N. edwardsonii* cells 10 d after inoculation showing fibrillar ring-like bodies (FR) located in the chromatin area adjactent to the inner nuclear envelope and associated with virus particles. Abbreviations are as in page X .

(fig. 4-9a). The chromatin distribution within the nuclei was not affected (figs. 4-9 & 4-10).

4.2.2. Ultrastructural Changes in the Chloroplasts:

For the first 4 d after inoculation, no ultrastructural changes were observed. By 5-6 d after inoculation, the time at which significant numbers of cells started to contain mature virions, several minor changes were observed in the chloroplasts of these virus-containing cells. The numbers of osmophilic globules increased and some disruption of the grana was observed. More obvious changes in the chloroplast ultrastructure were observed between 8 and 15 d after inoculation. About one quarter of the chloroplasts were swollen and spherical in shape with badly disrupted grana and large vesicles embedded in the stroma (fig. 4-11a). Double vesicles were observed centrally located in the stroma, often associated with smaller vesicles of various sizes (fig. 4-11b). In addition a large number of densely staining ribosome-like bodies were observed within the vesicles (fig. 4-11b). These bodies were similar in size, shape and degree of staining to ribosomes observed in the cytoplasm or chloroplasts of healthy cells. Chloroplasts from tissue at this stage of infection also developed large areas of starch (figs 4-11a,b).

During the period from 15 to 20 d after inoculation, a great many abnormalities were observed in chloroplasts from cells containing virions and also in cells in which virions were not observed (fig. 4-12). Inclusion bodies, varying in size, shape and internal location, were clearly seen embedded in the chloroplasts. These inclusion bodies contained aggregates of membrane-like and electron-dense material, sometimes surrounded by an envelope. Such structures were never

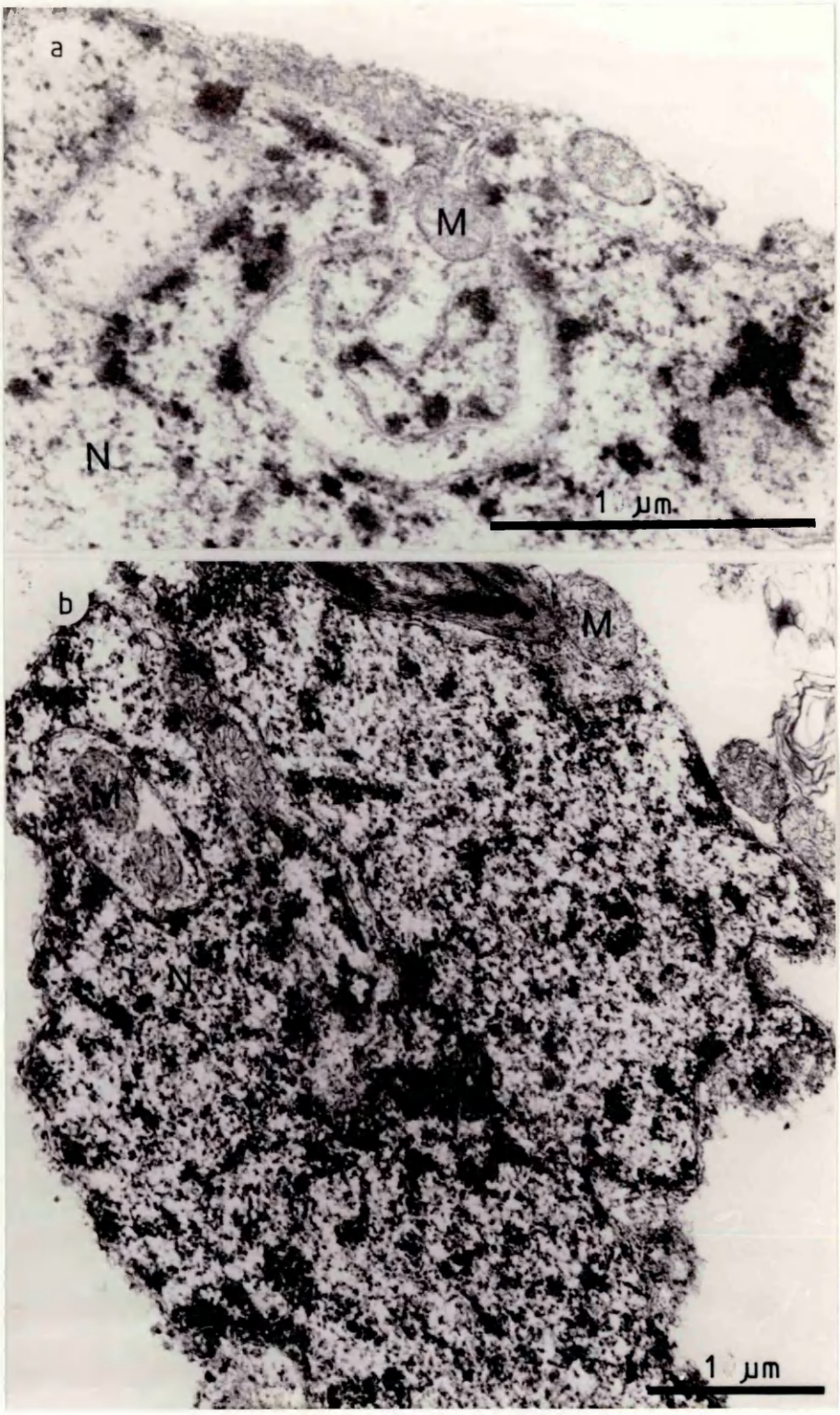


Figure 4-9:

Electron micrographs of infected *N. edwardsonii* cells (a) 35 d and (b) 40 d after inoculation showing deep cytoplasmic invaginations into the nuclei. Note in (b) the bound mitochondria invaginated within the nucleus. Abbreviations are as in page X .

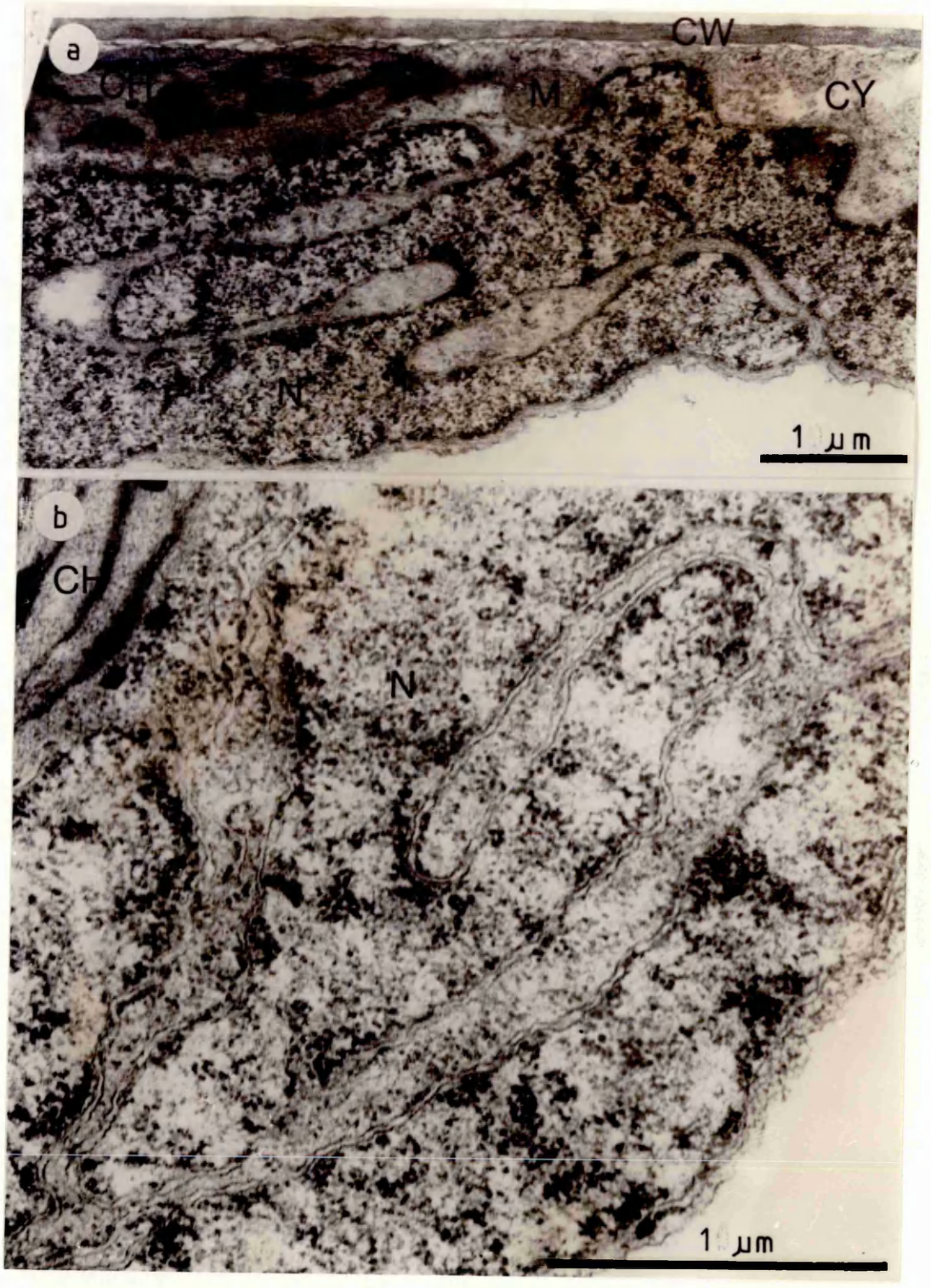


Figure 4-10:
Electron micrographs of infected *N. edwardsonii* cells (a) 75 d and (b) 90 d after inoculation showing deep cytoplasmic invaginations into the nuclei. Abbreviations are as in page x .

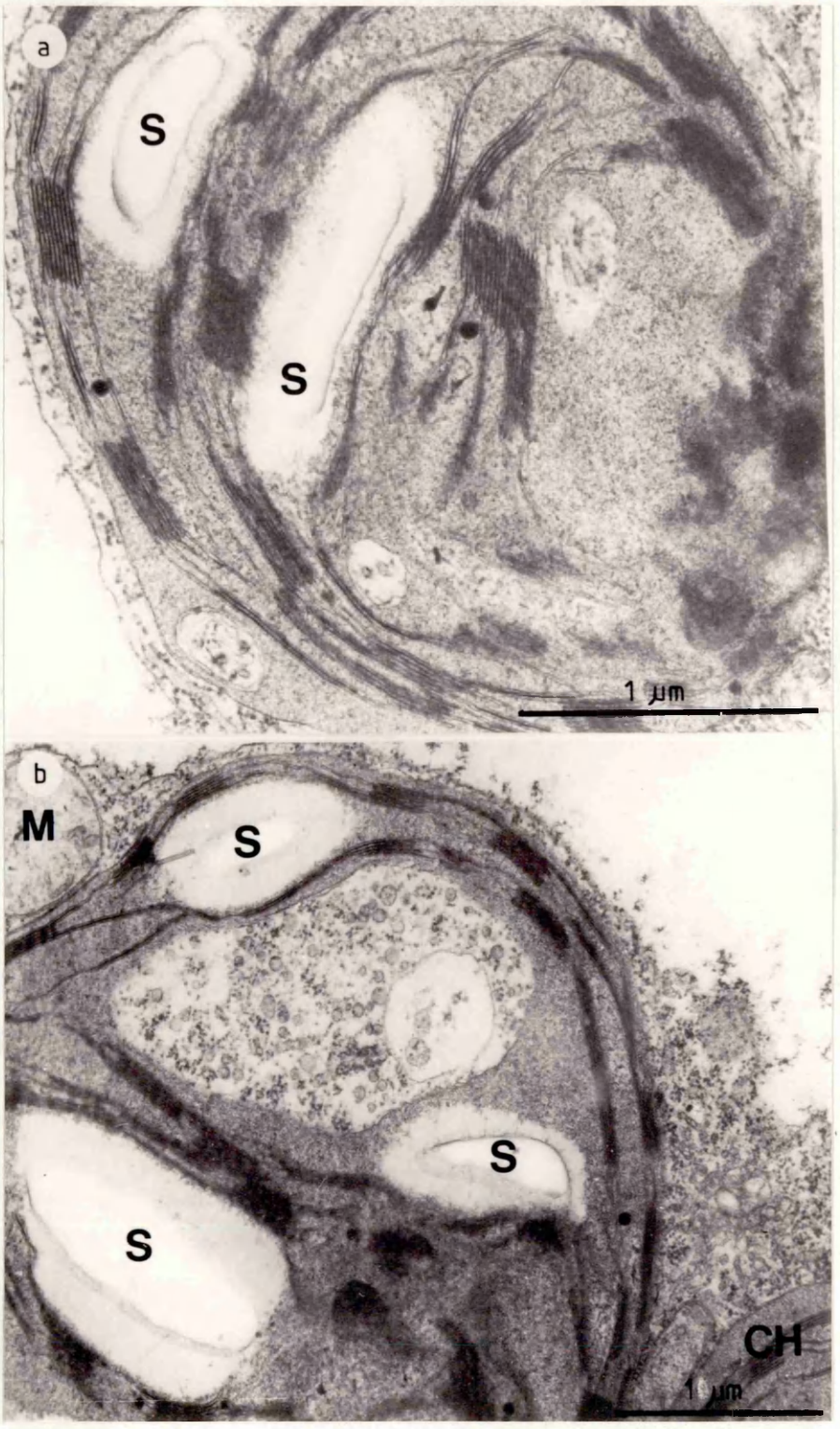


Figure 4-11:
Chloroplasts from SYNV-infected *N. edwardsonii* cells 8 d after inoculation, showing swelling and disruption of the thylakoids. starch grains are indicated by (S). Other abbreviations are as in page X

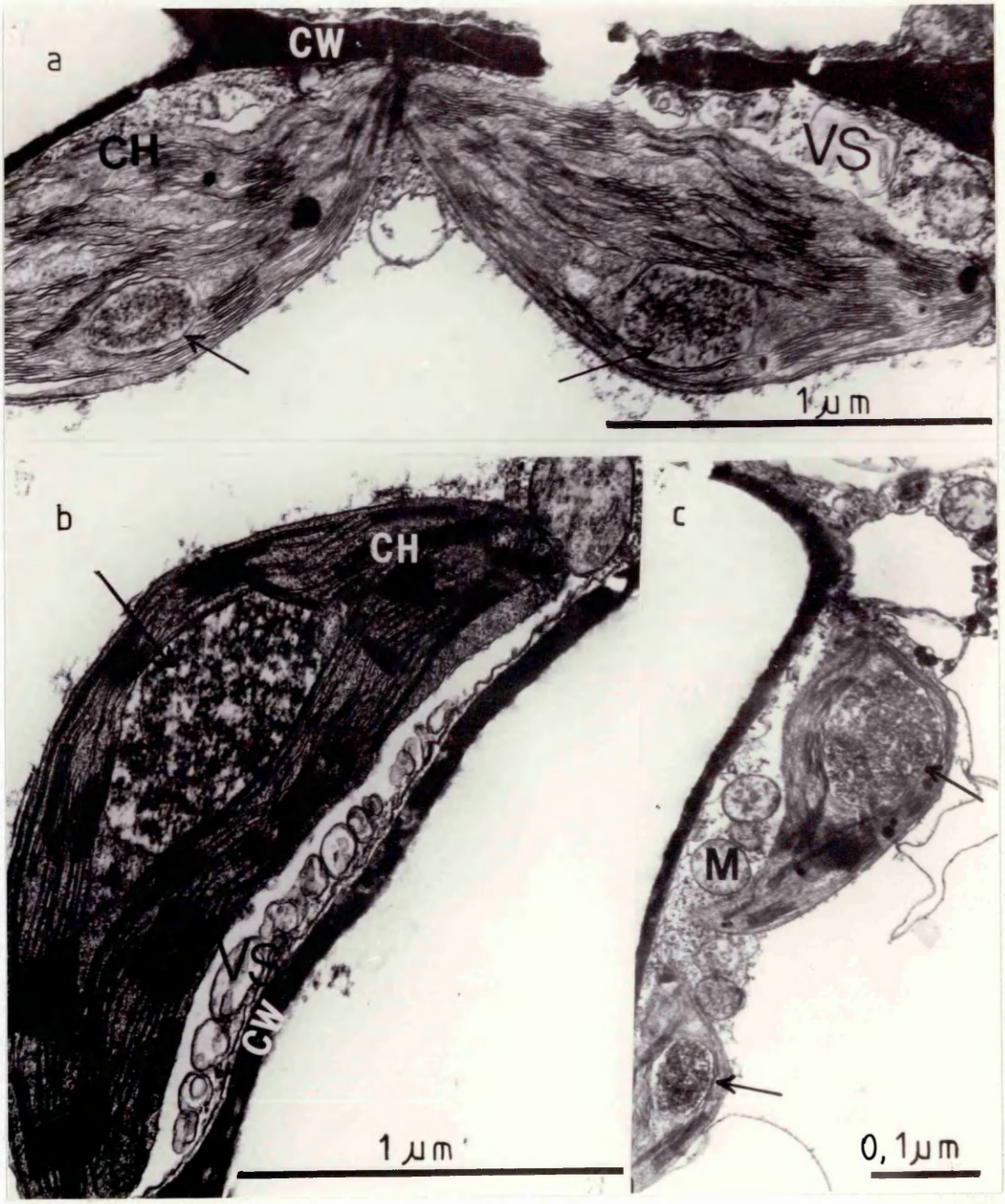


Figure 4-12:

Chloroplasts from SYNV-infected *N. edwardsonnii* cells 15 d after inoculation. Inclusion bodies are arrowed. Vesicles are indicated by (VS). Other abbreviations are as in page X

observed in the chloroplasts from healthy tissue. Despite some differences in the sizes and shapes of these inclusion bodies, they were generally located between the thylakoids either centrally or to one side of the chloroplast. Chloroplasts from cells at this stage of infection contained a variety of vesicles, differing in size, shape and number per chloroplast, mostly located near the chloroplast envelope on the side adjacent to the cell wall (fig 4-12b). A number of electron lucent areas were also observed (fig. 4-12a).

Later than 20 d after inoculation, at which stage recovery was well underway and few cells contained virions or nucleocapsids, chloroplasts exhibited extensive and gross abnormalities in their ultrastructure. Most were swollen, had loose networks of grana with convoluted membranes, and large areas of starch had developed (fig. 4-13a,b). Similar abnormalities in chloroplast ultrastructure were still evident 60-120 d after inoculation despite the apparent absence of virions in sections from these plants (fig. 4-13c).

In order to determine whether the changes in chloroplasts were brought about by the presence of virus protein, tissues from plants at various stages of infection were sectioned, treated with antiserum to SYNV and immunogold labelled. Chloroplasts from infected tissues bound extensive quantities of gold when either gold-conjugated goat anti-rabbit IgG or gold-conjugated protein A was used as label (fig. 4-14). When healthy tissues were used as controls or when non-immune rabbit IgG was substituted for the anti-SYNV IgG, at most only the occasional scattered gold particle was observed. The pattern of gold labelling varied little with the stage of infection.

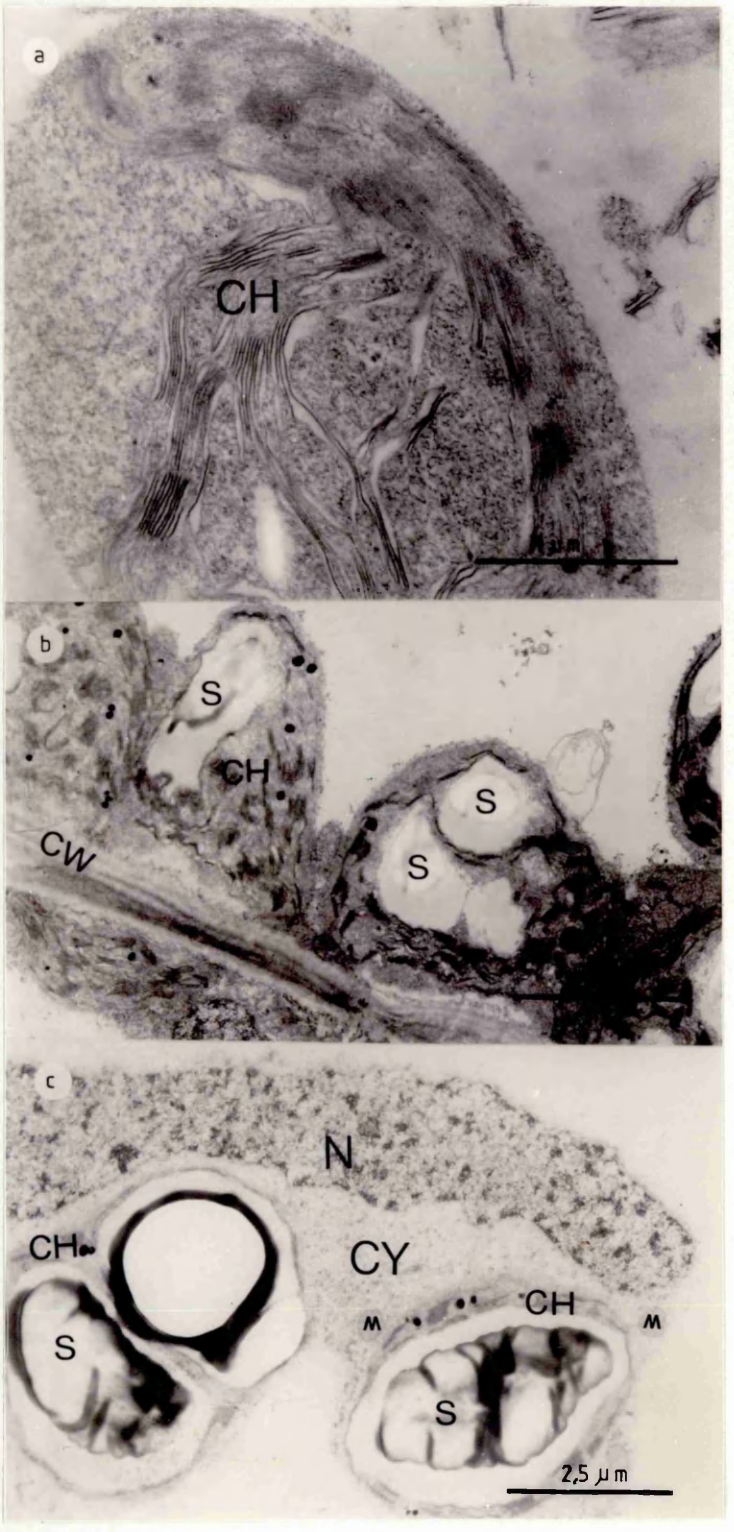


Figure 4-13:

Electron micrographs of SYNV-infected *N. edwardsonii* cells (a) 25 d; (b) 35 d and (c) 75 d after inoculation, showing swelling of the chloroplasts and the development of starch grains. Abbreviations are as in page X

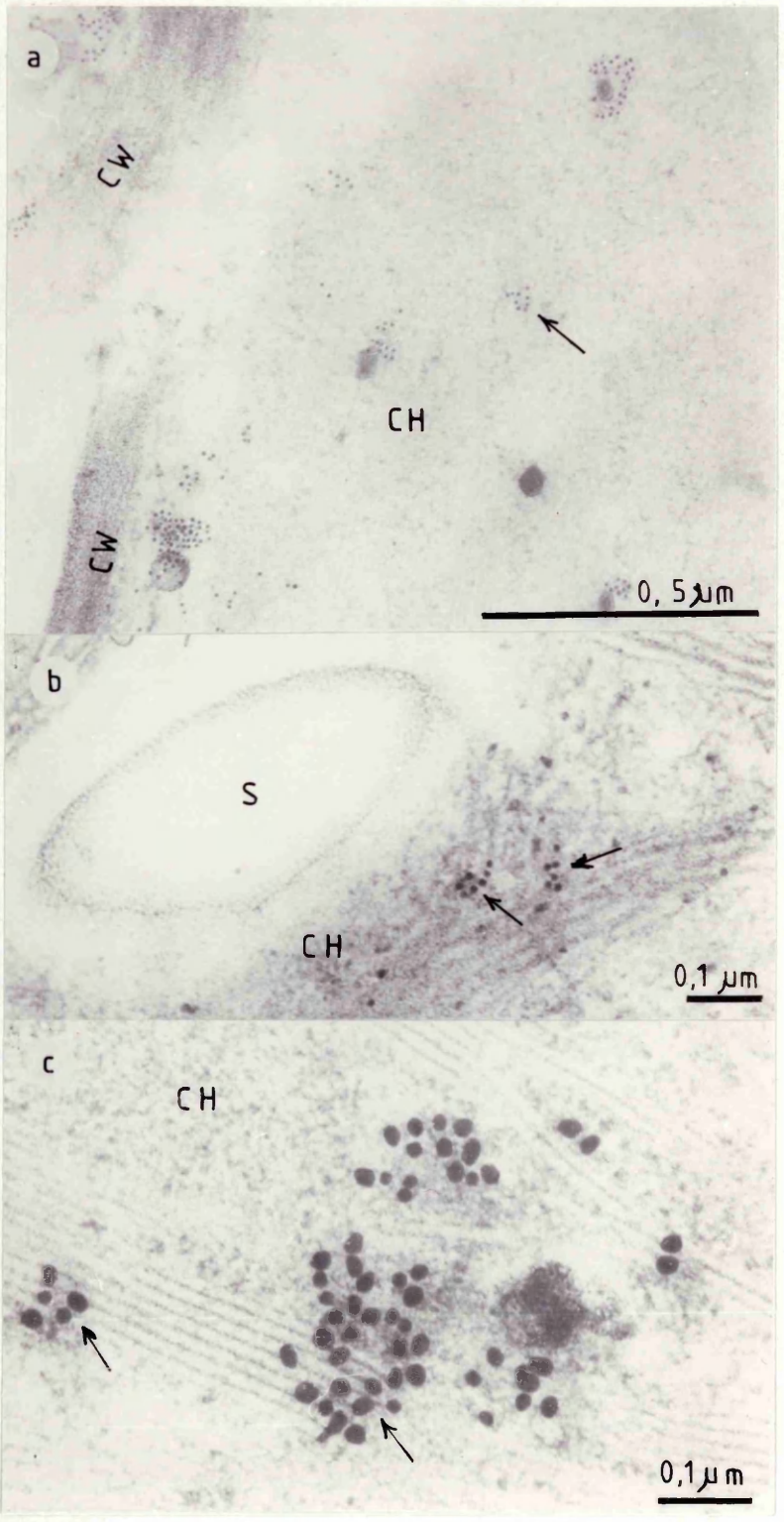


Figure 4-14:

Immunogold labelling of chloroplasts from SYNV-infected *N. edwardsonii* cells (a) 6 d; (b) 10 d and (c) 15 d after inoculation showing binding of gold particles to thylakoids (arrows). Abbreviations are as in page X

Gold particles were observed mostly in groups, strongly associated with the thylakoid membranes and grana. No label was associated with the lumen of the chloroplasts, the envelope or with the starch grains. Inclusion bodies in chloroplasts from cells 20 d or more after inoculation never bound any gold particles.

4.2.3 Ultrastructural Changes in the Mitochondria:

For the first 6-7 d after inoculation no ultrastructural changes were observed. However by 8-10 d after inoculation, the active phase of virus multiplication, obvious ultrastructural changes were observed in the mitochondria. At this stage, mitochondria in infected cells were clumped (fig. 4-15a). Clumping of mitochondria was also apparent in apparently uninfected cells from these plants when compared to healthy controls (fig. 4-15b). In infected cells, mitochondria appeared abnormal; they had usually lost most of their cristae and some of their mitochondrial matrix (fig. 4-15a). In uninfected cells adjacent to infected cells, clumping of mitochondria was sometimes observed but these mitochondria showed no internal abnormalities (fig. 4-15b). A particle of 253 x 56-72 nm embedded inside a mitochondrion, which had been invaginated by cytoplasmic matrix forming a horseshoe-like structure, was also observed (fig. 4-16a). Microbodies containing granular matrix, similar in size to mitochondria, and with a single bounding membrane were located next to diseased mitochondria. These microbodies either contained a single crystalline object embedded in the granular matrix (fig. 4-16b) or fibrillar matrix (fig. 4-16b).

Abnormalities were observed in infected cells 12-20 d after inoculation. Mitochondria containing clusters of electron dense

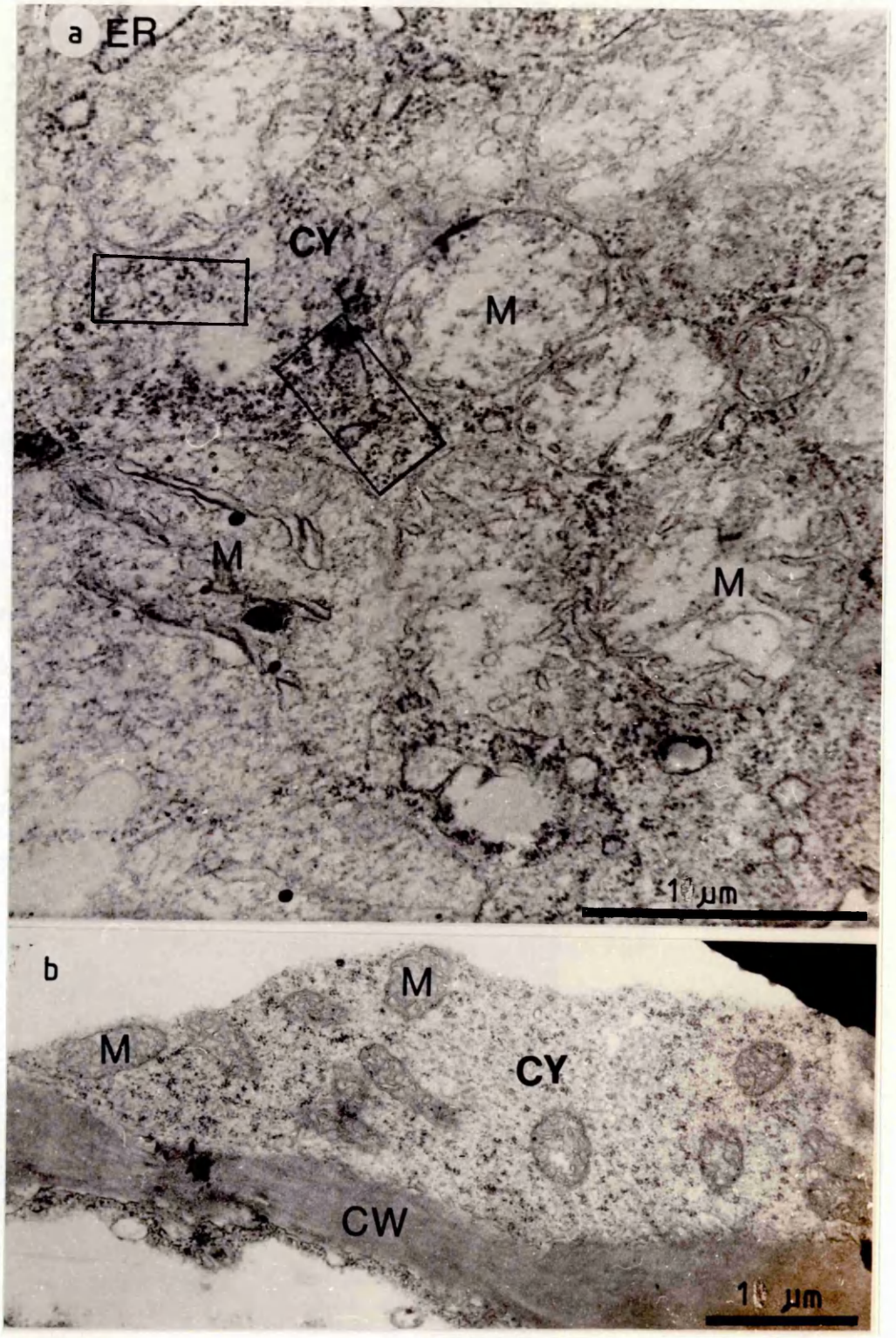


Figure 4-15:

Electron micrographs of infected *N. edwardsonii* cells 8 d after inoculation. (a) Aggregation of abnormal mitochondria in infected cell. Examples of aggregated ribosomes are boxed. (b) Aggregation of healthy looking mitochondria in cell adjacent to (a). Abbreviations are as in page X

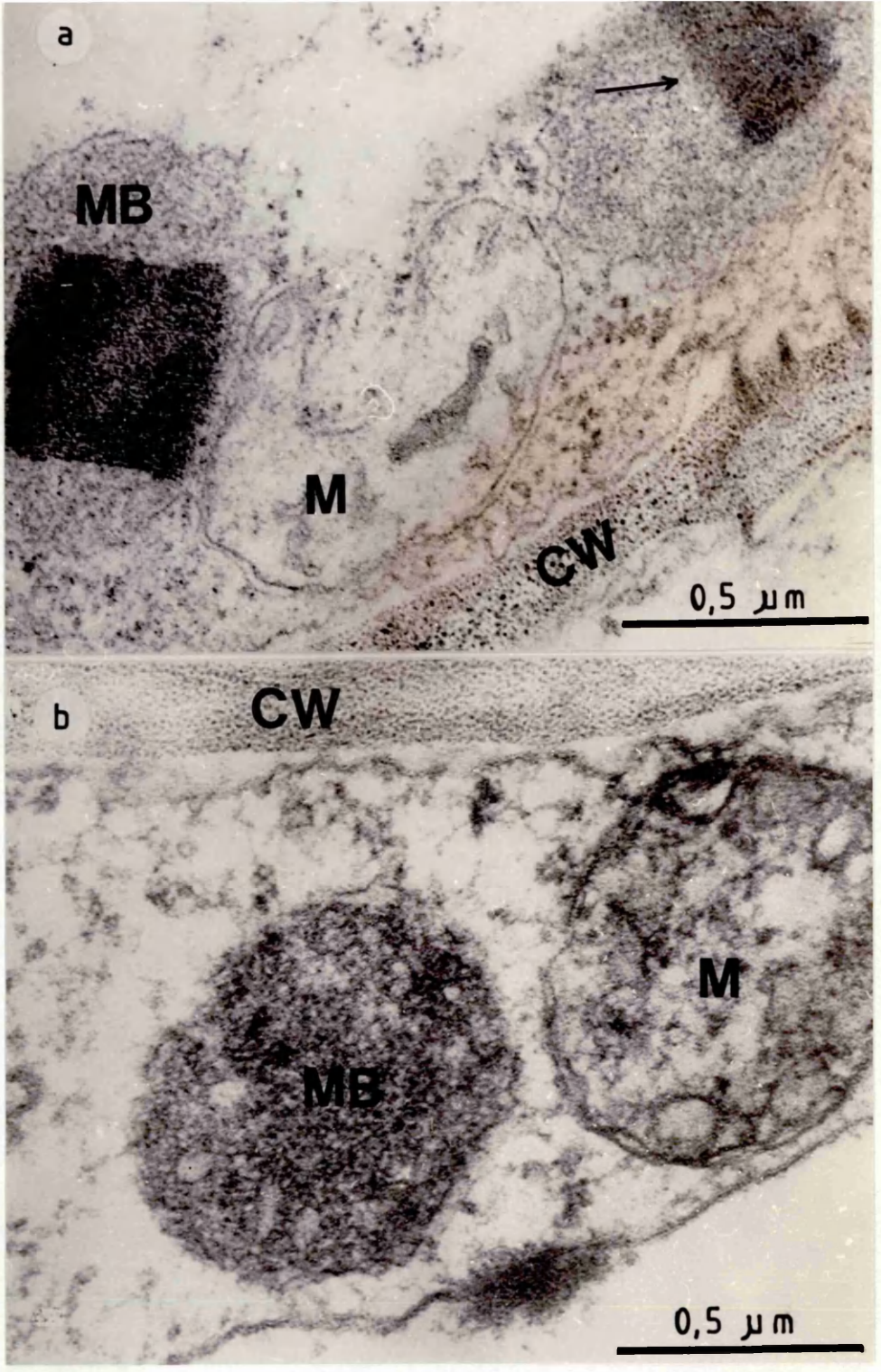


Figure 4-16:

Electron micrographs of infected *N. edwardsonii* cells 10 d after inoculation showing (a) cytoplasmic invagination into a mitochondria containing a bacilliform-like particle and (a & b) microbodies (MB) bounded by a single membrane. The microbody in (a) contains a single crystal embedded into granular matrix and in (b) is free of crystal material but contains fibrillar matrix. Abbreviations are as in page X

finely granulated material within the matrix, were observed in infected cells (fig. 4-17a,b). In cells without virions, swollen mitochondria containing double-membrane-bounded vesicles were observed. These cells had abnormal invaginated nuclei suggesting that they had been infected. These vesicles contained fibrillar (fig. 4-17c) or granular (fig. 4-17d) matrix. A crescent-like structure was also found embedded in the mitochondrial matrix (fig. 4-17d). About 20-25% of mitochondria in infected leaf tissue up to 20 d after inoculation appeared distinctly abnormal.

By 30 d and on after inoculation, mitochondria were highly altered and were often difficult to recognize. Enlarged and very irregularly shaped mitochondria were evident (fig. 4-18b). Multiple vesicles, differing in size, were seen in the mitochondria. Some had their mitochondrial membranes separated from each other forming a perimitochondrial space (fig. 4-18a). At this stage of infection about 50-65% of mitochondria appeared abnormal.

In immunogold labelling studies, no gold particles ever reacted to any mitochondrion in any tissue at any stage of infection.

4.2.4 Ultrastructural Changes in the Plasma Membrane and in the Cell Wall:

An extensive series of electron micrographs were taken at a variety of times after inoculation in order to investigate the effects of SYNV-infection on the plasma membranes and the cell walls. A variety of changes, described in detail below, were observed. Similar abnormalities were never observed in the plasma membranes or cell walls of healthy controls maintained under the same conditions.

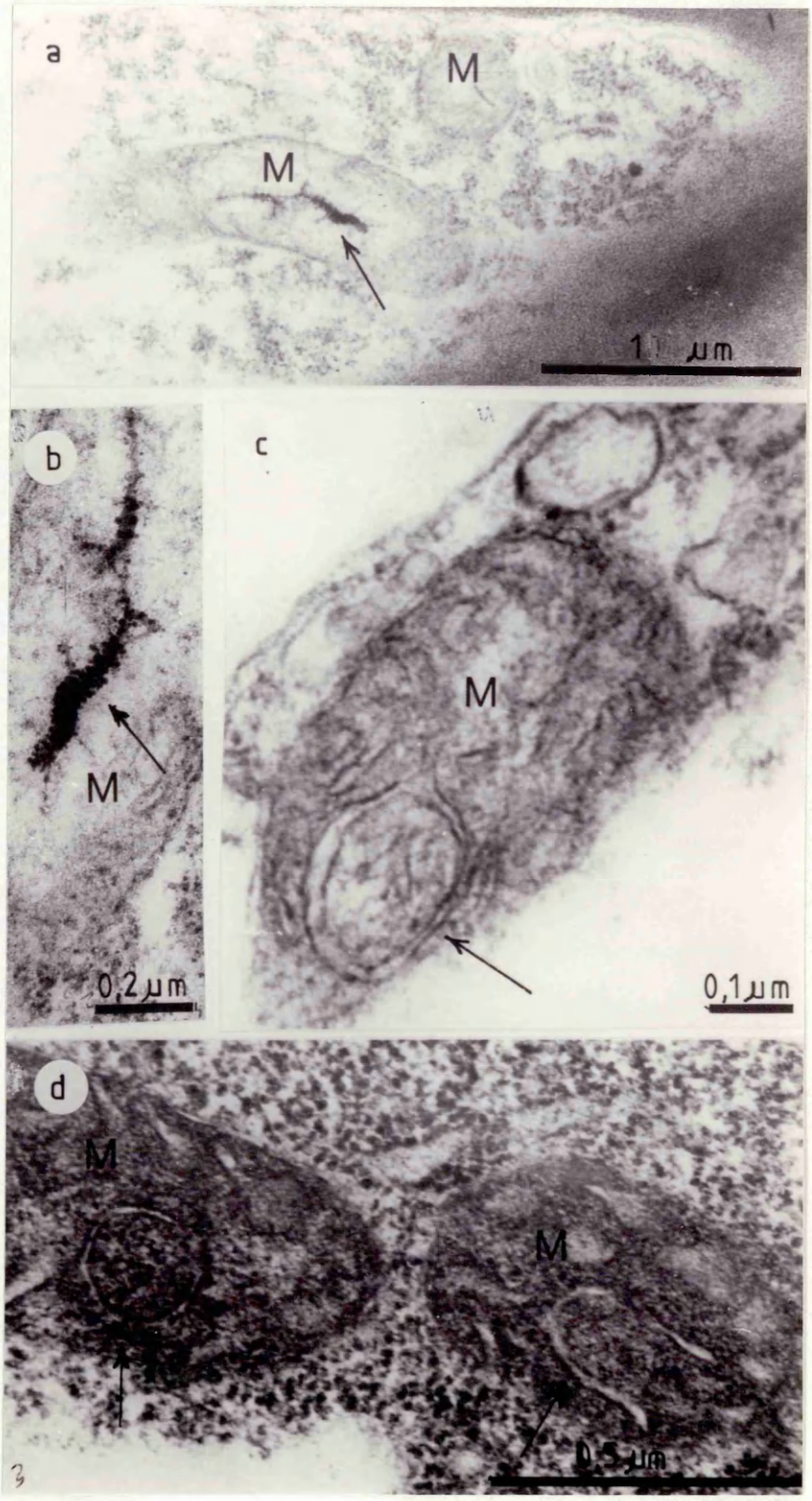


Figure 4-17:
Electron micrographs showing various abnormalities (arrows) in the mitochondria in infected *N. edwardsonii* cells. (a) Infected cell 15 d after inoculation. (b) Higher magnification of the internal part of (a). (c & d) Micrographs from cells, apparently free of virus particles, adjacent to highly infected cells 12 d after inoculation. Abbreviations are as in page X

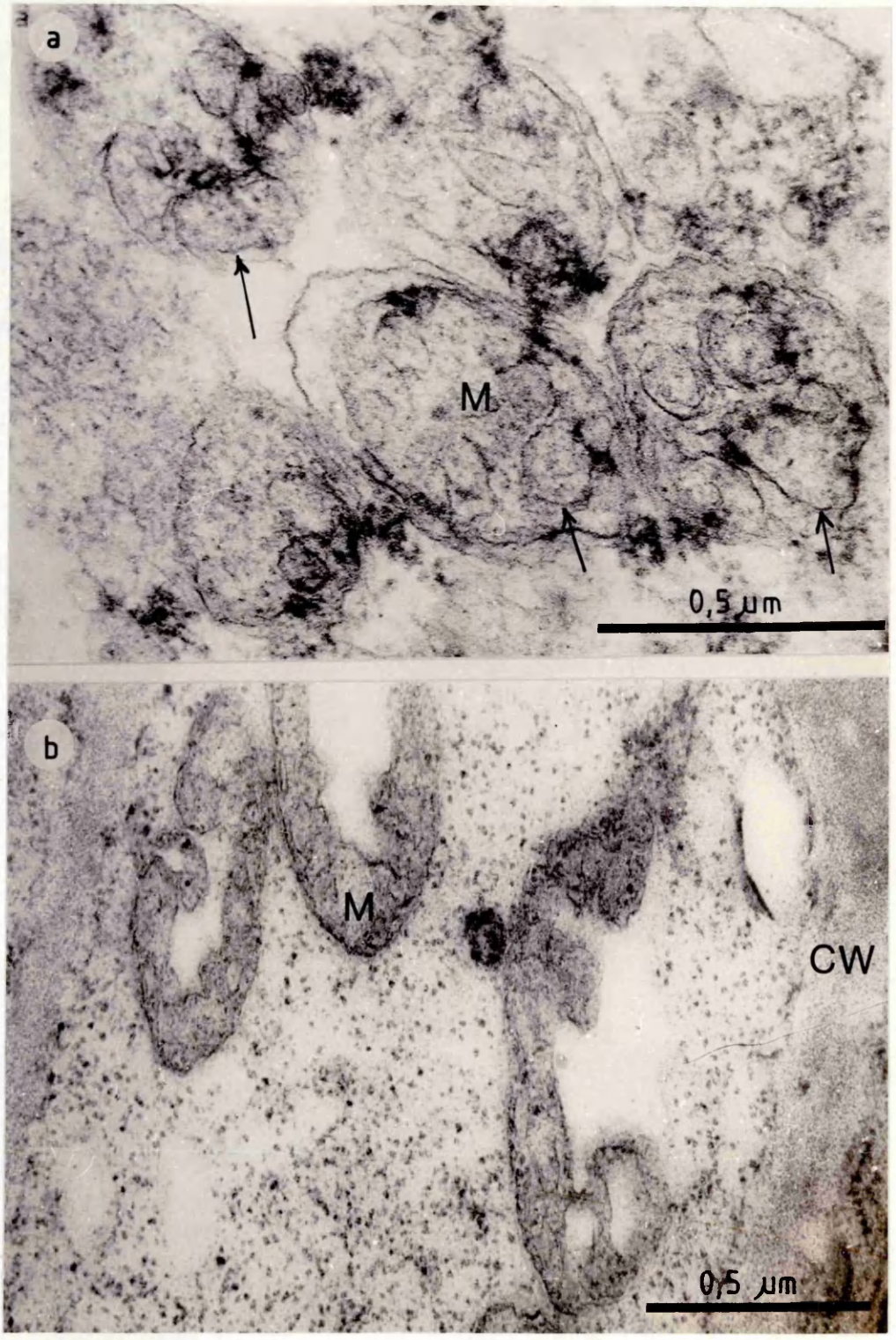


Figure 4-18:

Electron micrographs of infected *N. edwardsonii* cells showing highly altered mitochondria. Note (a) the existence of multivesicles within the mitochondria (arrows) and the highly developed perimitochondrial space; (b) the inlarged mitochondria. Abbreviations are as in page X

Five to eight days after inoculation, the plasma membranes of SYNV-infected *N. edwardsonii* cells were outgrown, forming numerous paramular bodies (these will be subsequently referred to as plasmalemmasomes) (fig. 4-19a & 4-20). In a variety of sections taken at this stage of infection, differences in number, shape and location of these plasmalemmasomes were observed. Outgrowths of the plasma membrane, forming plasmalemmasomes, were seen in a variety of tissues. Figures 4-20a & 4-20b show irregularly shaped plasmalemmasomes filled with densely-stained fibrillar material in epidermal cells and cells of the xylem. Plasmalemmasomes were commonly observed opposite one another separated by the cell wall in adjacent cells from the vascular bundle (fig. 4-21b) and in mesophyll cells (fig. 4-20b & 4-21c). Plasmodesmata were sometimes, but not always, observed between these plasmalemmasomes (cf. fig. 4-20b to figs. 4-21b & 4-21c). Virus particles were often clearly seen in cross-section in the region between the outgrown plasma membrane and the tonoplast, usually surrounded by a membrane (fig. 4-19a).

Pear shaped plasmalemmasone-like structures, filled with irregularly shaped granular material, were sometimes observed in the cytoplasm of mesophyll cells (see fig. 4-19b). It is not clear whether these were formed by the outgrowth of plasma membrane or from the tonoplast. These structures were sometimes associated with crescent shaped objects in the region between the plasma membrane and the tonoplast (fig. 4-19b).

Plasmalemmasomes and plasmalemmasome-like structures which were apparently detached from their growth-sites appeared, with less fibrillar filling material, in the vacules from about 8 d after inoculation (fig. 4-19a,b) and in the cytoplasm of parenchyma cells

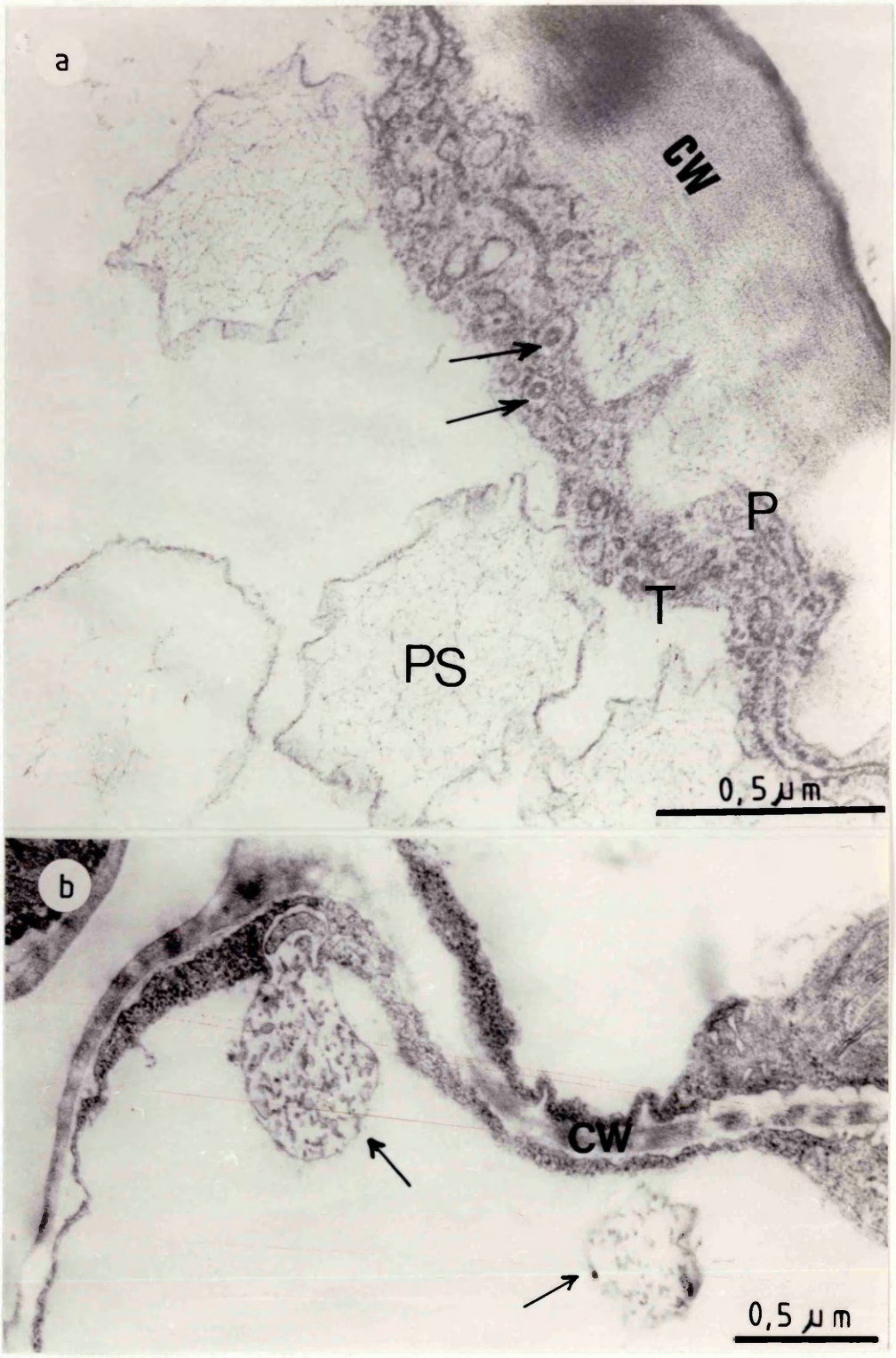


Figure 4-19:

Electron micrographs of cells from infected *N. edwardsonii* showing (a) plasmalemmasomes (PL) and cross-sectioned SYNV particles associated possibly with cross-sectioned channels (arrows) in an epidermal cell 8 d after inoculation. (b) Plasmalemmasome-like object (arrows) in a mesophyll cell 5 d after inoculation. Abbreviations are as in page X

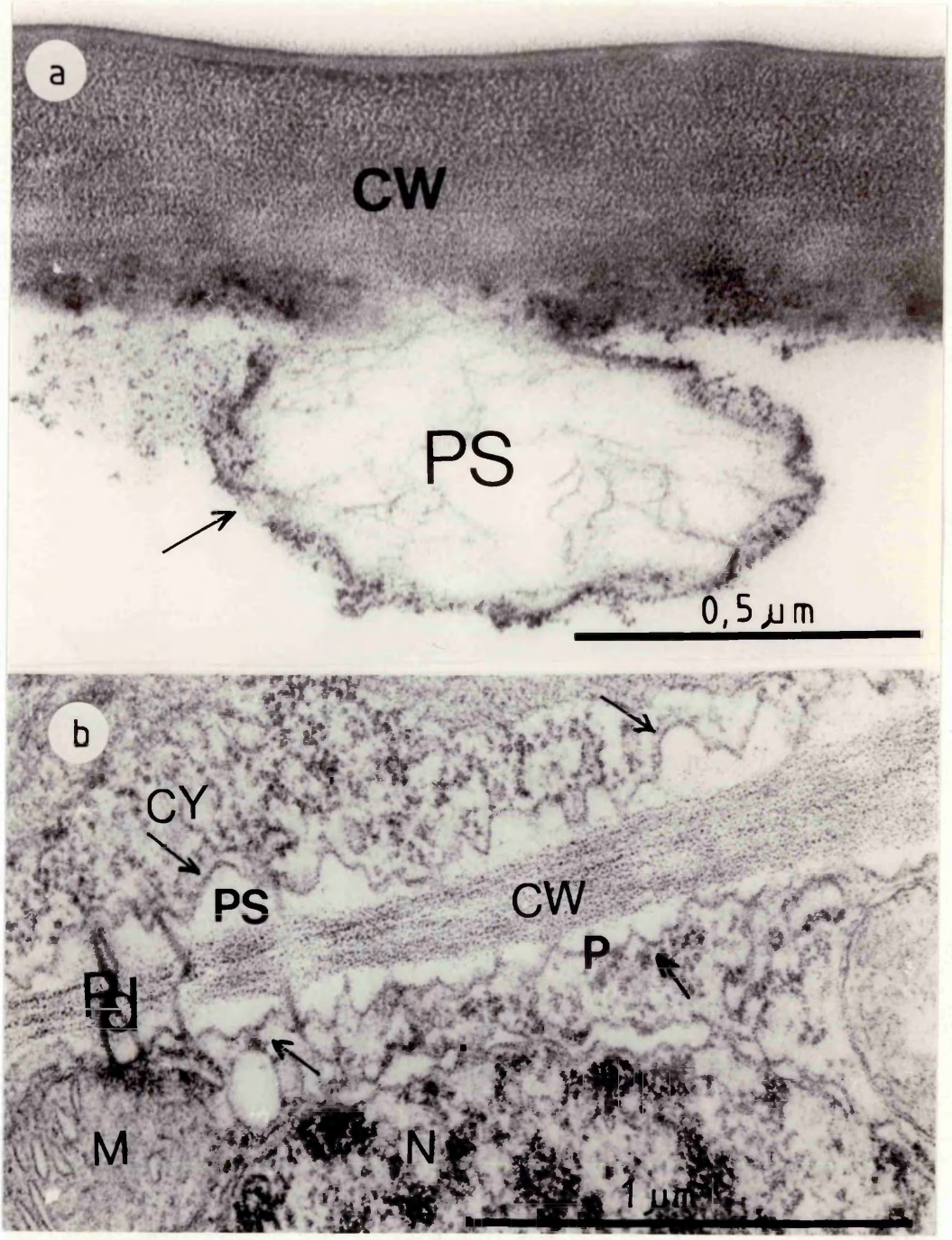


Figure 4-20:

Electron micrographs of cells from infected *N. edwardsonii* cells 6 d after inoculation showing plasmalemmasomes in (a) epidermal and (b) mesophyll cells (arrows). Note that the plasmalemmasomes in (b) are opposite each other in adjacent cells and separated by the cell wall. Abbreviations are as in page X

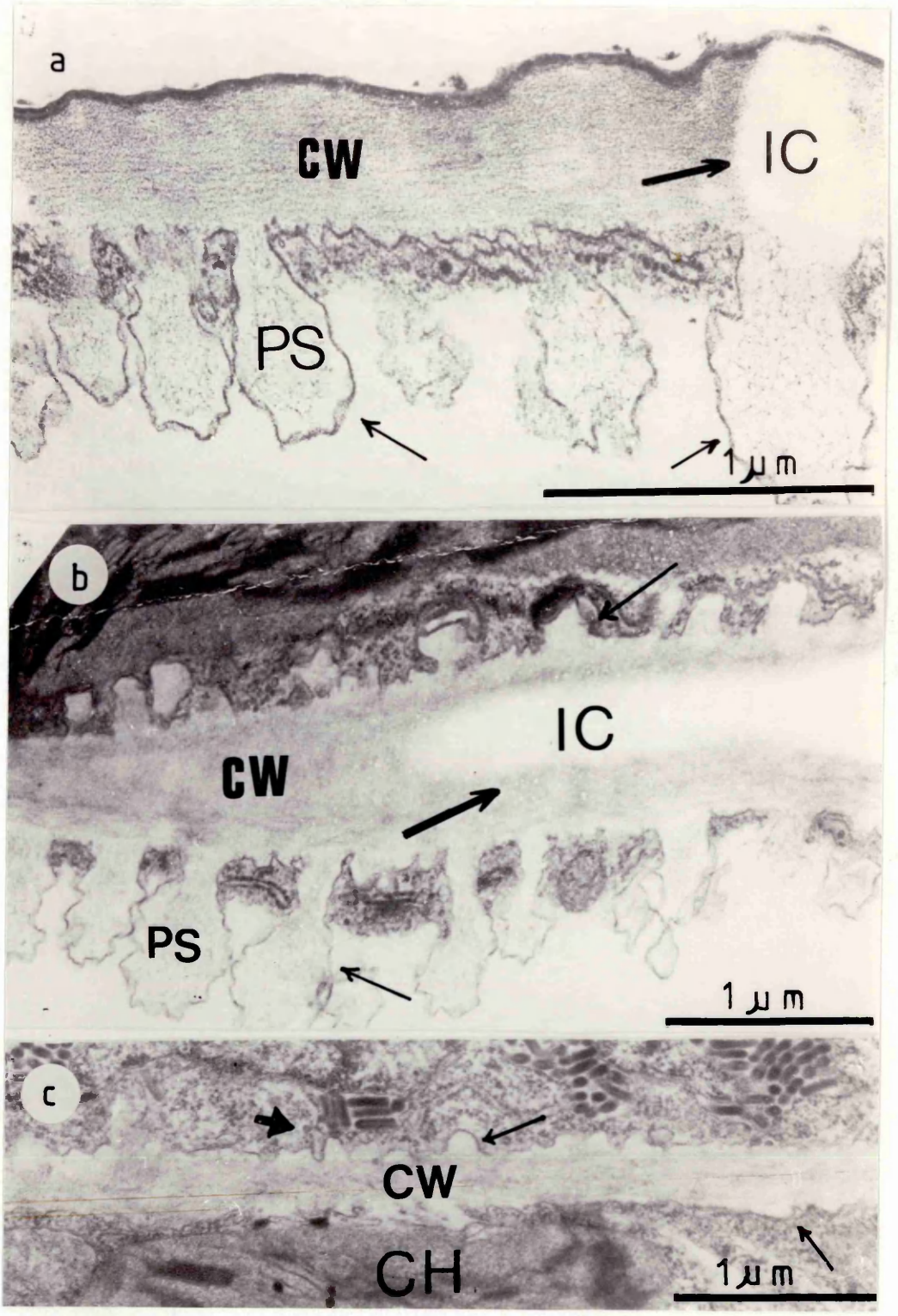


Figure 4-21:

Electron micrographs of cells from infected *N. edwardsonii* 8 d after inoculation showing various plasmalemmasomes in (a) epidermal and (b) & (c) mesophyll cells. Note the lucent area within the cell wall (bold arrows) in (a) and (b). Note the possible established channel (small bold arrow) in (c). Abbreviations are as in page X

from about 20 d after inoculation (fig. 4-22). Some detached plasmalemmasomes were seen adjacent to the tonoplast (fig. 4-19a). This detachment might be real or an artefact resulting from the plane of sectioning.

Multiply vesiculated plasmalemmasomes, resembling those reported by Hari (1980) were observed in the vacuole of mesophyll cells (fig. 4-21a). These were, perhaps, formed by multiple outgrowths of the plasma membrane from the same side as these seen in figure 4-23b, later becoming detached and appearing in the vacuole.

Plasmodesmata were observed in sections of both healthy control and infected tissues. No significant differences in the morphology and the number of plasmodesmata were observed in hundreds of sections of healthy and infected tissues. The diameters of plasmodesmata in infected tissue (at various times after inoculation from 5 d to about 150 d) were generally about 20-32 nm (fig. 4-24a). This is about 3 times less than the diameter of nucleocapsids or mature SYNV particles. Thus neither mature virions or nucleocapsids would be able to move unimpeded from cell to cell through the plasmodesmata.

Channels of about 80-130 nm in diameter were observed in cross- and longitudinal sections in walls of mesophyll cells 10 d after inoculation and also in the cytoplasm (fig. 4-24b,c,d). Virus-like particles were usually seen embedded in some of these channels (fig. 4-24b,c,d). These channels, which were never seen in the healthy controls but were common in infected cells, were quite different in size and morphology to plasmodesmata (cf. fig. 4-24b,c,d to figs. 4-24a & 4-26a,b). In cells of the vascular bundle 10 d after inoculation up to 5 channels per cell have been observed, each of

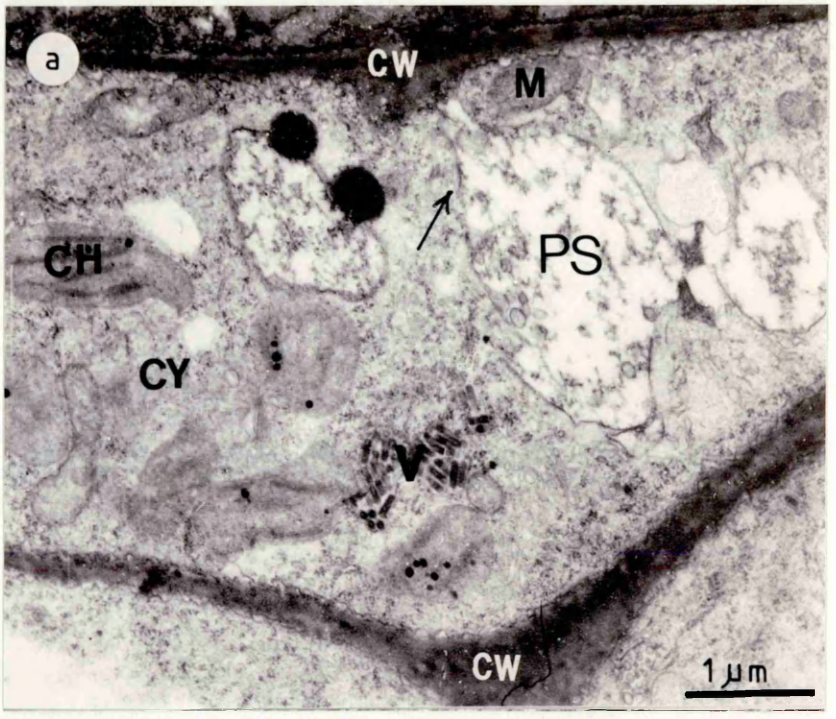


Figure 4-22:

Electron micrographs of cells from infected *N. edwardsonii* 20 d after inoculation showing large plasmalemmasomes which are apparently detached from the cell wall and embedded in the cytoplasm. Note that one of the plasmalemmasomes (arrow) remains connected with the cell wall protrusion and containes small vesicles. Abbreviations are as in page X

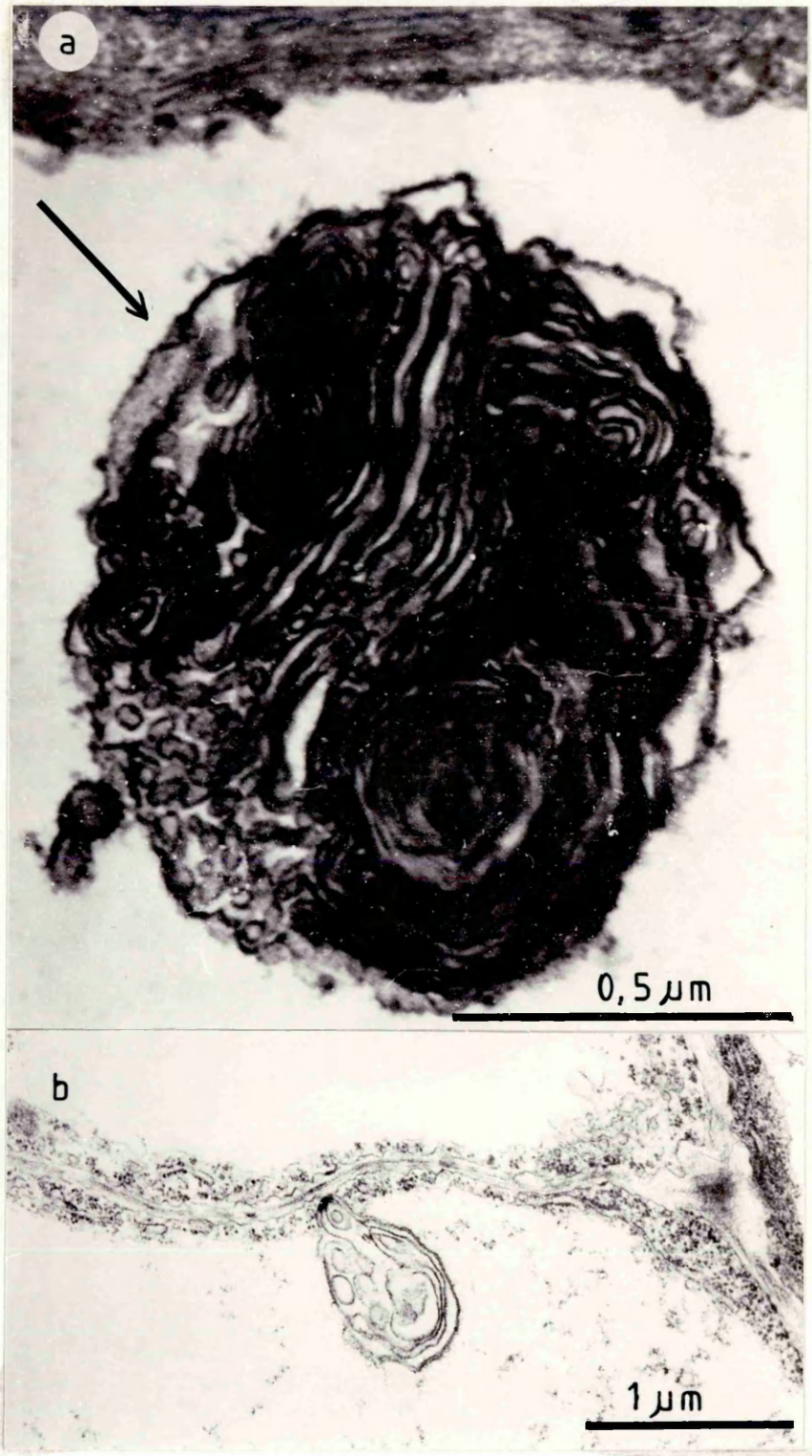


Figure 4-23:

Electron micrographs of cells from infected *N. edwardsonii* 15 d after inoculation showing (a) a free multiply vesiculated plasmalemmasome in the vacuole (arrow) and (b) a multiply vesiculated plasmalemmasome, possibly similar to that shown in (a) budding into the vacuole.

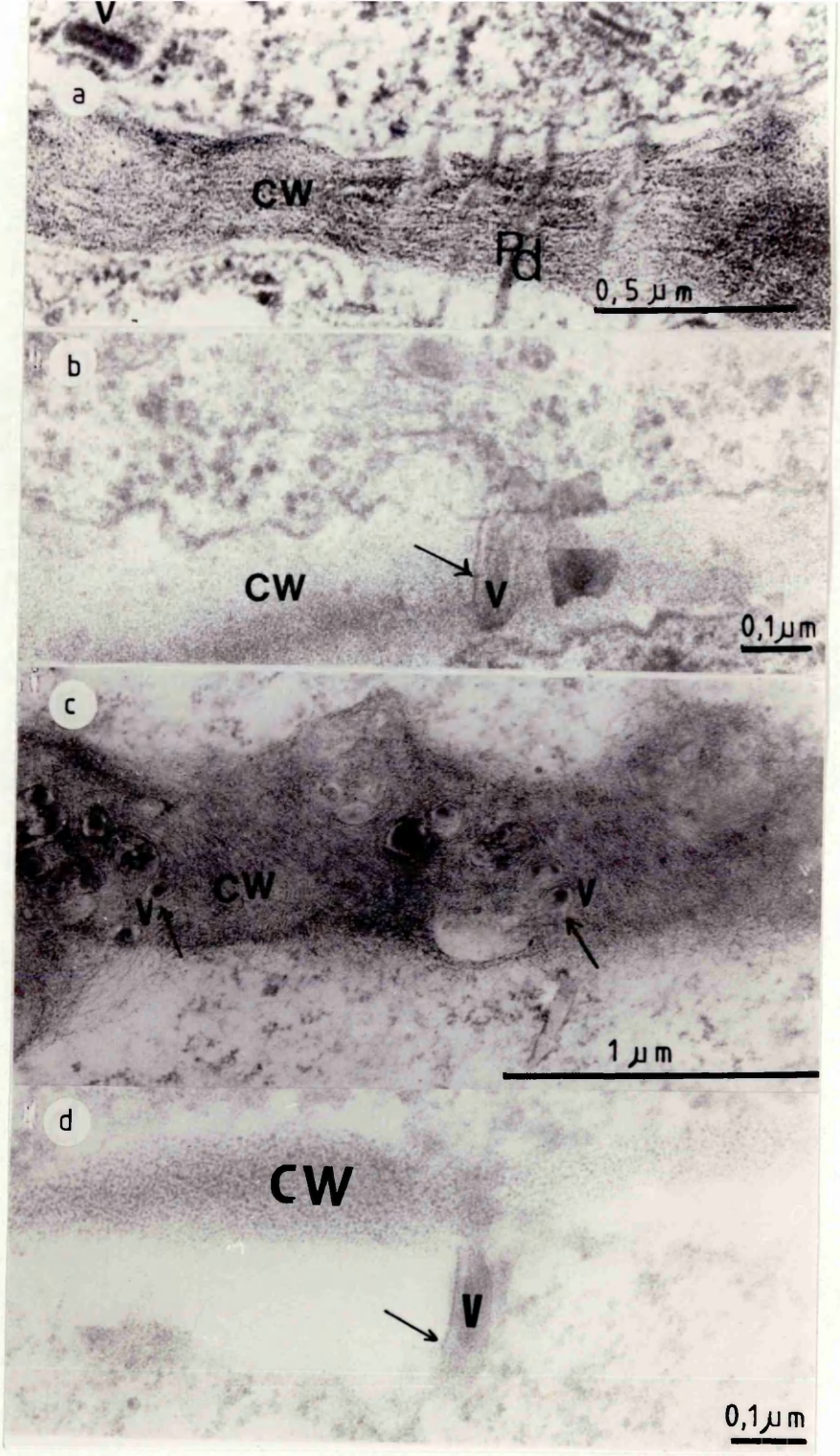


Figure 4-24:

Electron micrographs taken from one cell from SYNV-infected *N. edwardsonii* 10 d after inoculation. (a) Shows that the width of the virus particle is about three times the width of the plasmodesmata (Pd). (b) (c) and (d) Show virus particles in transit from cell to cell through the cell wall. Note that the SYNV particle in (b) embedded in the cell wall (arrow) whereas in (d) it has left the cell wall. (c) Cross-section of virus-associated channel (arrows). Abbreviations are as in page X

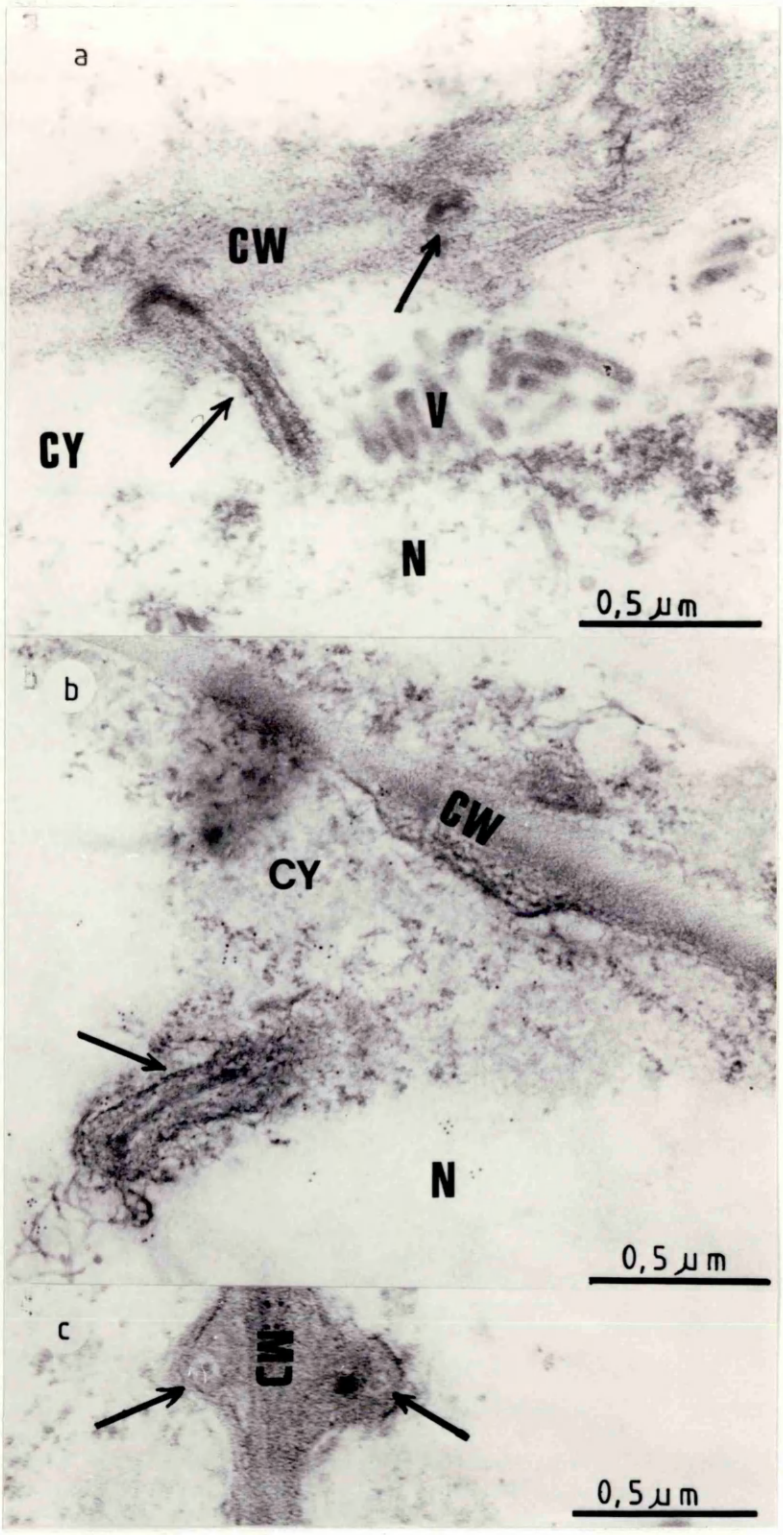


Figure 4-25:

Electron micrographs of cells from SYNV-infected *N. edwardsonii* 25 d after inoculation showing sheathed channels (arrows). (a) and (b) Channel connecting the cell wall to the nucleus. (c) Cross-section of one channel. Abbreviations are as in page X .

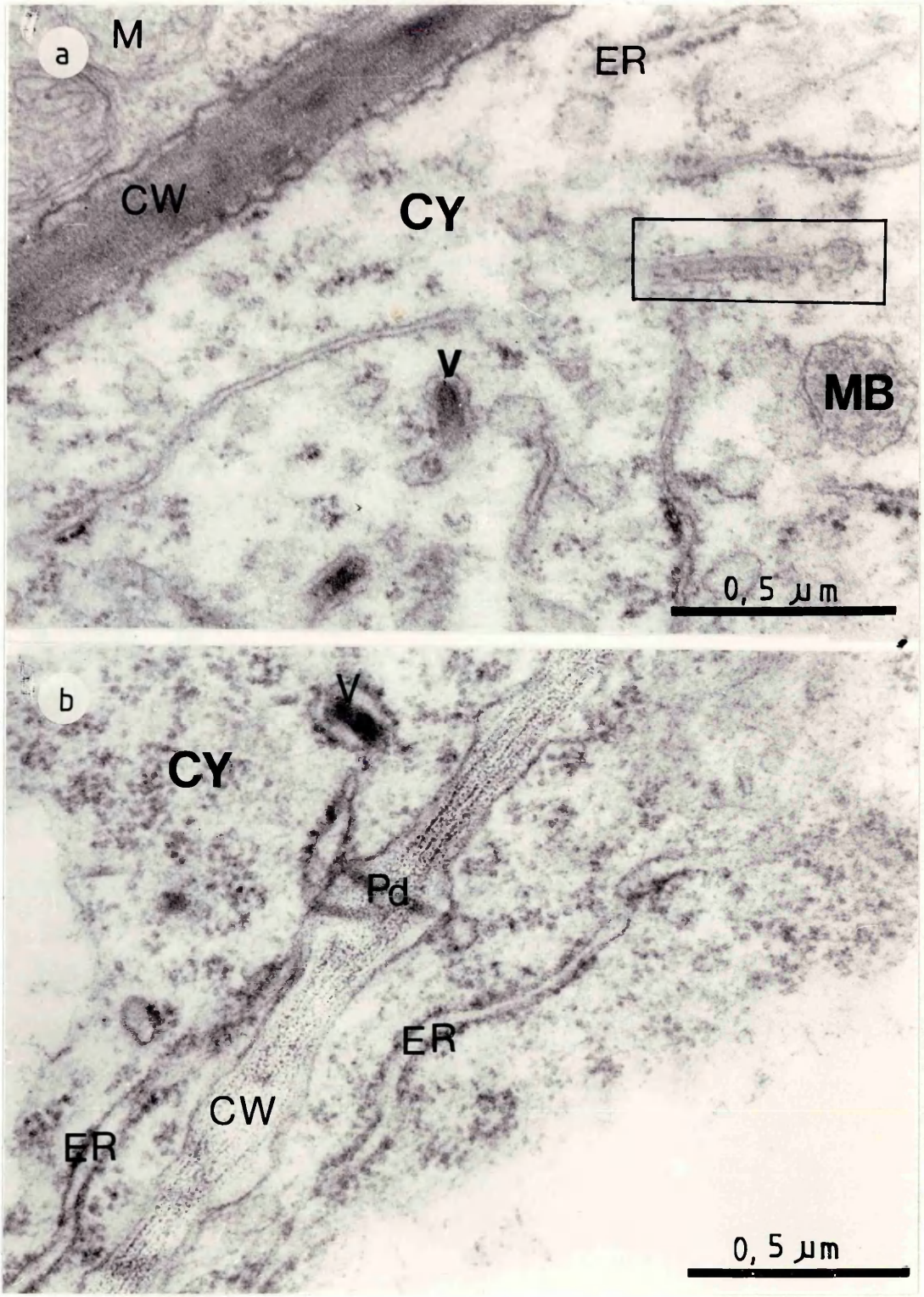


Figure 4-26:

Electron micrographs of cells from infected *N. edwardsonii* showing (a) a sheathed channel embedded into the cytoplasmic matrix (box) and (b) virus particles associated with ER which is still connected to the plasmodesmata in the cell protrusion. Abbreviations are as in page X

about 200-225 nm in diameter. Serial sections of these cells clearly show virus particles apparently in transit from cell to cell through these channels (fig. 4-28). The presence of structures such as those shown in figures 4-26 and 4-30 suggests that SYNV particles may be transported from cell to cell through specialized channels penetrating the cell walls. The mechanism of establishment of such channels, which are do not appear to be modified plasmodesmata, is not clear.

Additional support for the presence of specialized transport channels is given by the observation of sheathed channels connecting the cell wall protrusions to the nucleus of the infected cell (fig. 4-25a). Similar sheathed channels have been embedded in the cytoplasmic matrix of infected cells (fig. 4-26a) and others have been seen apparently detached from the cell wall but remaining connected to the nucleus of an adjacent infected cell (fig. 4-25b). Once again, the detachment may reflect the plane of sectioning. Evidently these channels have passed through the cell wall as seen in cross-sectioning, (fig. 4-25c,) and have penetrated into the cytoplasm (fig. 4-27).

Infected *N. edwardsonii* tissues, at various times after inoculation, were immunogold labelled in attempt to investigate whether viral protein(s) were involved in the establishment of trasport channels, and to localize this/ these protein(s) in the cell wall. The cell walls of infected plants, at various times after inoculation, but not controls, consistently bound large number of gold particles. Because OsO_4 was omitted from the embedding procedure for sections used for immunogold labelling it was not possible to visualize the plasma membrane. Attempts to include OsO_4 in the fixation procedure resulted in the failure of gold particles to bind

to the sections. Thus, it was not possible to determine whether the gold particles were bound to the plasma membrane, or to any other abnormal structure induced by SYNV-infection. A variety of patterns of labelling were observed. Clusters of gold particles were commonly seen opposite one another in adjacent mesophyll and vasular system cells (fig. 4-29) sometimes associated with apparent outgrowth of either the cell wall or associated structures. In some cases the clusters of gold particles completely traversed the cell wall (see e.g. fig. 4-30b,c). In other sections, although the clusters were interrupted, their topology suggests that they might be reacting to some structure which itself was continuous across the cell wall but which appeared by the plane of sectioning (see fig. 4-29a, boxed region). Virus particles were sometimes seen adjacent to such clusters.

Binding of gold particles to the inner surface of the cell wall (or possibly some associated but unstained structure) was also occasionally observed (fig 4-30a). It is possible that this binding reflects the presence of viral proteins in the plasma membrane which does not stain in this section. It is tempting to suggest that the gold particles were reacting to transport channels in the cell wall and that such channels are associated with at least one of the SYNV proteins.

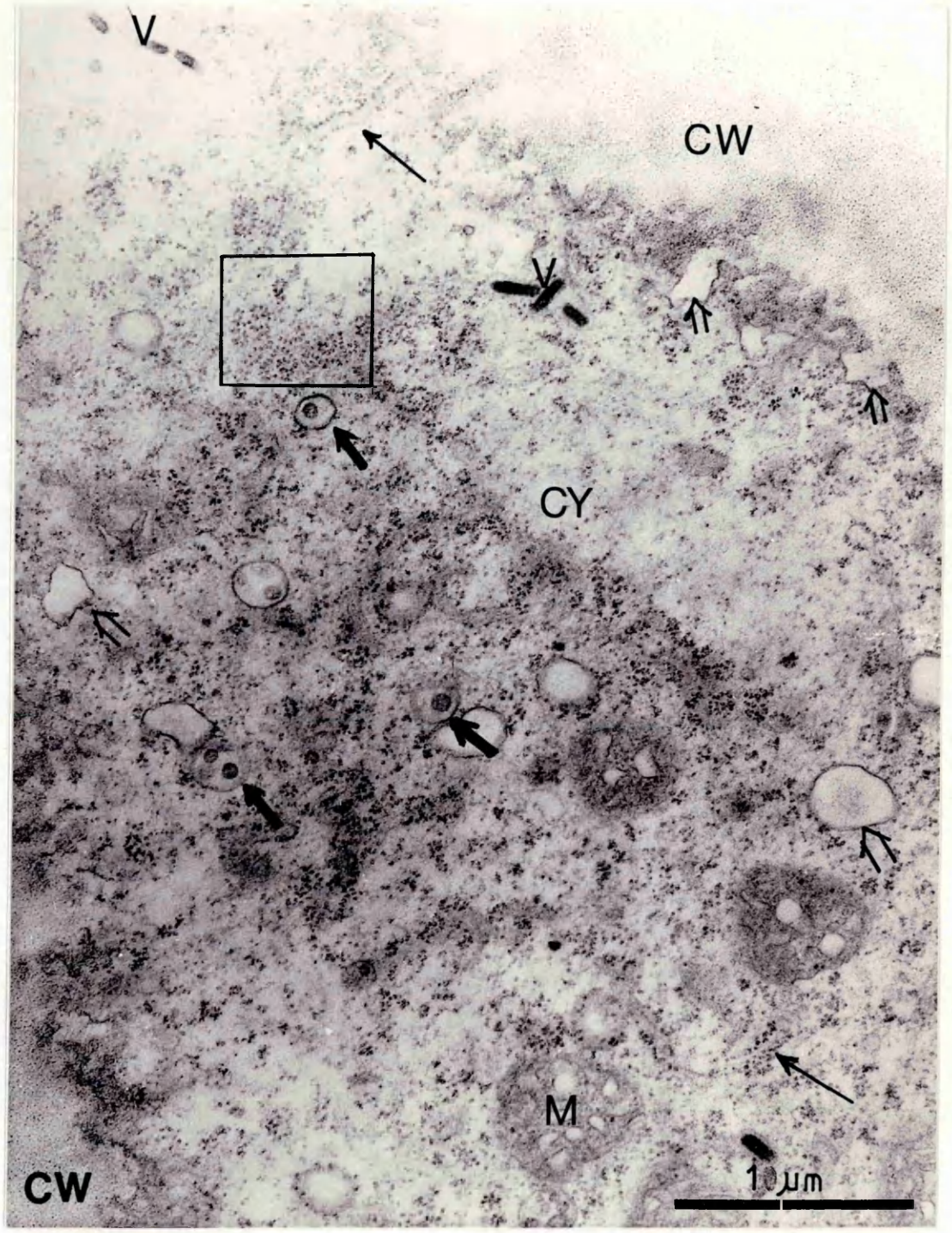


Figure 4-27:

Electron micrograph of a cell from infected *N. edwardsonii* 12 d after inoculation showing a cross-section of channels containing virus (bold arrows) and plasmalemmasomes (open arrows). Note the increasing numbers of ribosomes (box & thin arrows). Abbreviations are as in page X

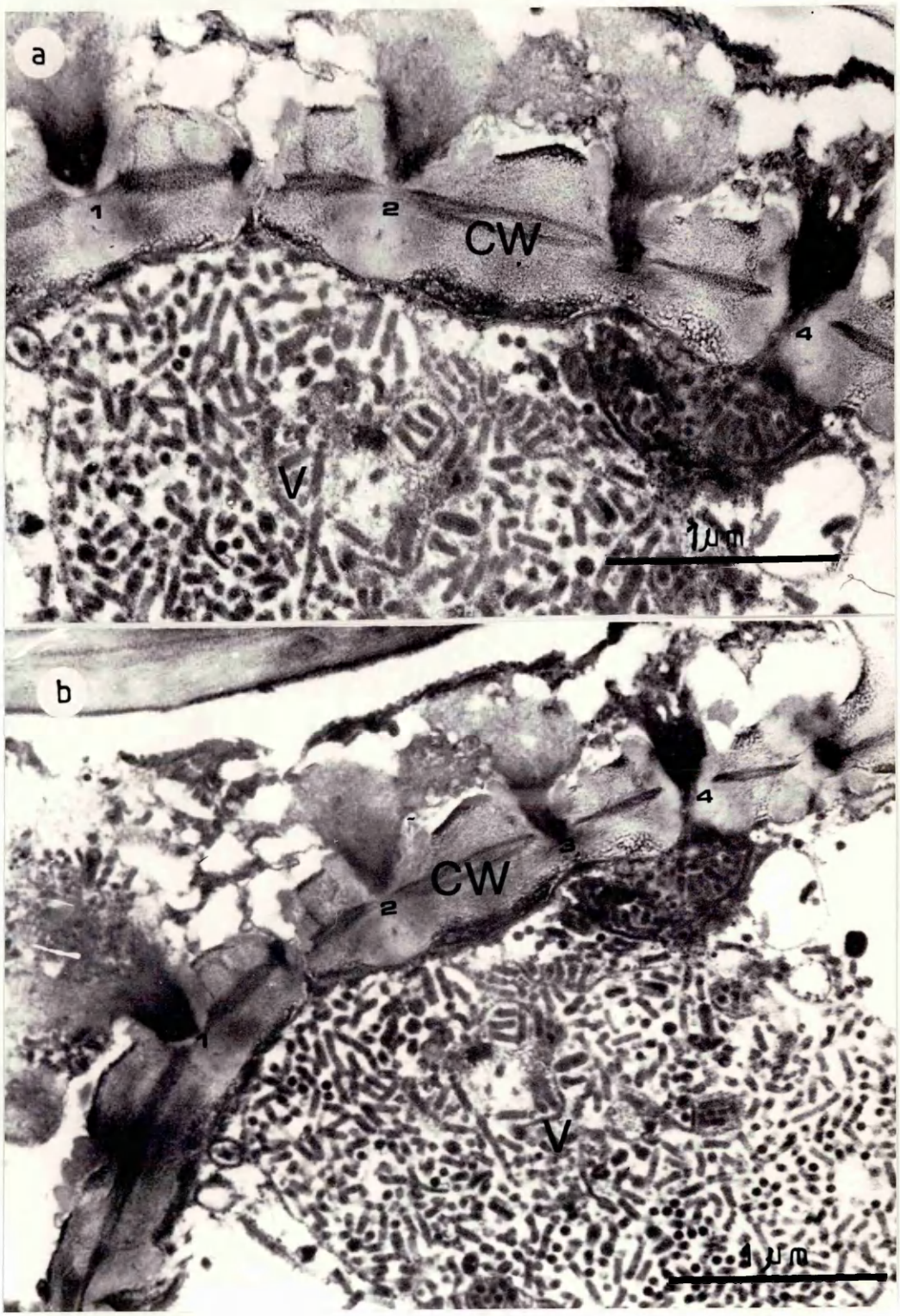


figure 4-28:

Serial sections of electron micrographs from vascular system of infected *N. edwardsonii* cell 10 d after inoculation. Note the virus particles apparently passing through specialized channel (1, 2, 3 & 4). Abbreviations are as in page X .

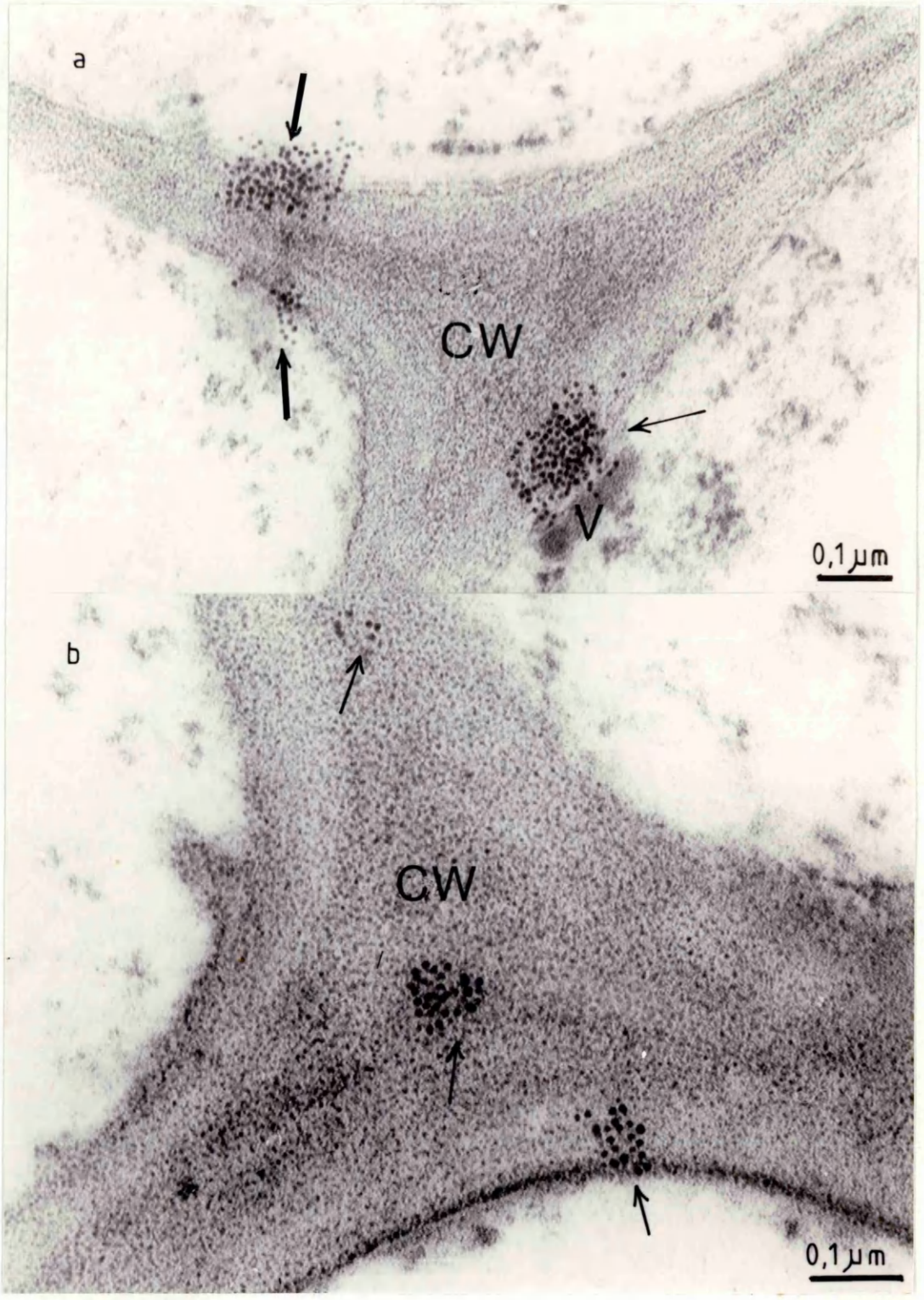


Figure 4-29:

Electron micrographs of immunogold labelled sections of infected *N. edwardsonii* cells 12 d after inoculation. Note the very strong binding of the gold particles to certain regions of the cell walls (arrows), and the outgrowth associated with the gold particles (bold arrow)

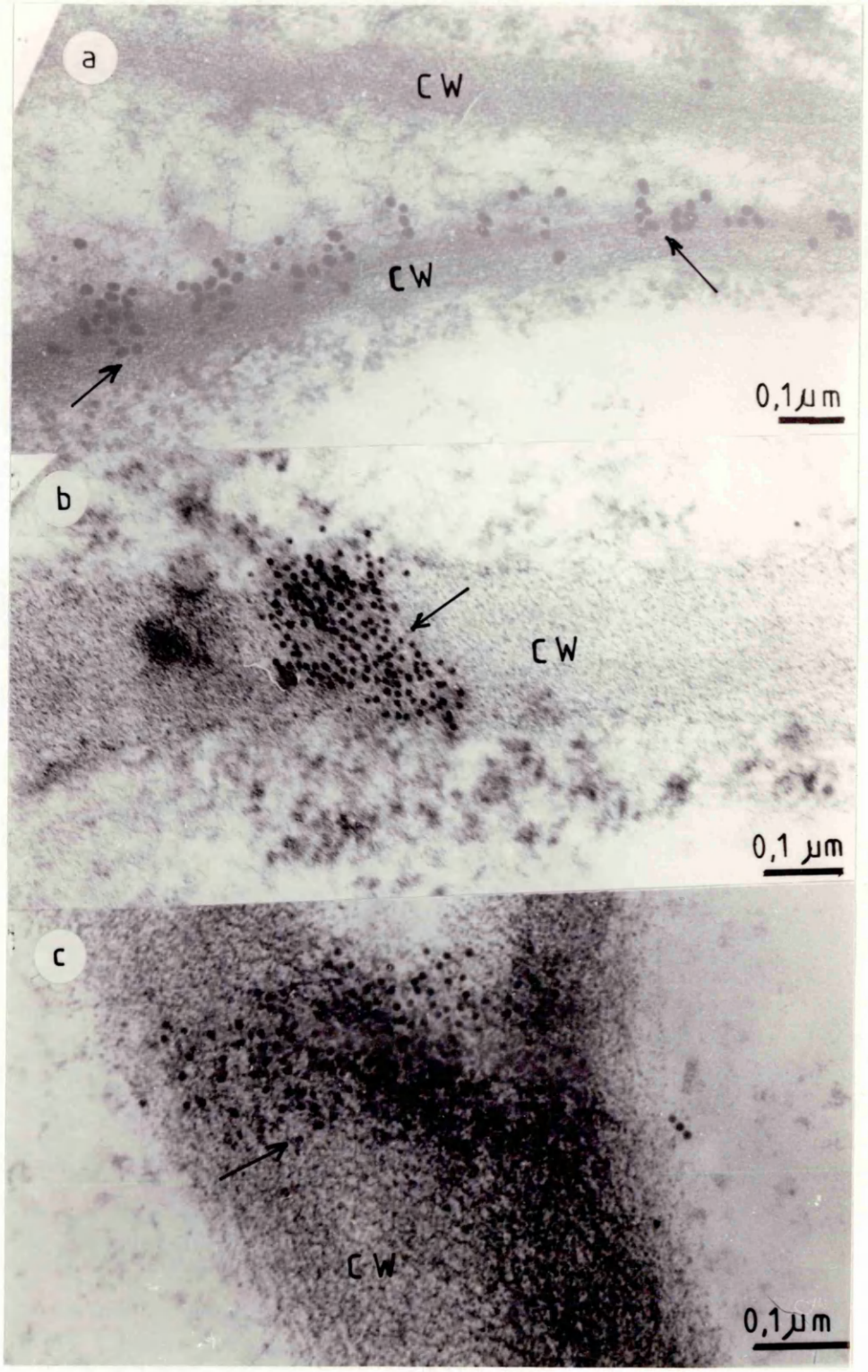


Figure 4-30:

Electron micrographs of immunogold labelled sections of infected *N. edwardsonii* cells (a) 5 d, (b) 10 d, (c) 15 d after inoculation. Note the very strong binding of the gold particles to certain regions on the cell walls (CW) and compare to that in figure 4-29.

4.3 CONCLUSIONS:

SYNV induces severe pathological changes in the cells of *N. edwardsonii*. These changes were very evident in the chloroplasts, mitochondria, nuclei, plasma membrane and the cell walls. They were first observed in the initial acute phase of virus multiplication and often became more marked during chronic stage of infection.

In infected cells during the phase of active virus multiplication, nuclei showed severe abnormalities, chromatin was abnormally distributed, areas of perinuclear space were enlarged and a great many cytoplasmic invaginations into the nuclei developed. Many nucleocapsids and membrane-bound inclusion bodies were observed in these nuclei, associated with the development of large viroplasms. That these viroplasm, are, indeed, the site of nucleocapsid assembly was revealed by immunogold labelling studies and by the association of a great many nucleocapsids with the patches of granular matrix in the viroplasm regions. In the chronic stage of infection the distribution of chromatin appeared similar to that in the nuclei of healthy controls. No viroplasms and no virus particles were observed. However the cytoplasmic invaginations were more marked and in immunogold labelling studies, gold particles still bound extensively to the nuclei.

Alterations were observed in the chloroplasts, such as loss of the thylakoid network. These changes may be connected with the presence of free virus protein(s) as demonstrated by immunogold labelling. However other abnormalities, such as the development of membrane-bound inclusion bodies and large areas of starch within the chloroplasts, did not seem to involve directly the presence of free virus proteins.

During the acute phase of infection, mitochondria showed some alterations such as the appearance of membrane-bound inclusion bodies embedded within them and a loss of mitochondrial matrix. Later, in the stage of chronic infection, large areas of perimitochondrial space developed. No viral protein seems to be involved directly in these alterations.

Plasma membranes in variety of infected tissues developed characteristic outgrowths, forming plasmalemmasomes. In the cell walls, channels of about 80-250 nm in diameter were observed interconnecting adjacent infected cells. These channels, which are not modified plasmodesmata, may be the route by which virus moves from cell to cell. Immunogold labelling studies showed a strong reaction between certain regions in the cell wall and the immunogold probe, suggesting the direct involvment of viral protein(s) in cell-to-cell virus transport or in the establishment of the transport channels.

CHAPTER 5

STUDIES ON *NICOTIANA EDWARDSONII* PLANTS CHRONICALLY INFECTED WITH SONCHUS YELLOW NET VIRUS

5.1 INTRODUCTION:

Infections by many plant viruses follow two phases. In the initial acute phase virus multiplies rapidly and spreads through the plant. The plants then show a partial recovery (Matthews, 1980). Little is understood about this recovery although the chronically infected state presumably represents the norm in virus infections.

Inoculation of SYNV to *N. edwardsonii* results in a typical acute infection in which virus spreads rapidly through all tissues reaching a maximum after 10 d. Plants then recover, with levels of virus antigen dropping rapidly (see chapter 3, fig. 3-15). By 60-75 d symptoms have disappeared and the plants appear similar to uninfected controls (see chapter 3, fig. 3-2). Plants at this stage of infection with SYNV, or for that matter with other plant rhabdoviruses, have not been the subject of previous studies.

The first aim of the work described in this chapter was to study the chronically infected plants in order to investigate the reason(s) behind the reductions in both the viral protein concentrations and the numbers of infected cells, which occur during this stage (see chapter 3, sections 3.2.5.2.2 & 3.2.5.4). The apparent recovery during chronic infection superficially resemblances the reduction in virulence caused by the formation of DI-particles of animal viruses during multiple passage at high multiplicities of infection (see chapter 1, section 1.7.1). Chronically infected plants have therefore been examined to see whether they contain defective form(s) of SYNV

5.2 RESULTS:

5.2.1 Synthesis of Virus Proteins in Chronically Infected plants:

Infected plants were identical in appearance to healthy controls by about 60-75 d after inoculation (chapter 3, fig. 3-2). Cells from acutely infected plants contain large numbers of virions (see chapter 3, section 3.2.5.4). In many hundreds of cells in 30-45 independent sections examined over a period of 2 years, no virus particles were ever observed in asymptomatic tissue from chronically infected plants. However cells showed the obvious and characteristic ultrastructural changes, typically invaginations of the nuclear envelope (fig. 5-1) and alterations in the chloroplasts and mitochondrial membrane, described in detail in chapter 4.

Despite the apparent absence of virions in chronically infected tissue, inoculation of sap from chronically infected to healthy plants resulted in typical symptoms of SYNV infection, 8-10 d after inoculation. Thus, infectious virus, or nucleocapsids, must be present, albeit at low concentration. Electron microscopy of leaf tissue from these systemically infected plants revealed the existence of SYNV particles. However, the number of infected cells and the number of particles per cell were about 30-45% lower than in plants inoculated with "standard" inocula and kept under identical conditions. Yields of virus prepared from these plants by the usual procedure (chapter 2, section 2.4.1), were 40-80% lower per gram of tissue than from leaves inoculated with "standard" inocula. When systemically infected leaves were taken from these plants 10-12 d after inoculation and used to inoculate a further batch of seedlings, the virus yield remained low.

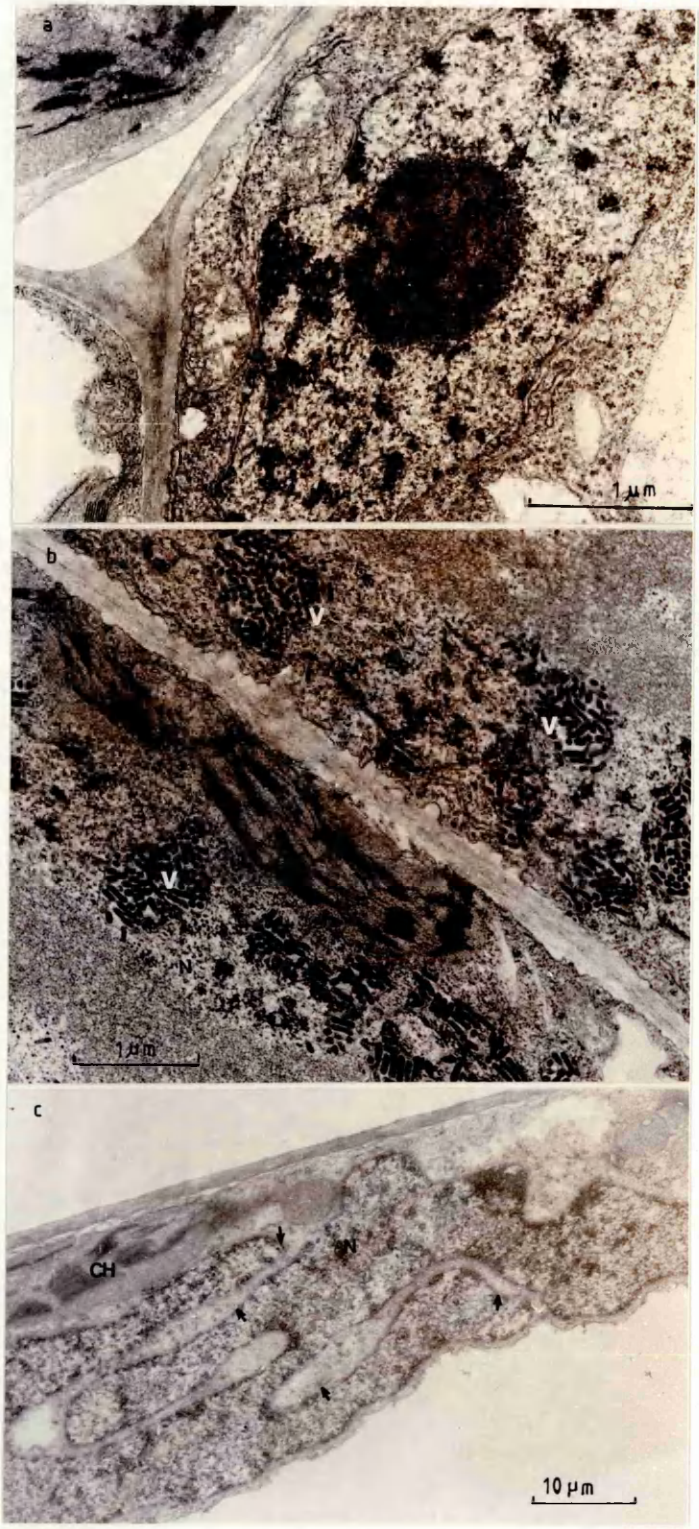


Figure 5-1:
Sections of leaf mesophyll cells from (top to bottom) healthy, 10 d infected & 75 d infected plants. Abbreviations are as in page X. Invaginations in the nuclear envelope of 75 d infected plants are arrowed.

These reductions in yield, numbers of virus particles etc. which were maintained on subsequent passage, suggest that the properties of the virus had changed. Chloroplasts from plants inoculated with inocula from chronically infected plants often contained virus nucleocapsids within chloroplasts (fig. 5-2a,b) as well as the usual virus clusters in the perinuclear space. These chloroplast-associated nucleocapsids, which could be identified by their dimensions and morphology and distinguished from mature virions by their lack of an envelope, were often centrally located in inclusion bodies within the chloroplasts. In some cases, nucleocapsids were associated with ribosome-like objects (fig. 5-2b). Nucleocapsids were never observed within chloroplasts from plants infected with standard inocula. Chloroplasts from the above plants were purified and fractionated into thylakoids and stroma. Proteins were separated by polyacrylamide gel electrophoresis, western blotted onto nitrocellulose and probed with anti-SYNV antiserum. Chloroplasts from healthy plants and purified SYNV were used as controls. Results are shown in figure 5-3. Infected thylakoids reacted strongly giving a strong band at 56-58 kD, comigrating with SYNV protein N, as well as a faint band of approximately 200 kD which may correspond to SYNV L protein. Infected stroma gave a weak band at 56 kD. Neither healthy stroma nor healthy thylakoids reacted with the antiserum.

SYNV contains one minor and four major structural proteins (Jackson, 1978). To determine which of these, were present in chronically infected plants, total protein extracts from leaves and roots were prepared from plants 70-90 d after inoculation. Leaf and root tissues were individually ground in 5% SDS (1g/1ml), clarified for 2 m by centrifugation and separated by SDS-PAGE. The proteins

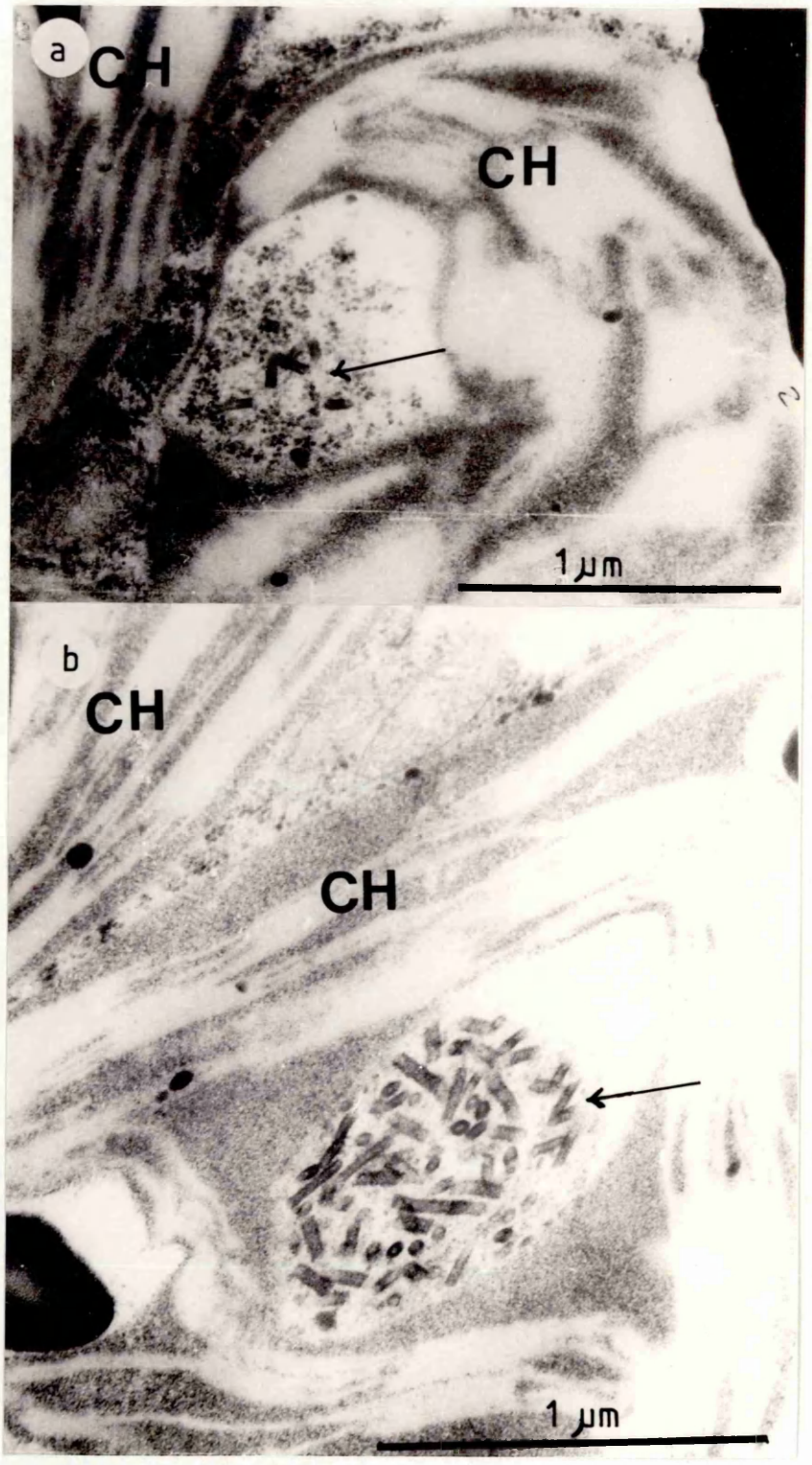


Figure 5-2:

(a) and (b) Chloroplasts from cells 10 d after inoculation with sap from chronically infected plants. Nucleocapsids are arrowed. (a) Nucleocapsids in association with ribosomes.

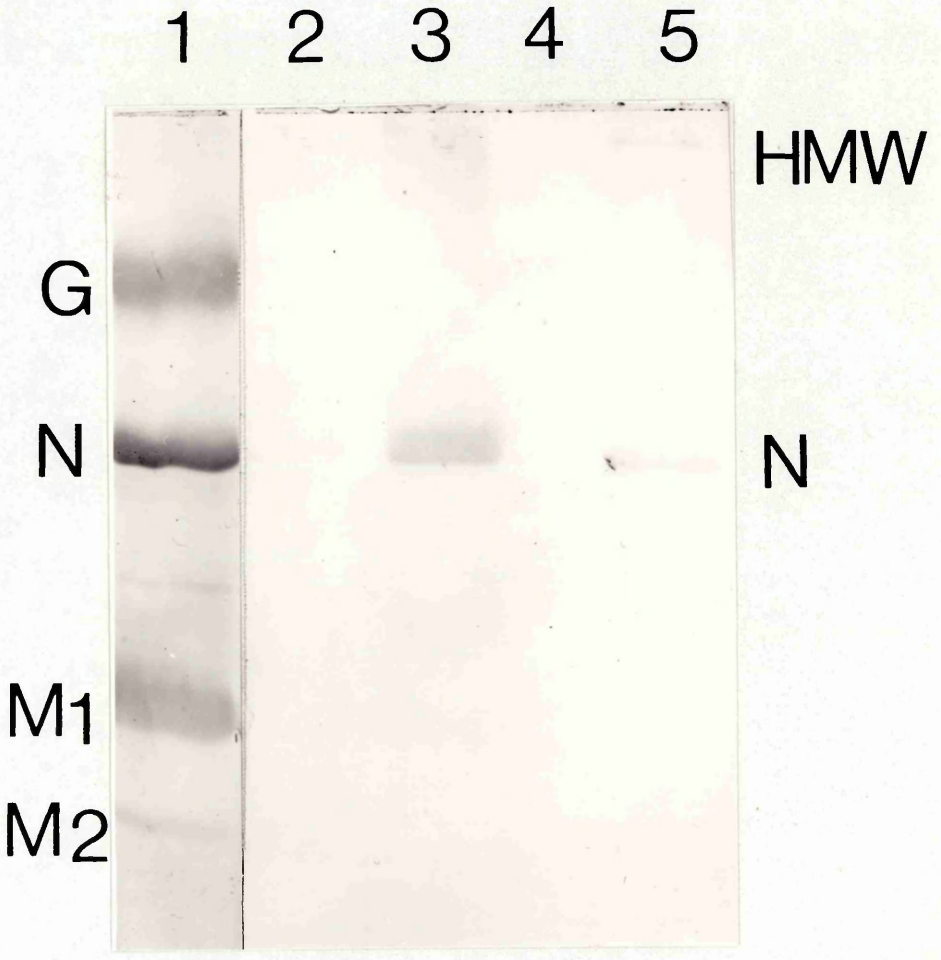


Figure 5-3:
Western blots of proteins extracted from chloroplasts 10 d after inoculation with sap from chronically infected plants and from healthy controls. (Lane 1) purified SYNV standard proteins; (lane 2) healthy thylakoids; (lane 3) infected thylakoids; (lane 4) healthy stroma; (lane 5) infected stroma. Virus proteins are indicated on the left.

were western blotted onto nitrocelluloce membrane and virus proteins were detected immunologically using antiserum to SYNV as described in chapter 2, section 2.13.8. Proteins from purified SYNV were run in a parallel lane as standard. Typical results are shown in Figure 5-4 . Three polypeptides reacted, the most prominant band corresponding to the nucleocapsid protein (N). The envelope-spike protein (G) was also present. Neither M1 nor M2 proteins were observed, even when gels were overloaded. However an extra major antigenically-cross-reacting polypeptide p41 (mol. wt. 41KD), was detected migrating slightly more slooly than the position of M1 in the standard lane. Similar results were observed with a large number of independent protein samples taken from plants between 60 d and 110 d after inoculation. A protein of 41KD, comigrating with p41, was also observed in western blots of purified SYNV preparations when the amount of purified virus per track was increased to 20-30µg, (fig. 5-5). This protein was not detected in infected tissues at the acute phase of infection (see fig. 3-4b) nor when less than 20µg of purified virus was loaded onto the gel (see fig. 5-5, lane 2).

To determine the intracellular locations of these viral proteins, sections of cells from chronically infected plants were immunogold labelled. Typical sections are shown in figure 5-6. Clusters of gold particles were found within the nuclei, associated with the nuclear envelope and to a lesser in the cytoplasm possibly associated with ER. Similar results were observed with a variety of independent sections.

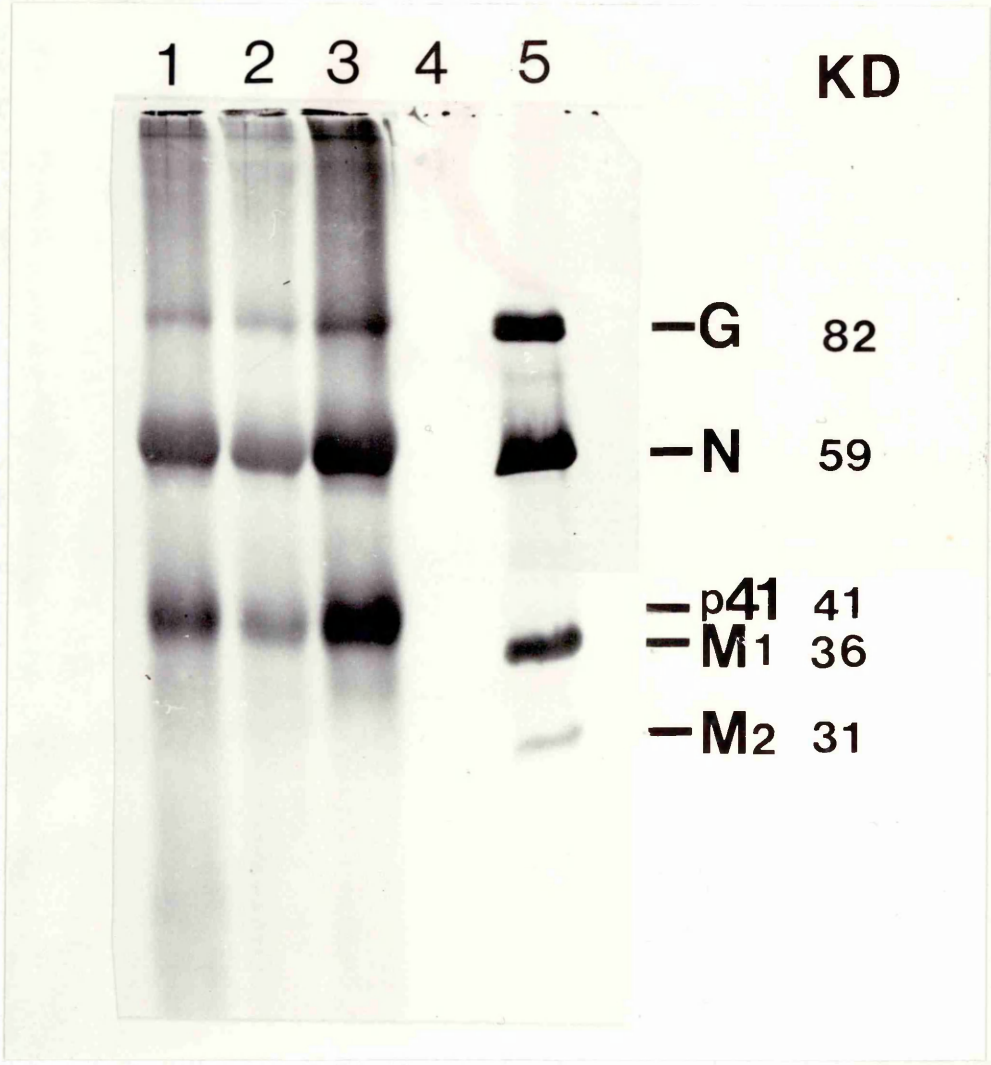


Figure 5-4:

Western blots, using anti-SYNV serum, of total proteins from healthy plants & plants 60 d after inoculation separated by SDS PAGE. (1,2,3) infected leaves from three separate plants; (4) healthy leaves; (5) purified SYNV. Virus proteins and molecular weights are indicated on the right.

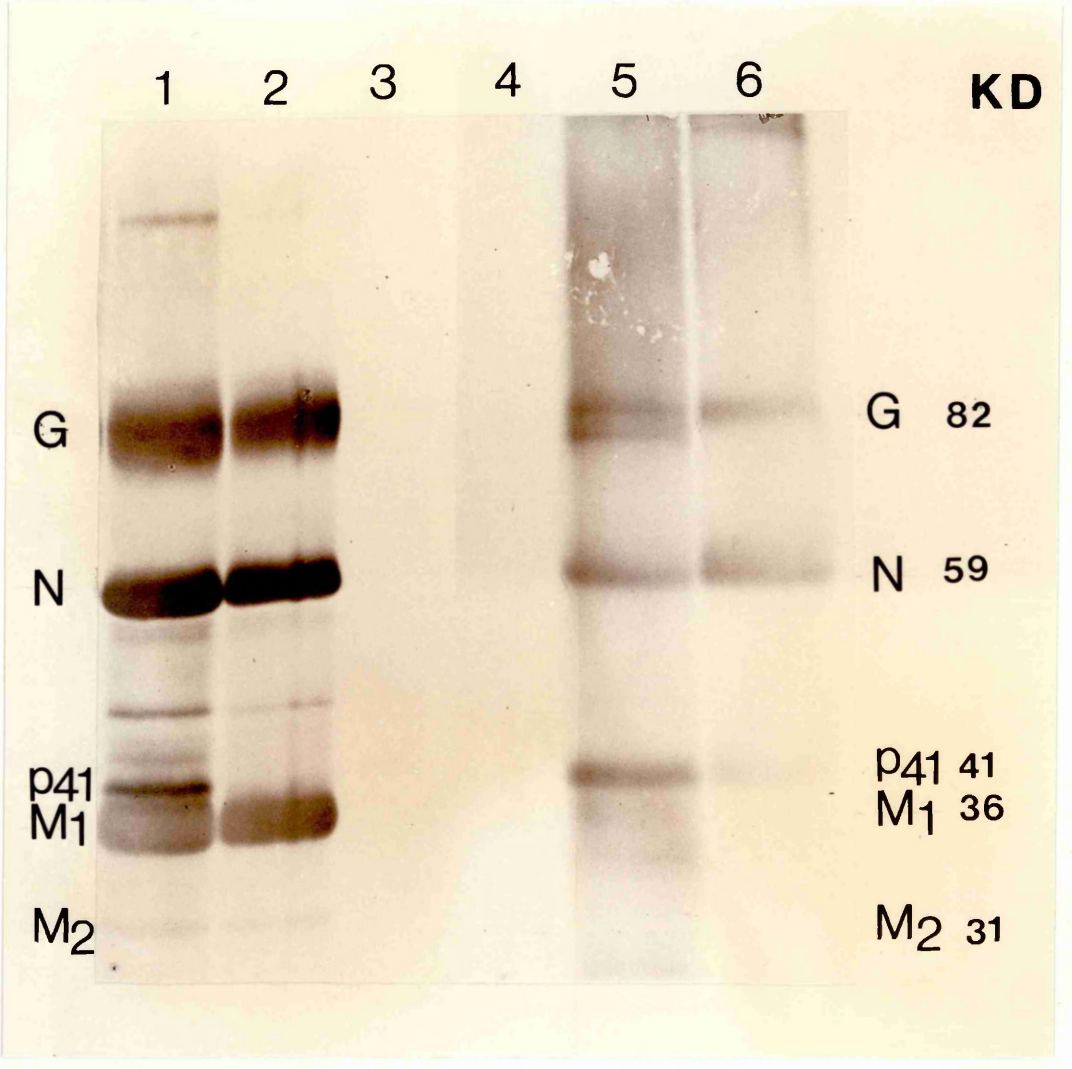


Figure 5-5:

Western blot, using anti-SYNV serum, of (1) 28µg (2) 16µg total virus proteins from purified SYNV preparations; total proteins extracted from healthy (3) leaves; (4) roots; Total proteins extracted from 75 d infected (5) leaves; (6) roots.

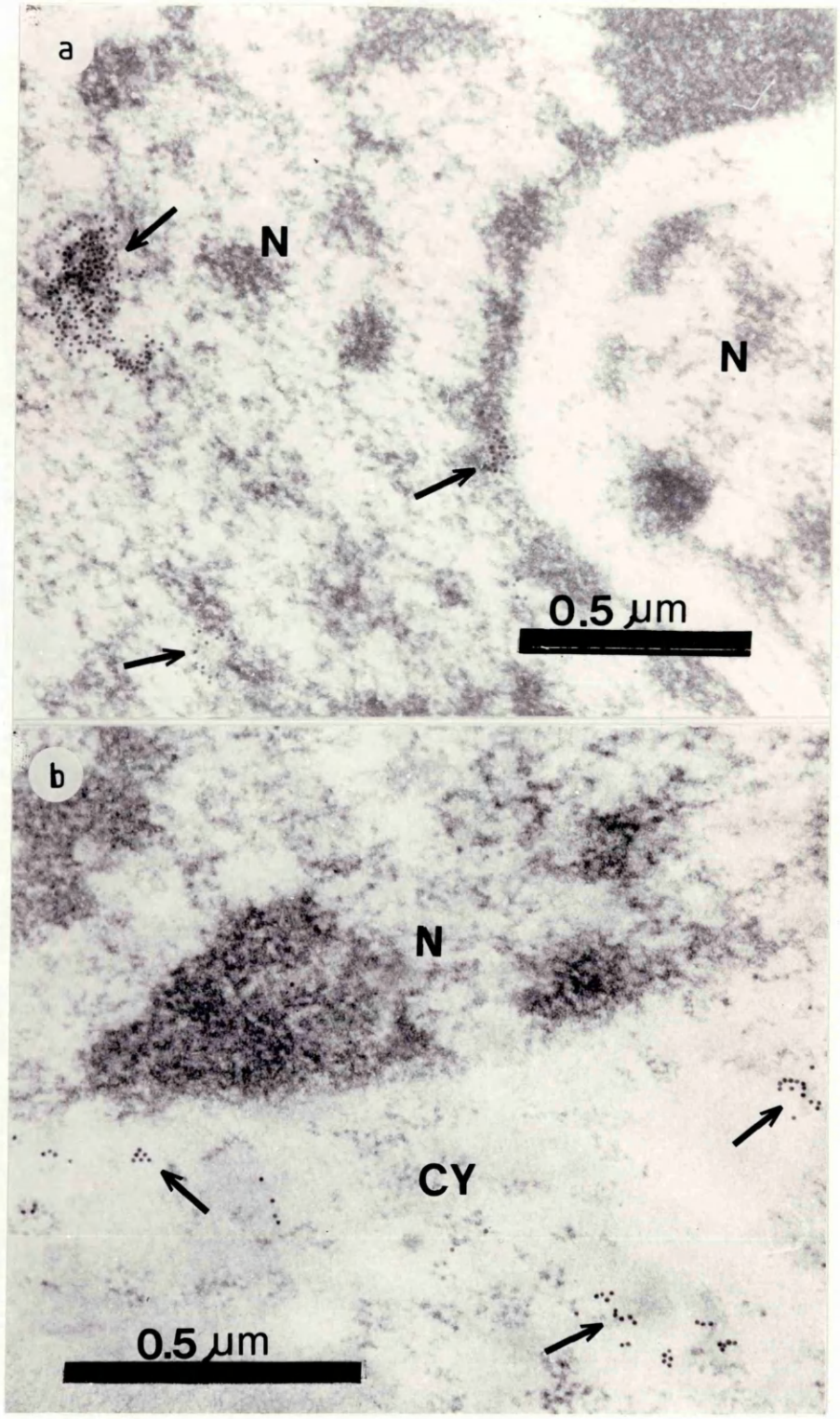


Figure 5-6:

Immunogold labelled sections of 90 d infected plants showing gold particles (arrowed) in (a) nucleus (N) and (b) cytoplasm (CY).

Poly(A)$^+$RNA was prepared from polysomes of both chronically infected and healthy plants and translated *in vitro* (see chapter 2, sections 2.8 & 2.11.2). The translation products were, in some cases, immunoprecipitated, fractionated by SDS-PAGE and viral proteins were detected by fluorography. A number of bands, presumably representing host proteins which cross-reacted with the antibodies or were otherwise non-specifically precipitated, were present in the translation products of both healthy and chronically infected poly(A)$^+$RNA. Most of the bands corresponded to the most prominent bands in the non-immunoprecipitated samples. Several additional polypeptides were present in the translation products of poly(A)$^+$RNA from chronically infected plants compared to healthy controls. Polypeptides comigrating with viral proteins N, M1 and M2 were specifically immunoprecipitated from the "chronically infected" translation products as well as a number of minor low molecular weight bands (fig. 5-7a). *In vitro* synthesis of G protein is very often difficult to detect even using mRNA from acutely infected plants, (in which the relative levels of viral compared to the host translation products are much higher (J.J. Milner, personal communication)). A prominent band of 41KD was present in the translation products of both "infected" and "healthy" mRNAs but was immunoprecipitable only from the "chronically infected" suggesting a possible viral product comigrating with a host polypeptide.

To investigate expression of these mRNAs *in vivo*, leaf discs were incubated with ^{32}S-methionine for 4-6 h as described in chapter 2, section 2.12. Viral proteins were extracted, immunoprecipitated

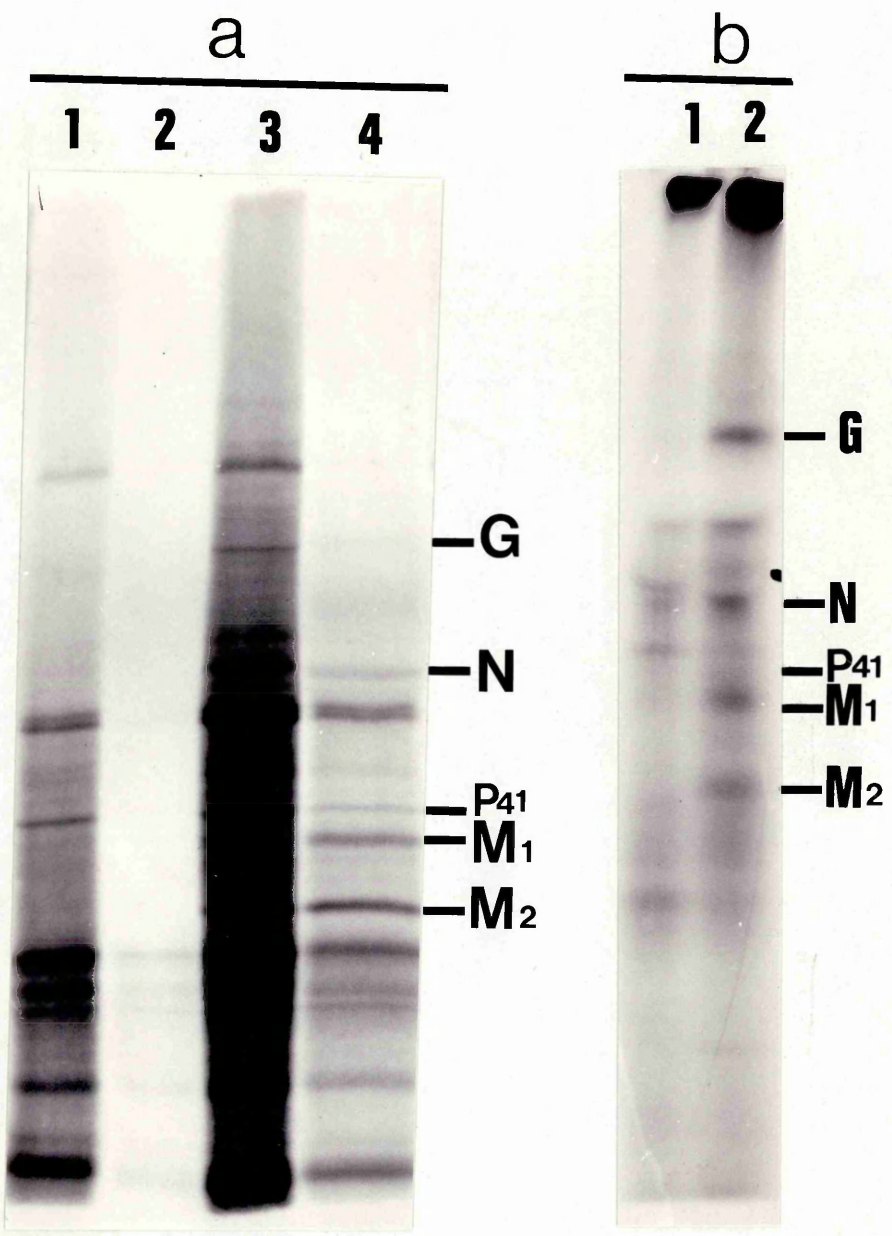


Figure 5-7:

(a) Fluorograph of SDS PAGE of proteins synthesised *in vitro* from poly(A)$^+$RNA from (1,2) healthy plants; (3,4) infected plants. Lanes (2) & (4) are immunoprecipates of the samples in lanes (1) & (2) respectively. (b) Fluorograph of SDS PAGE of immunoprecipitates of proteins synthesised *in vivo* in (1) healthy plants; (2) 90 d infected plants. Positions of SYNV proteins run in parallel as standards are indicated.

and analyzed by SDS-PAGE (fig. 5-7b). In lanes from chronically infected plants, four major radioactively labelled polypeptides which co-migrated with all four of the major virus structural proteins G, N, M1 and M2 were specifically immunoprecipitated. A few minor polypeptides were immunoprecipitated from both chronically infected and healthy plants but these were much less prominent than these corresponding to the virus proteins. A faint band of 41KD was immunoprecipitated from the chronically infected plants but was absent from the healthy plants. These results clearly demonstrate that all four major virus structural proteins are actively synthesized in the chronically infected tissue.

5.2.2 Isolation and Characterisation of DI-Particles:

While extensive investigations on the senescent chronically infected plants were underway to find an answer to the question of the virus disappearance, a large variety of tissues were examined at various times after inoculation. In plants 150 d after inoculation, large numbers of enveloped bacilliform particles were observed in sections of calyx tissue. These particles appeared shorter in length than standard SYNV.

Sections of calyx from five plants were examined. In all five, cells contained large numbers of enveloped, bacilliform virus-like particles in the nucleus, perinuclear space and cytoplasm (fig. 5-8a). A large numbers of these particles were also seen situated very close to the chloroplasts and possibly surrounded by the external membrane of the chloroplast (fig. 5-8b). Apart from that, the subcellular distribution of the virus particles was similar to that observed in cells from leaves 10 d after infection (see chapter

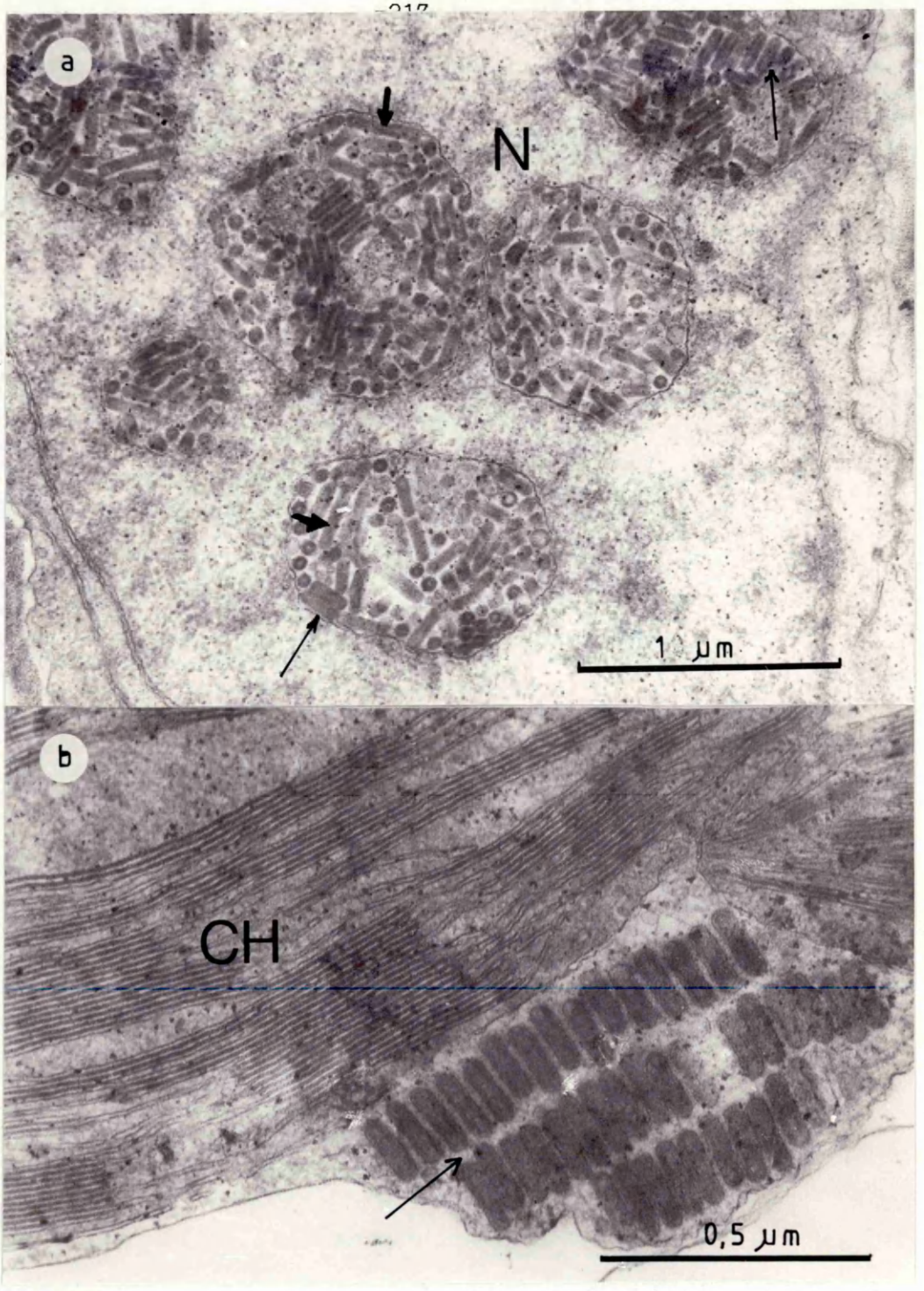


Figure 5-8:

Electron micrographs of 150 d chronically infected *N. edwardsonii* cells, from calyx tissue, with standard SYNV type, showing short particles (thin arrows) and standard virus particles (bold arrows). (a) Infected nucleus showing the location of short particles, (b) infected cytoplasm showing the location of short particles nearby the chloroplast.

3, section 3.2.4.5). However, most of the particles were shorter than the 217 nm expected for standard SYNV (fig. 5-9a).

In order to eliminate the possiblity that these short particles resulted from cross-infection with a different enveloped bacilliform virus, sections were immunogold labelled. Figure 5-9b shows gold particles extensively bound to the short particles as well as to the longer particles, indicating antigenic cross-reactivity with SYNV.

The size-distribution of the short particles was determined by measuring the lengths of 167 particles in several sections of the same tissue. Care was taken to exclude particles which were non-uniformly stained or without two rounded ends, in order to avoid the possibility of measuring oblique sections. Figure 5-10a shows the distribution of sizes of particles in calyx from a single plant (this was designated as isolate, DI-1). To confirm that calyx is the site of DI-particle generation, this experiment was subsequently repeated on a several independent sets of plants. In each case, short particles were observed in calyx 120-150 d after inoculation. The size-distribution of particles from one of these plants (designated isolate DI-2) is also shown (fig. 5-10b). As a control, the size-distribution of particles from the parental standard virus was measured in sections of leaf harvested 10 d after inoculation (fig. 5-10c). Sections of tissue infected with standard virus contained particles predominantly 210-220 nm long with very few apparently shorter species and a small number of longer particles. In contrast only 15% of the particles from isolate DI-1 were 210 nm or longer; the rest varied between 130 and 210 nm with the most

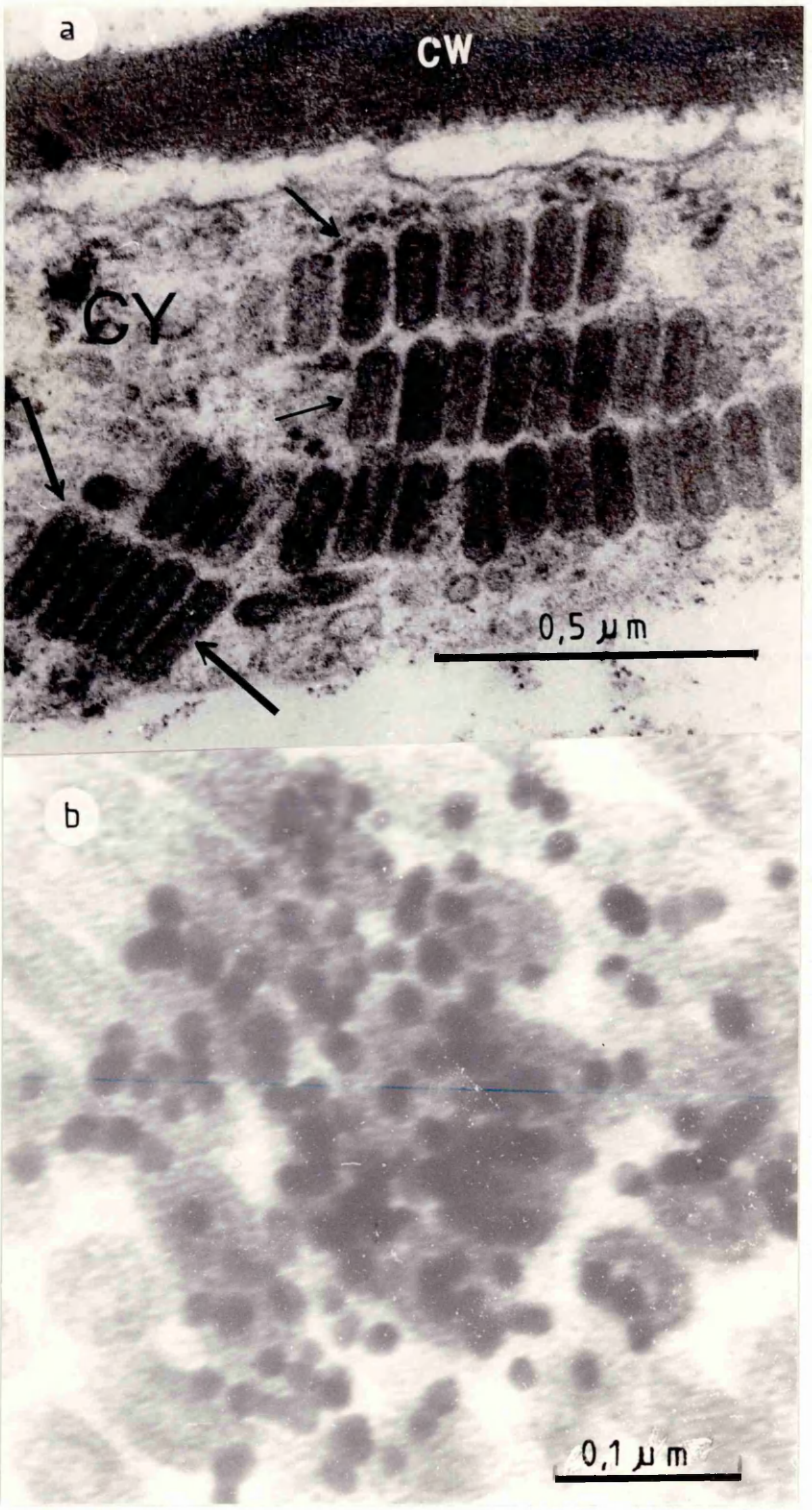


Figure 5-9:

(a) Electron micrograph of section of calyx from plants 150 d after inoculation with standard SYNV type showing DI-particles (thin arrows) and standard virus particles (bold arrows). (b) Similar section, immunogold labelled with anti-SYNV antiserum.

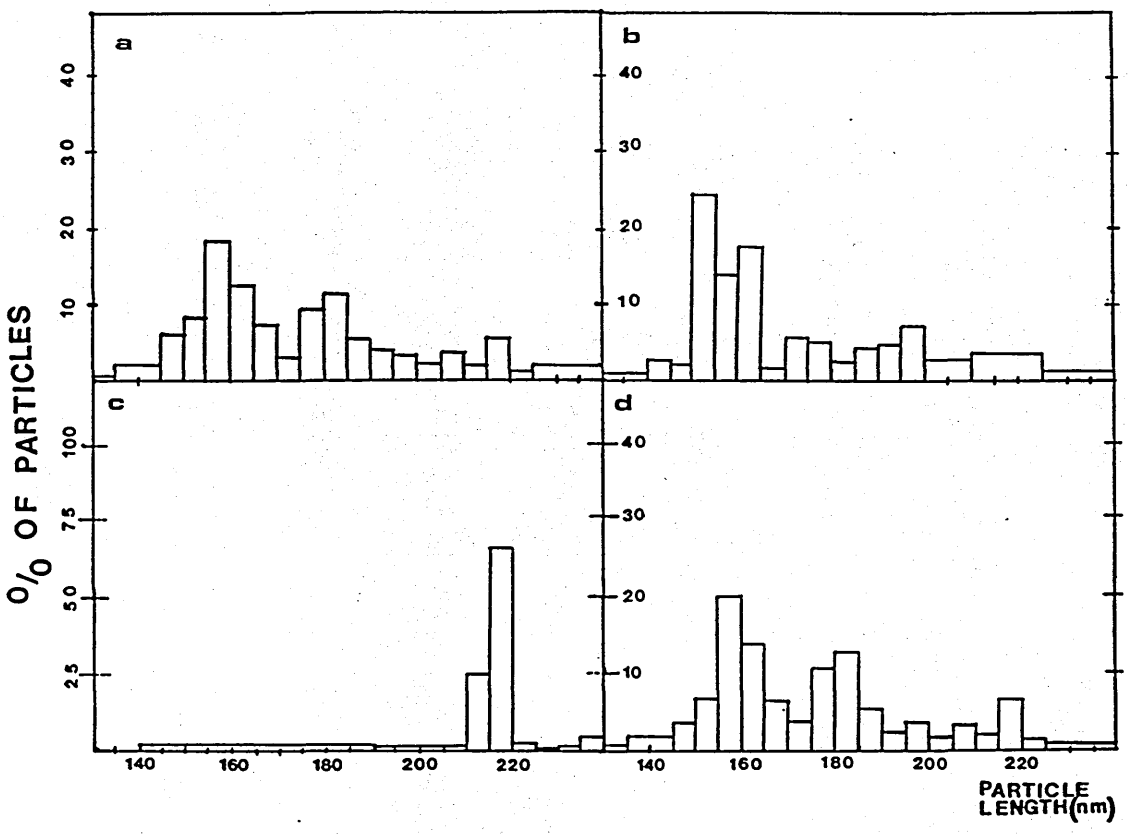


Figure 5-10:
Size-distribution of virus particles within sections of (a) isolate DI-1, calyx from plants 5 months after inoculation with standard SYNV, (b) isolate DI-2, calyx from plants 5 months after inoculation with standard SYNV, (c) systemically infected leaf from plants 10 d after inoculation with standard SYNV, (d) systemically infected leaf from plants 10 d after inoculation with DI-1.

abundant species measuring 160 and 180 nm. Particles from DI-2 showed a similar but not necessarily identical distribution of sizes.

Calyx which contained short particles (isolate DI-1) was used to inoculate *N. edwardsonii* plants. Symptoms developed on 80 of 115 plants 8-10 d after inoculation. Inoculated leaves showed distinct chlorotic local lesions, uninoculated tip leaves (unexpanded leaves) developed a chlorotic mottling. These symptoms were quite different to the systemic vein-clearing which follows inoculation with standard SYNV (fig. 5-11). In tissue sections of systemically infected leaves, cells contained numerous bacilliform particles within the nucleus and perinuclear space. The range of particle sizes was identical to the range of sizes observed in the calyx tissue from which isolate DI-1 was derived (fig. 5-10d).

In order to test whether the generation of short particles was a peculiarity of the inoculum of the isolate of SYNV-type used (perhaps as a result of its long history of maintenance in the glasshouse by successive mechanical transfer) plants were inoculated with an isolate, SYNV-L85 (Falk *et al.*, 1986), which had been subjected to only 4 successive mechanical transfers since its identification in the field (B. Falk, personal communication). Ten days after inoculation, plants exhibited similar if somewhate more severe symptoms to these inoculated with SYNV-type and plants subsequently recovered. In calyx tissue from plants 120 d after inoculation, short enveloped bacilliform particles varying in size from 130 nm to 165 nm were always present (fig. 5-12a). Standard SYNV-lettuce particles were 190-200 nm in length (fig. 5-12b).

One hundred g of leaves from plants inoculated with isolate DI-1 were homogenised and virus purified. The final stage of the

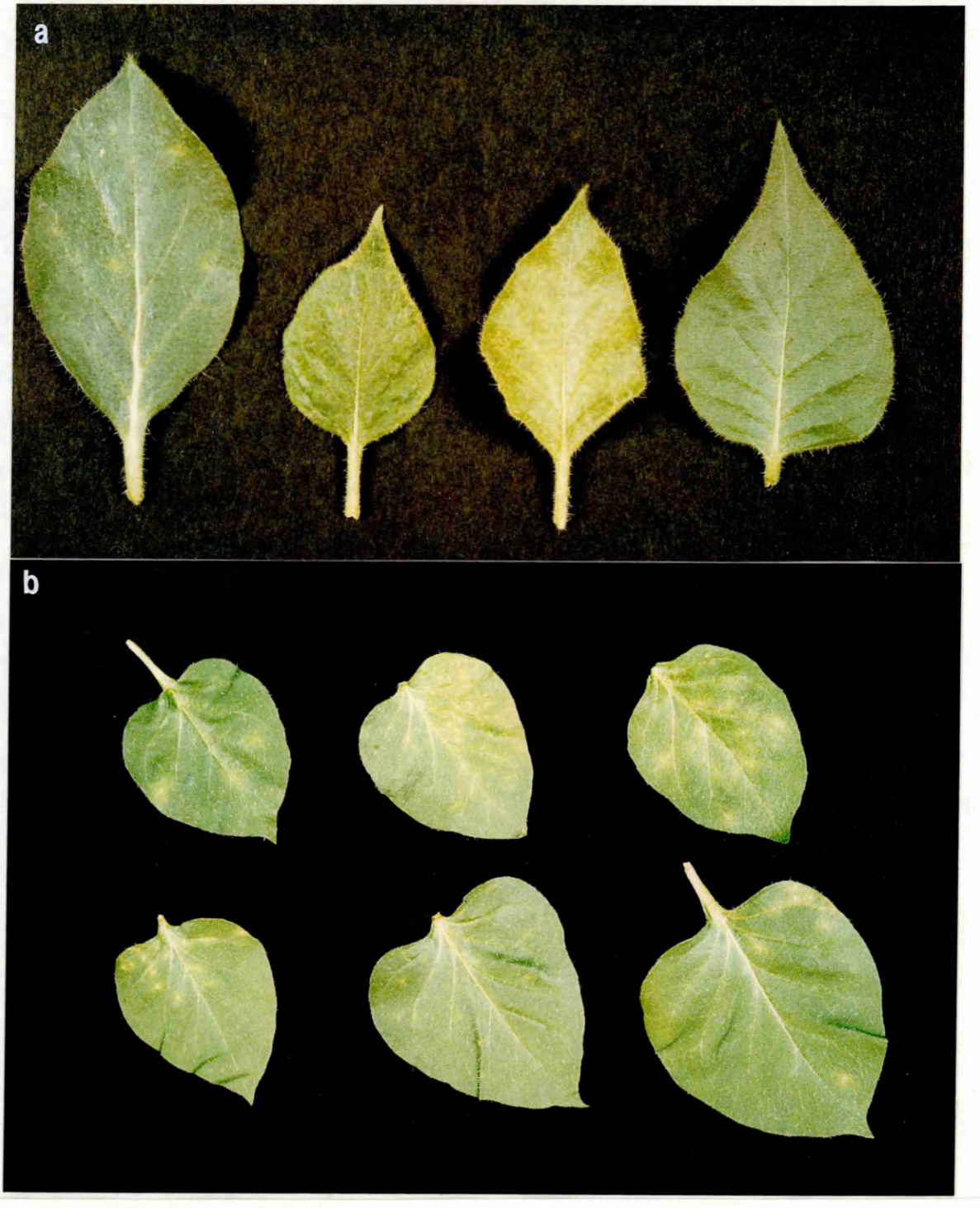


Figure 5-11:

(a) Leaves from *N. edwardsonii* showing symptoms of infection: (from left to right) inoculated leaf showing local lesions induced by DI-1; leaf systemically infected with standard SYNV; leaf systemically infected with DI-1; healthy leaf. (b) Variety of leaves showing local lesions induced by DI-1.

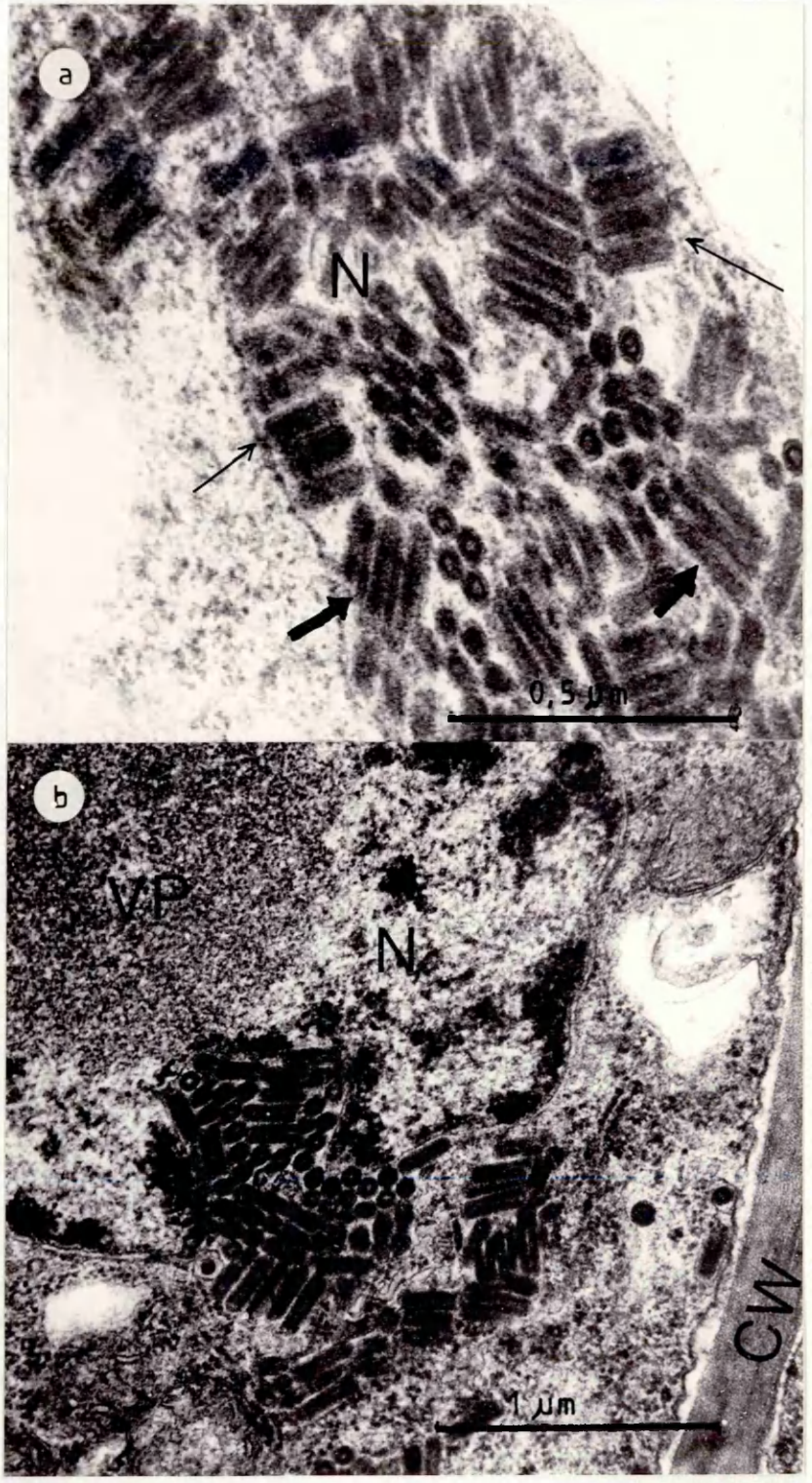


Figure 5-12:
Electron micrographs of inoculated *N. edwardsonii* with SYNV-L85 isolate. (a) Section of calyx cell 120 d after inoculation showing DI-particles (thin arrows) and standard virus particles (bold arrows). (b) Section of leaf cell 10 d after inoculation with standard SYNV-L85 showing the full length virus particles (V). Abbreviations are as in page X

procedure involves rate-zonal sedimentation on 5-30% sucrose gradients (see chapter 2, section 2.4.1). Virus purified from plants infected with isolate DI-1 sedimented as a single broad peak but did so more slowly than standard virus centrifuged in a parallel gradient (fig. 5-13). The yields of short particles, as estimated by the relative peak areas, were consistently lower than yields of standard virus prepared from similar quantities of infected leaves. The fraction corresponding to the peak was saved, pelleted by centrifugation at 100,000 g for 30 m and resuspended in 0.5 ml maintenance buffer.

Ten µl of purified short particles (equivalent to the yield from 2g of infected leaves) were diluted in 5 ml of 1% Na_2SO_3 and used to inoculate *N. edwardsonii*. No plants developed symptoms although control plants inoculated with a similar quantity of standard virus all became systemically infected. When plants were inoculated with the same quantity of short particles mixed with 10µl of purified standard SYNV (the yield from 2g of leaves and about a three fold excess based on absorbance) 3 plants out of 24 developed symptoms of systemic infection similar to those following inoculation with calyx containing the original DI-1 isolate. An additional 12 plants developed distinct chlorotic local lesions on inoculated leaves but showed no symptoms of systemic infection. Tissue sections were examined by electron microscopy. Uninoculated leaves from the three systemically infected plants contained large numbers of predominantly short virus particles associated with the nuclei. Virus particles were seen neither in uninoculated leaves from the plants which developed only local lesions nor in asymptomatic areas of inoculated leaves from the same plants.

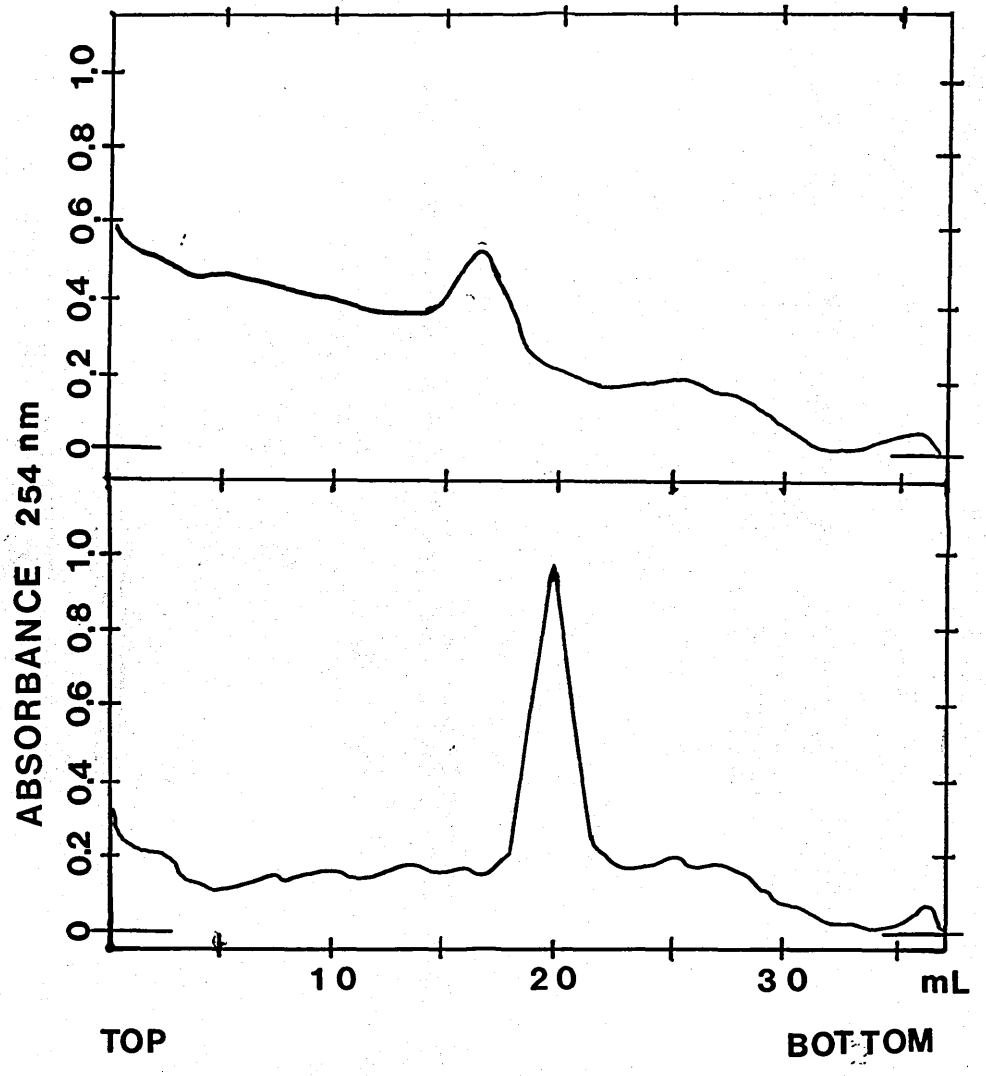


Figure 5-13:
Sedimentation of (upper) DI-1 and (lower) standard SYNV in 5-30% (v/w) sucrose gradients centrifuged for 20 m at 25.000r.p.m. in a Sorvall AH629 rotor. Virus particles were extracted from 100 g leaf tissue 10 d after inoculation. Gradients were analysed at 254 nm using an ISCO gradient fractionator and UA5 absorbance monitor.

Proteins purified from short and standard particles were analysed by SDS-PAGE (fig. 5-14). In gels stained with coomassie blue, short particles gave four major polypeptide bands which comigrated with the four major authentic SYNV polypeptides. In western blots, using anti-SYNV serum, all four of these polypeptides reacted strongly giving an identical pattern to standard virus.

RNA samples were prepared from purified short and standard particles and the sizes analysed by agarose gel electrophoresis. In stained gels, RNA from short particles clearly migrated faster than that from standard virus (fig. 5-15a). The sharpness of the RNA band from short particles also varied somewhate from preparation to preparation. Assuming that standard SYNV RNA is 13 kb (Jackson & Christie, 1977) and using RNAs from TMV and brome mosaic (BMV) viruses as additional molecular weight markers, the size of RNA from short particles is estimated to be 10.5 kb. Although the DI RNA appears to migrate as a sharp band, some degree of heterogeneity might not be apparent because of the limited resolving power of the gel for such comparatively large RNAs. The RNA gels were northern blotted and hybridized to a mixture of ^{32}P labelled plasmid DNAs pSYN302, pSYN402 & pSYN503 containing sequences derived from the mRNAs for N, M1 & M2 proteins (chapter 2, section 2.14.3.1). Both the RNAs from standard and short particles hybridized strongly to the probe (fig. 5-15b).

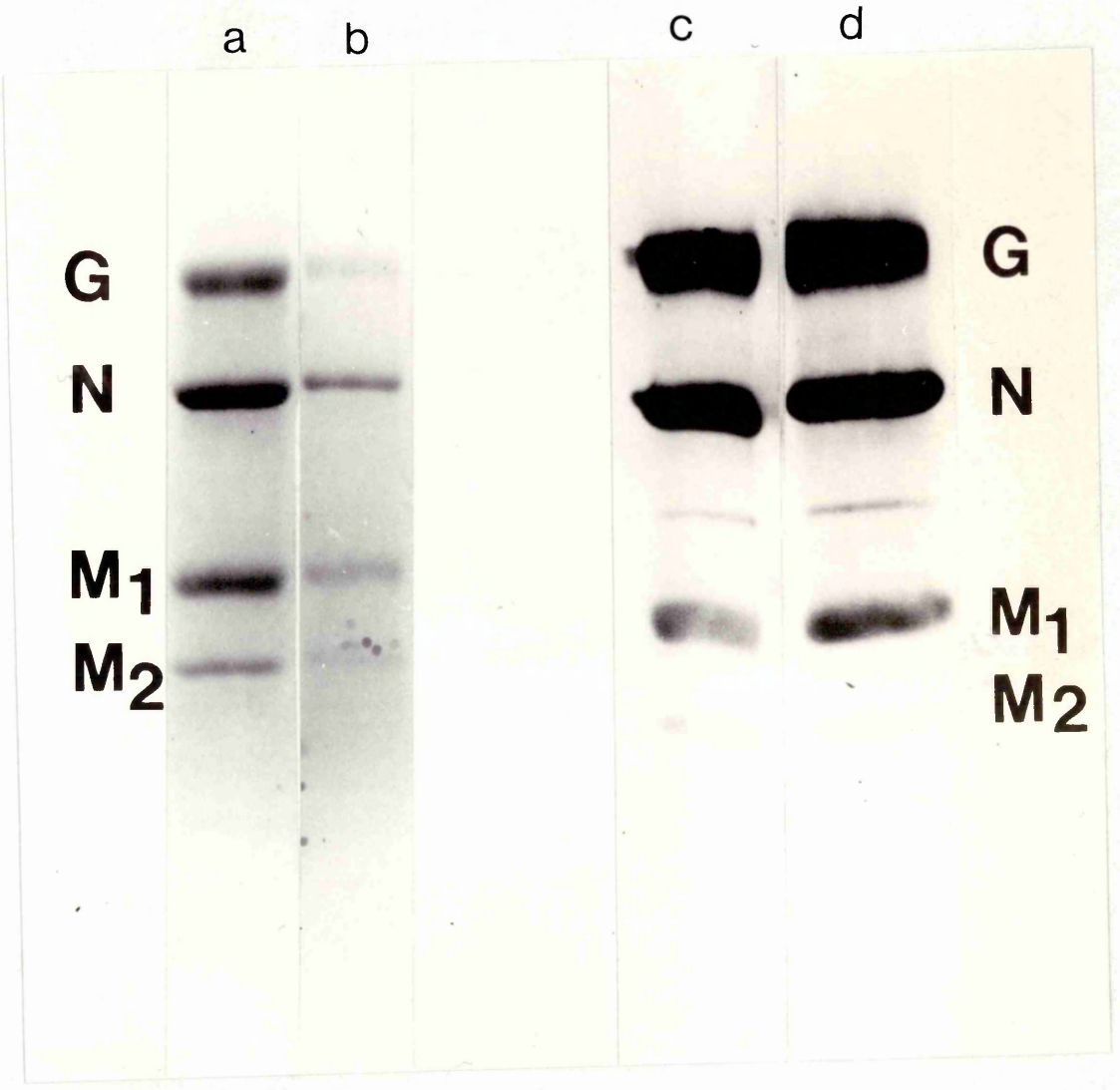


Figure 5-14:

Polyacrylamide gel electrophoresis of viral proteins. (Lane a) standard SYNV and (lane b) DI-1, stained with coomassie blue. (Lane c) standard SYNV and (lane d) DI-1, western immunoblotted with anti-SYNv serum.

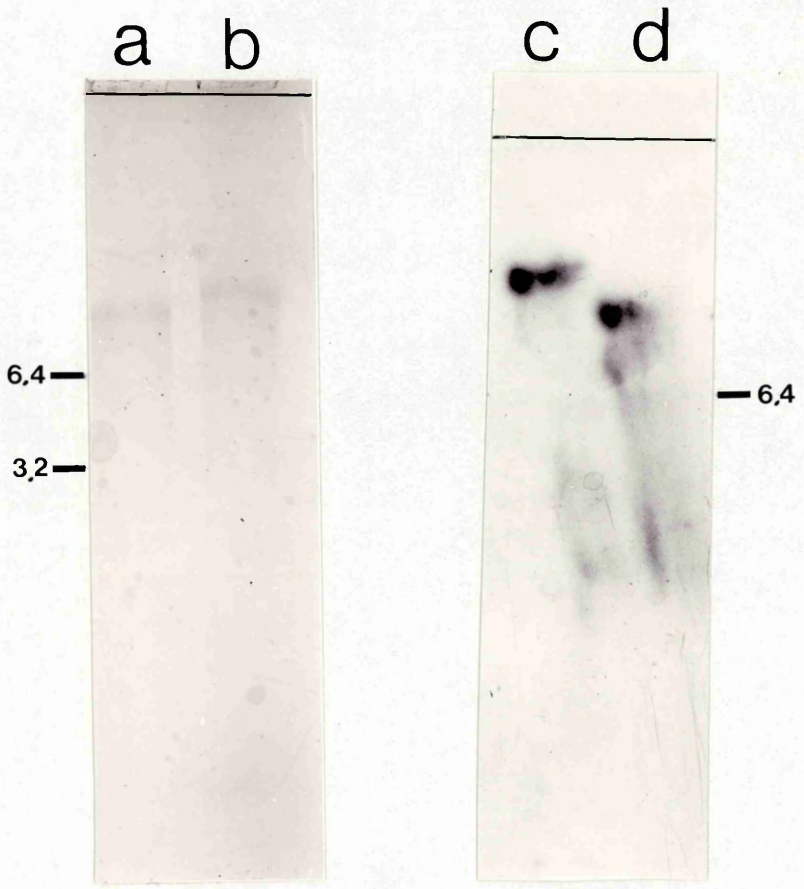


Figure 5-15:
Agarose gel electrophoresis of viral RNA. (Lane a) DI-1 RNA and (lane b) standard SYNV RNA, stained with toluidine blue. (Lane c) standard SYNV RNA and (lane d) DI-1 RNA, transferred to Biodyne and hybridized to a mixture of ^{32}P-labelled pSYN302, pSYN402 & pSYN503 DNAs. The bars indicate size standards (in Kb).

5.3 CONCLUSIONS:

Despite the fact that no virus particles were detectable in plants more than 60-75 d after inoculation, plants were still infectious. Thus, virus must be present at very low concentrations in chronically infected plants. This virus may be present in an altered form since yields of virus were greatly decreased and viral nucleocapsids were observed within the chloroplasts of plant tissue inoculated with chronically inoculated plants. Incorporation of ^{35}S-methionine *in vivo* into virus proteins, and translation *in vitro* of mRNAs from these plants indicates that viral mRNAs are present and that all four major virus structural proteins are synthesised. Western blotting of sap extracted from these plants indicates the presence of G & N proteins and an immunologically cross-reactive polypeptide, p41. However M1 & M2 proteins were undetectable. The failure of M1 & M2 proteins to accumulate, despite being synthesised, may be a result of rapid turnover. The origin of p41 is not clear. This protein is detectable in the preparation of purified virus when 20-30µg per track is subjected to SDS-PAGE. It may be a novel virus protein, a modified form of one of the four major virus proteins or possibly a host protein which accumulates specifically in chronically infected plants and which cross-reacts with anti-SYNV serum.

The results described in section 5.2.2 demonstrate that short virus particles are found within calyx about 150 d after inoculation and that they have all the characteristics of DI- particles. The DI-isolates of SYNV have apparently been generated naturally during long-term chronic infection of individual plants. This is supported by the ability of the isolate of SYNV-L85 (which had been transferred

mechanically to *N edwardsonii* four times only) to generate a similar short particles.

CHAPTER 6

DISCUSSION

During the phase of rapid virus synthesis, virions and unenveloped nucleocapsids are present in large numbers within tissue sections. The slight differences in size compared with those reported by Jackson & Christie (1977) presumably reflect differences in preparation techniques and between tissue sections and negatively stained purified virus. Bullet-shaped particles of SYNV have been reported by Christie *et al.* (1974) using potassium phosphotungstate stain containing BSA. Similar bullet shaped particles have been reported in chapter 3 but using phosphotungstic acid stain. In the case of another type II plant rhabdovirus RTYV, the bullet shaped particles dominated the preparations whereas the bacilliform particles were occasionally observed in both, host cells and purified preparations (Chen & Shikata, 1971). Similar results to those obtained for SYNV, have been published for BNYV, a type I plant rhabdovirus, using the same stains (Campbell & Lin, 1972). Bullet shaped particles of *Sonchus* virus (SV) have been reported using phosphotungstate as a negative stain, although the bacilliform particles were reported in thin sections from the same infected plant tissue (Vega *et al.*, 1976). These alterations in the morphology of the virus particles may be due to the manipulation in negative stain mounts for electron microscopy. Striking examples of particle variability due to preparative artifacts have been reported for LNYV (Wolanski & Francki, 1969). It seems likely that the integrity of the morphology of SYNV particles has been preserved in such a way that the real particle morphology would be seen when the grids-containing the purified virus preparation were decorated with anti-SYNV antiserum before being negatively stained with either uranyl acetate or phosphotungstate. Since virions seen in tissue sections are also

invariably bacilliform, this suggests that the bullet-shaped SYNV particles are an artifact of the negative-staining procedure.

Using ELISA and the immunoblotting assay, the movement of SYNV away from the initial site of inoculation into the other parts of the plant was detectable after as little as 24 h. Although the levels of antigen detectable within roots and uninoculated leaves were low, the absorbances in ELISA were consistently threefold above the background obtained with healthy sap. Conclusive evidence that virus movement was occurring was furnished by the developement of systemic infections in plants from which the inoculated leaves had been removed 24 h after inoculation but not in plants from which the removal of infected leaves had taken place 12 h after inoculation. Synthesis of viral proteins can be detected between 9 and 13 h after inoculation of cowpea protoplasts (Van Beek *et al.*, 1986). Mature virus is assembled between about 12 and 56 h after inoculation (Van Beek *et al.*, 1985a,b). This implies that virus starts to spread systemically, presumably via the vascular system after no more than one cycle of replication if multiplication occurs at similar rate in cells of infected *N. edwardsonii*. Indeed it is possible that virus from the inoculum is transported directly through the vascular system. Özel (1973) has reported spread of SYVV to the roots two days after inoculation by aphid vectors. However he used electron microscopy as a detection system and would not have seen low levels of virus occuring sooner after inoculation.

Rising levels of virus antigen within roots and unexpanded leaves between 1 and 4 d after inoculation suggest that virus multiplication is occuring within these tissues. Within expanded leaves, the lack of increase in levels over the same period may

indicate that antigen detected after 24 h comprises virus circulating within the vascular system and that this virus multiplies less efficiently within expanded leaves. No virus was detectable by ELISA in inoculated leaves for 4 d after inoculation when virus must *a priori* be present from the time of inoculation. It is possible that virus is restricted to a few initially infected cells and that tissue samples did not contain these particular cells.

During the period between 5 and 10 d after inoculation, the virus appears to multiply and spread rapidly, as indicated by levels of virus antigen, viral RNA and the proportion of infected cells observed by electron microscopy. Neither ELISA nor the dot-blot assays indicate how much of the protein or RNA is encapsidated. In immunogold labelling studies (discussed in detail below) a considerable quantity of label bound to extensive regions of the nucleus and cytoplasm rather than to virions or nucleocapsids. Thus at this stage of infection large amounts of free, or unassembled, virus protein(s) must be present. The probes used to detect RNA would be expected to detect both genomic (minus sense) and messenger (plus sense) RNA. It is of interest to note that the estimates suggest that 10 d after inoculation, unexpanded leaves contain 200 times more viral proteins than viral RNA by mass. The relative proportions found in the mature virion are approximately 140:1 (Jackson & Christie, 1977).

During the phase of rapid virus synthesis, nucleocapsids are consistently observed within electron dense nuclear viroplasms containing scattered patches of granular matrix. The viroplasm regions, containing the granular matrix, are indeed the sites of the assembly of the nucleocapsids as a great many gold particles reacted strongly to such regions. Comparison of the numbers of gold particles

binding to granular matrix and the areas reacting suggests that the amount of free virus protein within the viroplasms is greater 8 d after inoculation than 10 d after inoculation. This suggests that the *rate* of nucleocapsid assembly 8 d after inoculation is actually higher than 10 d after inoculation, although the total amount of accumulated protein is maximal 10 d after inoculation. The decline in numbers of nucleocapsids in infected cells from day 10 and on after inoculation, coincided with a reduction in the amount of the granular matrix in such cells, additional evidence that the granular matrix is the site of nucleocapsid assembly.

Immunogold label reacted with nuclear viroplasms in PYDV-infected *N. rustica* (Lin *et al.*, 1987), but the distribution of the gold particles in infected cells was different to that observed in SYNV-infected *N. edwardsonii*. In the PYDV-infected cells, scattered single gold particles reacted to a wide area of the viroplasm region, the most heavily labelled area having only about 11 to 18 gold particles. The differences between the results of Lin *et al.* and those presented in chapters 3 & 4 may reflect either the differences between the labelling technique used or most likely, the different ways in which nucleocapsids are assembled and perhaps the differences between the host plants. Most importantly, the results presented in this thesis demonstrate that in SYNV-infected *N. edwardsonii* ultrastructural changes in host cells and intracellular location of virion are dependent on time after inoculation. This is most likely to be true for other rhabdoviruses, a fact insufficiently appreciated in earlier studies.

Viroplasms have been observed in the cytoplasm of cells infected with BYSMV (Conti & Appiano, 1973) and NCMV (Toriyama, 1976).

Both these type I rhabdoviruses assemble in the cytoplasm. Van Beek *et al.* (1985b) report the presence of a dense-staining granular matrix within the nuclei of cowpea protoplasts infected with SYNV. This structure is presumably similar to the viroplasms which have beem observed in infected *N. edwardsonii*.

After 10 d nearly every cell examined contained virus. SYNV thus resembles the majority of other members of the rhabdovirus group in being able to infect a wide variety of cell types (Peters, 1981; Jackson *et al.*, 1987). Jones *et al.* (1974) have observed limited infection of xylem parenchema cells in raspberry infected with RVCV although in this case the virus was cofined to these cells. The maximum levels of virus (approximately 2.0-7.3 µg/g depending on tissue) can be compared to yields of some of the positive-stranded viruses, e.g 2mg/g for TMV and 0.7mg/g for potato virus X (Bawden, 1964). The low yields of SYNV and presumably some other rhabdoviruses must therefore reflect the numbers of virus particles per cell rather than a failure of the virus to spread to the majority of cells.

Following the peak of virus concentration 10 d after inoculation, the levels of virus antigen and viral RNA fell dramatically in most parts of the plants. This fall is accompanied by changes in the location of most virus particles, from nuclei to the cytoplasm, and a marked reduction in numbers of particles per cell. The considerable decrease in the amount of virus in the nuclear or perinuclear inclusions suggests an active mechanism whereby virus is either degraded or released/transported into the cytoplasm.

Assembly of the type II rhabdoviruses is believed to occur in the nucleus (Francki & Randle, 1980; Peters, 1981; Jackson *et al.*, 1987). Although these observations, as well as previous reports

(Jackson & Christie, 1977; Van Beek *et al.*, 1985b), demonstrate that this is the case for SYNV. In this study, virions are consistently observed scattered throughout the cytoplasm sometimes within small membrane-bound inclusions. Similar observations have been made for other type II plant rhabdoviruses including WSMV (Sinha, 1971) and clover enation mosaic virus (Vela & Rubio-Huertos, 1974). Van Beek *et al.* (1985b) detected nucleocapsids within the cytoplasm of SYNV-infected cowpea protoplasts; such species are also observed in this study. The numbers of both enveloped and unenveloped particles within the cytoplasm increased, between 10 and 20 d after inoculation presumably as a result of rupture or vesiculation of the outer nuclear envelope surrounding perinuclear inclusions of the virus (e.g. fig. 3-26).

Although, SYNV is reported to be one of the poorer yielding plant rhabdovirus among all of those studied so far (Jackson *et al.*, 1987), the yield on purification was high as measured by ELISA. About three quarters of the expected amount of the virus were recovered from infected leaf tissues harvested 10 d after inoculation using the standard procedure of Jackson & Christie (1977).

The association of the maturation of some plant rhabdoviruses with the endoplasmic reticulum including; LNYV (Wolanski & Chambers, 1971), BNYV (Garrett & O'Loughlin, 1977), and unidentified viruses associated with *Orchid* spp (Peters, 1977), *Iris germanica* (Rubio-Huertos, 1978a) and *Zea mays* (Rubio-Huertos, 1978b), led Francki & Randles (1980) to group all the plant rhabdoviruses behaving in a similar way into third subgroup. Van-Beek *et al.* (1985b) reported the association of SYNV particles with endoplasmic reticulum in SYNV-infected cowpea protoplasts. They proposed that as has been suggested

by Francki (1973), the enveloped particles had entered the lumen of the endoplasmic reticulum from the perinuclear space. However, the presence of viral nucleocapsids as well as enveloped SYNV particles within endoplasmic reticulum in SYNV-infected *N. edwardsonii* cells could suggest a similar story. It is possibly that, the nucleocapsids, after being assembled in the nuclear viroplasm, moved to the perinuclear space and then escaped into the lumen of the endoplasmic reticulum before being enveloped, utilizing the connection between the nuclear envelopes and the endoplasmic reticulum (Clowes & Juniper, 1968). Thus, the envelopes of those SYNV particles associated with endoplasmic reticulum, would be derived from the endoplasmic reticulum itself. The explanation given by Van-Beek *et al.* (1985b) that the nucleocapsids occur in the cytoplasm of infected cowpea protoplast as a result of fusion of the envelopes of mature virions within the endoplasmic reticulum, is equally likely to be the case.

Increased numbers of ribosomes in the cytoplasm of SYNV-infected *N. edwardsonii* clearly support the results observed in SYNV-infected cowpea protoplasts (Van-Beek *et al.*, 1985b) indicating a possible acceleration of translational activity.

The involvement of the nucleus in the assembly and maturation of the virus has been dicussed earlier in this chapter. In addition to the development of viroplasm, discussed above, other ultrastructural changes are appearent in the nuclei of infected cells from about 5 d after inoculation. These include swelling of the nuclei, cytoplasmic invaginations into the nuclei, nuclear invagination into the cytoplasm, alterations of the chromatin distribution and notably, the enlargement of the perinuclear space. Cytoplasmic and nuclear,

invaginations each as reported here in SYNV-infected *N. edwardsonii* have been reported only briefly in the past in rhabdovirus-infected plants, although it has been suggested that they may be occur generally in plant rhabdovirus infections (Martelli & Russo, 1977a). The mechanism(s) of development of the cytoplasmic and/or the nuclear invaginations is(are) not clear, but evidently these were formed as a result of the virus infection. The enlarged perinuclear space has been widely reported in plant hosts infected with type II plant rhabdoviruses, and is believed to take place as a result of the rapid multiplication and maturation of the virus (for review see Francki, 1973; Francki *et al.*, 1981; Jackson *et al.*, 1987).

Nuclei, from about 5 d after inoculation until the end of acute phase of infection, were notably swollen possibly because of the presence of large viroplasms. The altered distribution of the chromatin in such nuclei has presumably occurred as a consequence of, at least, two factors: (1) The formation of chromatin-free viroplasm regions and (2) the swelling of the nuclei as a result of the first factor. These resulted from SYNV infection as no similar observations have been seen in healthy controls. Similar alteration in chromatin distribution have been reported for other type II plant rhabdoviruses such as EMDV, SYVV and PYDV (Martelli & Russo, 1977a; Lee & Peters, 1972; Lin *et al.*, 1987), but these authors reported the disappearance of chromatin rather than altered distribution.

The swelling of the nucleolus in infected cells has also been reported with the other type II plant rhabdoviruses, EMDV (Martelli & Russo, 1977a) and SYVV (Lee & Peters, 1972). Nucleoli with several patches of unidentified matrix were observed in SYNV-infected *N. edwardsonii*, resembled those which can be observed in micrographs of

SYNV-infected *N. edwardsonii* cells published by Christie *et al.* (1974) and Jackson & Christie, (1979) although the authors have not mentioned this in their reports.

Hypertrophy and the segregation of the nucleolus, and the formation of fibrillar rings have been reported for BGMV-infected *Phaseolus vulgaris* (Kim *et al.*, 1978). Kim *et al.* (1978) implied that they comprised intermediate in the replication of the viral DNA. However, in SYNV-infected *N. edwardsonii* cells, fibrillar ring-like structures, similar in morphology and location to those reported by Kim *et al.* (1978), have been observed. It is not clear how these fibrillar ring-like structures were generated. However there is no possibility that the SYNV-infected plants were also infected with a geminivirus. Neither the patches in the nucleoli nor the fibrillar ring-like structures reacted to the anti-SYNV antiserum.

The segregation of the nuclear matrix into three distinguishable areas in some nuclei of cells of the vascular system has not been reported in any plant rhabdovirus-infected plant tissue. The significance of this observation is unclear.

The effects of SYNV infection on the plasma membranes including the cell walls of mechanically inoculated *N. edwardsonii* have been studied. The common observations were; the formation of plasmalemmasomes, plasmalemmasome-like structures and multivesiculated plasmalemmasomes, and the establishement of transport channels associated with SYNV particles. The observation of detached plasmalemmasomes, free in the cytoplasm and/or in the vacuole away from the cell walls, may be an effect of the plane of sectioning of the samples. Plasmalemmasomes and multivesiculated plasmalemmasomes do not appear to be directly involved in cell wall deposition (Flowek &

Setterfield, 1969) but may reflect an alteration in the physiological conditions of the tissue (Semancik & Vanderwoude, 1976). The frequency of occurrance of plasmalemmasomes and plasmalemmasome-like structures in CEV-infected *Gynura aurantiaca* is reported to be directly correlated with the initiation of symptoms, as well as the recovery of pathogenic RNA, suggesting that alterations in cell-surface properties may constitute a significant phase in viroid replication or pathogenesis (Semancik & Vanderwoude, 1976). Wahn *et al.* (1980) reported the observation of plasmalemmasomes and the cell wall protrusions in both, symptomatic leaves of *G. aurantiaca* infected with CEV and healthy control plants. They reported that the alterations of the internal structures of the tubular and vesiculated plasmalemmasomes, which were always combined with corrugation of the adjacent cell wall, were amongst the effects of CEV on infected *G. aurantiaca*. However, these kinds of alterations in plasma membranes and in the cell walls were neither seen in healthy controls of *N. edwardsonii* (chapter 4) nor in healthy controls of *N. glutinosa* compared to that infected by PVCV (Di Franco *et al.*, 1980).

The involvement of the altered plasma membranes in the process of replication and/or movement of SYNV in infected *N. edwardsonii* is not fully evident. Attempts were made to immunogold label the plasma membranes in SYNV-infected tissue, to determine the possible involvement of altered plasma membranes in the events of SYNV replication and/or movement. Unfortunately these attempts were not successful because the plasma membranes in both healthy and infected tissues were not visible because of the omission of OsO_4 during the sample fixation. OsO_4 must be omitted from the fixation of the samples for immunogold labelling since it prevents the binding of gold

particles to the viral proteins (chapter 2, section 2.9.1.2) (I.M. Roberts, personal communication). The observations of scattered gold particles along the cell wall (fig. 4-30a), in the area between the possible position of the plasma membrane and the cell wall, may suggest the possible direct involvement of the plasma membranes in the cell-to-cell movement of the virus.

The observation of tubular channels in SYNV-infected *N. edwardsonii* is of particular interest. These could be "transport channels" through which the viral nucleacapsids, or mature virions, move from cell to cell. Several observations support this hypothesis: (1) Tubular channels observed by transmision electron microscopy, in cross and longitudinal sections, were associated with nucleocapsids and/or virus particles. These channels were more obvious in the vascular system-cells where masses of virus particles and nucleocapsids were seen evidently travelling from cell to cell through wide channels. (2) The strong binding of clusters of gold particles to certain parts of the cell wall suggests the existence of localized region of viral protein(s). The circular areas of the cell wall which bound gold particles in cross-sectioned samples of infected-tissue, and the transver clusters in longitudinaly sectioned ones are strongly indicative of presence of underlying structures traversing the cell wall and containing virus protein(s). Such structures may be involve in the cell-to-cell movement of the virus.

The tubular channels observed in SYNV-infected *N. edwardsonii* nuclei, especially those channels containing nucleocapsids, are also of particular interest. The association of the nucleocapsids with these channels suggests that they may be directly involved in virus-movement in infected cells. This is supported by the observation of

similar tubular channels interconnecting two adjacent cells. No similar observation seems to be have been reported for any of the other type II plant rhabdoviruses. However, similar tubular channels were observed in the cytoplasm of cells infected with members of type I plant rhabdoviruses, having been observed in RVCV-infected raspberry (Jones *et al.*, 1974) and in *Denddrobium* virus-infected *Denddrobium phalaenopsis* (Lawson & Ali, 1975). The first authors believed that they were large virus-like particles whereas the second authors believed that they were rigid, sinuous tubular particles. Long particles have been reported associated with several plant type I & II rhabdoviruses. The explanations given for them were the envelopment of two nucleocapsids in an end to end configuration (for review see, Martelli & Russo, 1977a; Francki & Randles, 1980). However, this was certainly not the case for the tubular channels reported here, because the channels are as long as 10-15 nucleocapsids, the diameter of the channels were about three to fivefold of the diameter of the nucleocapsid and several nucleocapsids have been observed within these channels.

The observation of the tubular channels connecting the cell walls to the nuclei combined with the observation of the channels within the infected nuclei, also suggests the involvement of channels in the movement of the virus. The observation of apparently detached channels could be a result of the plane of sectioning.

It is unlikely, the tubular channels reported here are a modified form of plasmodesmata since no alteration of plasmodesmata have been detected or observed during the extensive immunological and electron microscopical studies of both healthy and infected tissues.

In the case of other plant rhabdoviruses, long virus particles (LV) of PVCV (Di Franco *et al.*, 1980), which appeared to originate from the nucleus, were observed associated and embedded in the cell wall protrusions of naturally infected *pittosporum* and in graft infected *pittosporum* and *N. glutinosa*. The authors believed that it was unlikely that cell wall protrusions containing LV represented transitional stages of a process by which virions are transferred from cell to cell. They, however favoured the idea that LVs are trapped within the outgrowths, the development of which were stimulated by infection. However a comparison of the results presented here to those results reported by Di Franco *et al.* (1980), taking into acount their figure, 2a,b,c and the diameters of the tubular membranes surrounding LV which varied, in their report, from 120 to 190 nm suggests that the protrusions reported by Di Franco *et al.* are another form of the transport channels. Cell wall projections and long core-like structures have also been observed in RVCV-infected raspberry (Jones *et al.*, 1974), however these projections did not contain virus particles.

Ultrastructural changes were apparent in chloroplasts in infected cells from 5 d after inoculation, the earliest stage after inoculation at which cells containing virions were detected using electron microscopy. These changes became more striking and widespread over the following 10 d, the period during which virus levels reach a maximum in all parts of the plant. Some of the changes, notably swelling of the chloroplasts, the formation of marginal vesicles and disruptions in the grana have been commonly observed in chloroplasts of plants infected with a number of unrelated viruses including TYMV (Hatta & Matthews, 1974), tomato spotted wilt

(Mohamed, 1973) and the viroid, potato spindle tuber (Hari, 1980; Kojima *et al.*, 1983). Changes such as the appearance of osmophilic granules, starch grains and vacuoles, are similar to those reported in *N. glutinosa* infected with the rhabdovirus LNYV (Wolancki, 1969).

From 20 d after inoculation and onwards, despite the apparent recovery of the plants and the absence of virions within the cells, abnormalities in the chloroplasts were greater than at earlier stages in infection. In addition to the formation of vesicles noted earlier, changes in the membranes were particularly apparent, with gross abnormalities in the grana.

Although there have been numerous reports detailing the effects of a variety of viruses on cellular ultrastructure, there is little convincing evidence as to the mechanisms by which such changes are induced. Reinaro & Beachy (1986) have recently reported the association of TMV coat protein principally with the thylakoids but also with the stroma of chloroplasts of infected tobacco. The quantity of coat protein within the chloroplasts was many times greater during infection with a severe strain of TMV than with an asymptomatic strain. There have also been reports of the accumulation of TMV pseudovirions within chloroplasts (Rochan & Siegel, 1984; Schalla *et al.*, 1975). In this study, a strong binding of anti-SYNV antibodies to the thylakoids and grana were observed. These antibodies were clustered in patches. Because these antibodies are prepared to purified virus, it is not clear whether all or only some of the virus proteins are present. However, significant quantities of free virus protein must be associated with these structures.

It is possible that the presence of free virus protein within chloroplast membranes might be directly responsible for the

ultrastructural abnormalities, perhaps by promoting membrane fusion or otherwise altering the biophysical properties of the membrane. However, this scenario is likely to be an oversimplification, since many abnormal structures e.g. chloroplast inclusion bodies, vacuoles and marginal vesicles fail to bind any antibodies. Nevertheless, these data demonstrate that the virus protein within the chloroplasts is apparently specifically targetted and these results, taken with those of Reinaro & Beachy (1986), support the hypothesis that symptom expression and ultrastructural changes may be linked to the accumulation of free virus protein within cellular structures.

Four types of alterations appeared in mitochondria of SYNV-infected *N. edwardsonii* cells: (1) Clumping of mitochondria, which usually had lost most of their cristae and some of their mitochondrial matrix. (2) The appearance of unusually shaped mitochondria, located very close to large microbodies; these mitochondria had also lost most of their mitochondrial matrix. (3) Enlargement of mitochondria containing either clusters of electron dense material, double-membraned vesicles or horseshoe-like structures. (4) Overdevelopment of mitochondrial membranes forming large areas of perimitochondrial space and vesicles.

Aggregated mitochondria, similar in morphology and location to these observed in SYNV-infected *N. edwardsonii* cells, have been reported in *Datura* cells infected with henbane mosaic virus (HMV) (Kitajima & Lovisolo, 1972), but not in *N. tabacum* cv. White Burley infected with HMV or in *N. tabacum* cv. Xanthi-nc infected with Atropa mild mosaic virus (Plumb & Vince, 1971; Harrison & Roberts, 1971). How the mitochondria get clumped, and why they lose some of their interior membrane organisation is not clear, but "the cell's

metabolism must be deranged by the synthesis of virus RNA, and by the synthesis of virus-coded protein(s)" (Harrison *et al.*, 1970).

Small vesicles bounded by a membrane, induced by cucumber green mottle mosaic virus (CGMMV) in cells of a range of host species, have been reported lying within the perimitochondrial space, and in the cristae (Hatta *et al.*, 1971; Hatta & Ushiyama, 1973; Sugimura & Ushiyama, 1975). In this study on SYNV-infected *N. edwardsonii*, similar double membrane bound-inclusion bodies were observed within the mitochondria. Inclusion bodies with double membranes have been observed lying within inside the diseased mitochondria, it is possible that they result from cytoplasmic invaginations. The inclusion bodies observed in CGMMV-infected cells could also be the result of cytoplasmic invaginations because they are mono-membrane bounded and they have been observed within the perimitochondrial space. Similar invaginations of the mitochondria have been no reported for other plant rhabdoviruses.

Enlarged and irregularly shaped mitochondria containing several clusters of a finaly granulated electron-dense material have been reported in SV-infected *N. glutinosa* (Vega *et al.*, 1976). Also, although not specifically mentioned by the authors, in electron micrographs of mitochondria from LNYV-infected *N. glutinosa* published by Chambers *et al.* (1965), two clusters of an electron-dense material can be seen. All these abnormalities resemble those observed in this study. Swollen mitochondria have been reported in plants infecting with BNYV, RVCV, EMDV (Campbell & Lin, 1972; Jones *et al.*, 1977; Martelli & Russo, 1973), similar to those observed in SYNV-infected *N. edwardsonii*. In SV-infected *N. glutinosa*, very distorted mitochondria containing single and double-membraned vesicles and also,

the total disruption of the mitochondria have been reported (Vega *et al.*, 1976). Similar alterations on the mitochondria of SYNV-infected *N. edwardsonii* have observed in this study.

In tobacco cells infected with tobacco rattle virus (TRV), mitochondria have been reported to be associated with the virus particles (Harrison & Roberts, 1968; Kitajima & Costa, 1969). Harrison and Roberts (1968) suggest that the mitochondria are the site of TRV assembly or that TRV particles become adsorbed to the mitochondria after assembly eleswhere. However, there are several lines of evidence which indicate that this is not the case for SYNV: (a) The site of the virus assembly is the perinuclear space. (b) No virus particles have ever been seen associated with the mitochondria during the course of this study. (c) No free viral protein associated with the inside or outside of the mitochondria was ever detected when the latter were immunogold labelled with anti-SYNV antibodies. These observations exclude any direct role by the mitochondria in SYNV replication or assembly. Although, Vega *et al.* (1976) suggest that the structural alterations of mitochondria in SV-infected *N. glutinosa* may be implicated in the replication process of the SV particles, there is no evidence that this is the case for SYNV.

The stage of apparent recovery of infected plants is characterized by reductions in the levels of the virus proteins and viral RNA, reductions on the numbers of infected cells and the numbers of particles per cell, migration of the virus particles from the nuclei into the cytoplasm and continued cytopathological changes in cells of plant. Infected plants, following this stage, enter the stage of chronic infection.

This is the first report to examine specifically plants chronically infected with a plant rhabdovirus. In the nuclei of chronically infected plants (in which no virus or nucleocapsids were observed) the distribution of the chromatin was similar to that observed in the healthy control but the cytoplasmic invaginations into the nuclei were actually more pronounced than earlier in infection. Thus, despite the apparent disappearance of virions, pathological changes were still apparent reinforcing the idea that chronically infected plants, despite their normal appearance, are still affected by the presence of the virus. The apparently normal distribution of chromatin perhaps reflects the disappearance of the viroplasms.

Chloroplasts in chronically infected plants also exhibite structural abnormalities. There does not seem to be any direct connection between the numbers of virions present within infected cells and the severity of the cytopathological changes in the chloroplasts and nuclei. Thus, the connection between the ultrastructural abnormalities and symptom expression must be complex, since chronically infected plants appeared outwardly indistinguishable from healthy controls.

The observations that nucleocapsids accumulate in the chloroplasts when inocula are taken from chronically infected plants are surprising. That the bacilliform particles are indeed authentic nucleocapsids is supported by several lines of evidence: Their dimensions (185-200 nm x 48-52 nm) are identical to those of authentic nuclocapsids observed within nuclei; no envelope can be observed and only protein N and possibly L, which are nucleocapsid-associated (Jackson, 1978), are detectable in significant quantities in western blots of proteins from chloroplasts isolated from infected

plants. There is no evidence to suggest that virus replication is occurring within chloroplasts. Enveloped SYNV virions and membrane bound clusters of virions are capable of fusing with membranes such as endoplasmic reticulum and do so actively during the later stages of infection. It is possible that the nucleocapsids may enter the chloroplasts following a similar fusion of the cluster membrane and/or virion envelope with the chloroplast envelopes. Although calyx from chronically infected plants can be a source of DI particles, the inocula from chronically infected plants do not apparently contain significant quantities of such DI particles since their virions are full length. They do however give consistently reduced virus yields; whether these are due to low levels of DI particles or to other subtle mutations is not clear. There is no explantion as to why nucleocapsids are found in chloroplasts only when such inocula are used.

SYNV particles contain one minor and four major structural proteins (Jackson, 1978). The four major proteins all reacted to the anti-SYNV antiserum used in this study when purified SYNV preparations were immunoblotted. Immunoblots of total proteins from infected plants at the acute phase of infection demonstrated the presence within plants of all four major structural proteins. In contrast, in chronically infected plants only two of these proteins, G & N were detectable. The possibility that low concentration of M1 & M2 are present cannot be excluded. However, even if this is the case, the levels of these proteins, relative to G & N, is much reduced when compared to virus particles or acutely infected plants. Interestingly, in leaf discs from chronically infected plants incorporation of ^{35}S-methionine into M1 & M2 as well as G & N is

detectable and the relative levels of these proteins appears similar to the relative levels in virions or acutely infected plants. Moreover, mRNAs extracted from chronically infected plants direct synthesis of M1 & M2 proteins. Thus, the apparent absence of M1 & M2 in immunoblots cannot be a result of either the absence of their mRNAs or by the failure of their mRNAs to be translated. Since these proteins are synthesised at apparently "normal" rates relative to G & N but fail to accumulate they must be turned over or degraded much more rapidly than G & N proteins. There are no previous reports of any similar phenomenon occurring with any other plant virus.

The origin of the p41 polypeptide is not clear. This polypeptide accumulates to high levels in chronically infected plants. However, attempts to demonstrate synthesis *in vivo* in leaf discs or in *vitro* using mRNAs from chronically infected plants were not entirely conclusive. Small amounts of an appropriately sized immunologically cross reactive polypeptide were detected but levels were low compared to the levels of the major virus structural proteins.

Recent analysis of the sequence of SYNV RNA showed that SYNV has six genes (Heaton *et al.*, 1987), the gene order being;

'3-leader RNA - N - M2 - p4 - p6 - G - L -'5

The p6 & p4 genes could code for polypeptides of 39-42 KD (A.O. Jackson & L. Heaton, personal communication) one of which is certainly M1 protein and the other is an unidentified viral structural protein, which could be p41. In this study, a polypeptide of 41 KD has been detected at low levels when 20-30µg/track of purified virus protein was immunoblotted, suggesting that p41 could be the extra

viral structural protein. Two other hypotheses on the origin of polypeptide p41 are also possible: (1) p41 could be a modified form of one of of the other virus proteins. (2) p41 could be a host protein which accumulates specifically in chronically infected plants and which cross reacts with the anti-SYNV antiserum.

Differential accumulation of virus structural protein in animal and man have been documented (Yougner & Preble, 1980) associated with the presence of defective forms of viruses in certain slow virus diseases. Although SYNV is capable of generating DI particles (see below) this does not seem to be the cause of the apparent absence of M1 & M2 and such plants, despite the apparent absence of virions in electron microscopy, must contain predominantly normal virus. The evidence for this is; (1) Plants inoculated with extracts from these plants gave typical SYNV-symptoms and contained typical SYNV particles when examined by electron microscopy; (2) polysomes from chronically infected plants contain mRNAs for all four major virus proteins; (3) The observation of DI-particles in the calyx tissue 120-150 d after inoculation, is in fact strong evidence for the existence of standard SYNV particles in these plants since DI-particles can not replicate in the absence of the standard virus. Although inocula from chronically infected plants appear normal there is some evidence that the virus may be subtly altered, induces (1) the ability of the nucleocapsids to gain entry into the chloroplasts of inoculated plants only when such inocula were used and (2) the amount of virus which could be purified from such plants was much lower than from plants infected with standard inocula.

Short bacilliform particles were found in the calyx of *N. edwardsonii* chronically infected with SYNV. According to Huang

(1973), DI-particles are identified by the following criteria: (1) They are deletion mutants of the parent virus; (2) they differ from the parent virus according to length and sedimentation coefficient; (3) they interfere with the replication and formation of the parent virus; (4) they contain the same structural proteins as the parent virus and (5) they cannot multiply without the parent virus. Proteins extracted from these short bacilliform particles appeared to be electrophoretically and antigenically identical to those of the standard virus. The short particles were heterogeneous in length, ranging from 130 nm to near full size (210-220 nm) although species of approximately 160 nm were the most abundant. They contained a genomic RNA of about 10.5 Kb, which shared at least some sequence homology with the standard RNA. Since the RNA hybridized to a probe containing sequences derived from genes located within 5-6 Kb of the 3' terminus of the standard genome (Heaton *et al.*, 1987), at least some of these sequences must still be present on the DI genome. Unexpectedly, the RNA appeared to migrate as a sharp band, although some degree of heterogeneity might not have been apparent because of the limited resolving power of the gel for such comparatively large RNAs. Possibly, passage of DI-1, subsequent to measurement of particle lengths but prior to virus preparation, might have resulted in enrichment for one component. Nevertheless, the relative size of the RNA of short particles compared to standard SYNV RNA (77%) was in close agreement with the relative length of the most abundant species of short particle to that of standard SYNV (73%).

Purified short particles showed little infectivity when inoculated to plants, suggesting that they are replication-defective. Inoculation with a crude extract of calyx from chronically infected

plants and containing both short and full length particles, causes a systemic infection but with different symptoms to those of standard infections. When plants were inoculated with an artificially reconstituted mixture of short and standard particles, fewer plants became systemically infected than with similar concentration of standard virus alone and the symptoms resembled those induced by naturally generated mixture of particles form calyx. Short particles predominated in sections from plants infected with mixed inocula. Thus, the short particles fulfil the criteria of Huang (1973) for authentic DI-particles.

There has been only one other report of potentially authentic DI-particles of a plant virus (Adam *et al.*, 1983). These authors did not measure the size of the particles of their defective isolate of PYDV, nor did they characterise its RNA. However it is likely that the isolate does represent an authentic DI-isolate. The defective isolate of PYDV was derived by multiple passage; in contrast, the DI-isolate of SYNV has apparently been generated naturally during chronic infection of individual plants.

The standard isolate of SYNV-type has been regularly passaged mechanically and may well have undergone some change. Indeed it is interesting to note that compared to the original report of the isolation of SYNV (Christie *et al.*, 1974), the current inoculum seems to induce symptom expression earlier in *N. edwardsonii* but plants subsequently appear to undergo a more completely recovery (chapters 3 & 5). However, the ability to generate DI-particles does not appear to be a consequence solely of repeated mechanical transfer since an independent isolate, SYNV-L85, which had been transferred four times only, also generates short particles under similar conditions.

Although short particles within plants infected with the standard strain were not detectable, the possibility that they are present at very low levels cannot be excluded. It may be significant that two independent experiments yielded similar although not necessarily identical sizes of DI-particles. Nevertheless, *de novo* generated DI-particles were detected in all plants examined, but only in calyx and only following long periods of infection. The standard isolate of SYNV still gives rise to "standard" infections 6-10 days after inoculation indicating that long-term infection is a prerequisite for the generation, or at least enrichment, of DI-particles. Thus, the mechanism of generation of DI-isolates must involve a potentially complex interaction between the virus and the host plant.

The connection between the recovery stage and the generation of DI-particles is not clear, but p41 could be a functionally involved in the generation of DI-particles and therefore it is detectable only in chronically infected tissue before or by the time of the appearence of DI-particles.

The generation of DI-particles may allow animal viruses to potentiate their cytolytic properties and establish persistent infection (Holland *et al.*, 1980). Although plant viruses are not cytolytic and routinely establish permanent infection of their hosts, partial recovery, as evidenced by sharp reductions in infectivity following the initial infection, occurs in LNYV (Crowley *et al.*, 1965), BNYV (Lin & Campbell, 1972), SYNV (Jackson & Christie, 1977; chapter 3) and may be common to, although not exclusive to, the plant rhabdoviruses (Francki & Randles, 1980) This recovery phenomenon may in some way parallel persistence by certain animal viruses with the concommittant generation of defective forms of the virus (Yougner &

Preble, 1980) . It is interesting to speculate whether the generation of DI-particles is common to plant virus infections in which the host undergoes a recovery.

On the basis of the observations presented in this thesis, a model for the spread of SYNV through mechanically inoculated tobacco can be proposed. Virus deposited at the infectible site(s) undergoes one or more cycles of replication. Nucleocapsids from these initially infected cells are assembled in the nuclear viroplasm, bud into the perinuclear space gaining an envelope and are then transported from cell to cell presumably through transport channels, and throughout the plant possibly via the vascular system. At this stage, nucleocapsids and/or enveloped virus particles can be found diffused within the ER and the ultrastructural anatomy of the cells is highly altered by virus infection. Virus multiplies in all or nearly all tissues until all susceptible cells have been infected. This acute phase of infection takes 10 d. The levels of both, viral RNA and virus protein then decline rapidly and the plants undergo a recovery phase which involves loss of virus from the perinuclear space of infected cells, marked reductions in infected cells and the migration of the virus from the nucleus and the perinuclear space into the cytoplasm. The extent of this loss must involve active degradation of virus and/or vesiculation or release of virus into the cytoplasm. Ultimately, the plants enter a phase of chronic infection in which number of virus particles within most infected cells has decreased to a level at which virions are no longer detectable by electron microscopy. In such plants, tissue extracts remain infectious, indicating that at least some virus is present. Synthesis of all four major virus structural proteins continues in such plants,

but M1 & M2 are turned over rapidly. Proteins G & N and a novel polypeptide p41 accumulated free throughout the nucleus, cytoplasm and chloroplasts. The ultrastructural anatomy of the cells remains altered, influenced by the virus infection. At some point during chronic infection DI-particles start to accumulate in calyx tissue.

AIMS FOR FUTURE:

During this research on SYNV-infected *N. edwardsonii* plants, several questions have arisen.

1- Where are each of the viral proteins synthesised? At which time after inoculation might synthesis take place?.

2- Why does virus migrate from the nuclei into the cytoplasm 20-30 d after inoculation, the time at which a marked reductions in the protein and RNA levels are apparent?.

3- What is the real origin of polypeptide p41?

4- Which of the viral protein(s) react to the cell wall? How are the transport channels established?.

5- Do the nucleocapsids observed within the chloroplasts gain entry there from the cytoplasm or are they synthesised there?.

6- Why do the M1 & M2 proteins fail to accumulate in the chronically infected plants?.

7- Why are DI-particles apparently confined to the calyx tissue of the chronically infected plants?.

8- Is there any connection between the failure of accumulation of M1 & M2 proteins and the presence of the polypeptide p41 in the chronically infected plants? Is there any connection between the presence of p41 protein and the generation of DI-particles in such plants?.

9- What types of deletions mutations are involved in the generation of SYNV DI-particles? Are all the deletions of one type or are different types of DI-particles present?.

10- How do the SYNV DI genomes compare with the genomes of animal rhabdoviruses DI-particles e.g. VSV? Do common mechanisms appear to be involved and if so what might they be?.

11- How do the different DI sequences compare? Are different clonal lines originally isolated from the same plant related to each other? Have DIs of different lengths arisen from single independent deletions or from a sequential series of several deletions? How do DIs generated from the two different isolates of SYNV compare?.

The above questions can be answered by using the following approaches :

(1) Antibodies should be prepared against individual virus protein. Immunogold labelling of infected tissue should be carried out at various times after inoculation using these antibodies; in this case, double-immunogold labelling of the same section could be used. The

immunoblotting experiments could be repeated using these antibodies.

(2) Immunoblotting, in combination with peptide mapping technique using e.g. V8 protease, could identify whether p41 protein is a modified form of one of the four major structural proteins or not.

(3) Purification of DI-particles through local lesions, although an old technique, would be a practical method for isolating clonal lines of DI-particles.

(4) Sequence analysis of the genomic RNA of the above purified DI-particles could answer many of the questions about their generation in plants.

PUBLICATIONS:

Some of the results presented in this thesis have been published:

ISMAIL, I.D., HAMILTON, I.D., ROBERTSON, E. & MILNER, J.J. (1987). Movement and intracellular location of Sonchus yellow net virus within infected *Nicotiana edwardsonii*. *Journal of General Virology*, *68*, 2429-2438.

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