Short title

Genomic masking of tobacco mosaic virus RNA

MASKING OF TOBACCO MOSAIC VIRUS RNA GENOME IN THE COAT PROTEIN OF BARLEY STRIPE MOSAIC VIRUS.

by

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ABSTRACT

Barley stripe mosaic virus (BSMV), tobacco mosaic virus (TMV) and genomically masked virions consisting of TMV-RNA in BSMV protein were detected in doubly infected barley plants grown at 30°. Methods which completely separated BSMV and TMV from artificial mixtures failed to remove all TMV infectivity, or TMV-RNA, from BSMV derived from natural mixtures. Such BSMV contained no TMV protein, and the TMV infectivity associated with it was neutralized by BSMV-antiserum but not by TMV-antiserum. As much as 8% of the RNA within BSMV-like particles from doubly infected Black Hulless barley leaves was TMV-RNA. Genomic masking was detected only in leaves which contained more TMV than BSMV. Most cells of these leaves were doubly infected. No genomic masking of BSMV-RNA in TMV protein was detected in these leaves. BSMV, but not TMV, was transmitted through seed collected from doubly infected plants.

RESUME

Une infection mixte du virus de la mosaique du tabac (VMT) et du virus de la striure d'orge (VSO) fut établie chez des plantes d'orge cultivées à 30°. On découvrit chez ces plantes des virions du VMT et du VSO, ainsi que des virions se composant de l'ARN du VMT et de la capside du VSO. Des méthodes qui séparent totalement ces deux virus mélangés in vitro ne réussissent pas à enlever tout l'ARN de VMT du VSO provenu de mélanges produits in vivo. De telles populations du VSO ne contiennent aucune protéine du VMT et leur capacité de provoquer une infection typique du VMT est éliminée uniquement par l'antisérum du VSO, et non pas par celui du VMT. Jusqu' à 8% de l'ARN des virions, en apparence du VSO, extraits de feuilles d'orge Black Hulless infectées par les deux virus est de l'ARN du VMT. Le masquage du génôme viral du VMT par la capside du VSO se découvre uniquement chez les feuilles qui contiennent plus de VMT que de VSO. Chez les mêmes feuilles, l'ARN du VSO ne se trouve jamais masqué par la capside du VMT. Le VSO est transmissible par la graine des plantes infectées par ces deux virus. Ce n'en est pas le cas pour le VMT.

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INTRODUCTION

A common aim when investigating plant diseases is to eliminate as many variable parameters as possible, including pathogens other than the one under investigation. While this is necessary for many kinds of problems, diseases in the field are not restricted to single pathogens, particularly on vegetatively propagated or perennial crops. Although the study of interactions between more than one pathogen has not been neglected by plant pathologists, it is certainly not a common problem for investigation. This is more the pity, since unexpected results often emerge from such studies.

The double infection investigated in the present study is a case in point. Tobacco mosaic virus (TMV) replicates poorly in inoculated leaves of barley plants, and can be detected rarely and with difficulty in systemically infected leaves. Infected plants look normal. When, however, barley plants are inoculated with both barley stripe mosaic virus (BSMV) and TMV, massive quantities of TMV are produced in the systemically infected leaves. The additional synthesis of TMV in doubly infected plants does not alter the symptomatology associated with the BSMV infection. The effect of BSMV on the repliction of TMV in barley was the subject of an earlier thesis (Dodds, 1972).

The present thesis concerns another aspect of this unique double infection, that of the encapsulation of some TMV-RNA by BSMV protein (i.e. genomic masking). Section I is an investigation of barley leaves doubly infected with BSMV and TMV at the ultrastructural level, to

determine the frequency and nature of double infection of single cells. If cells are not doubly infected, the chances of structural interaction between BSMV and TMV are minimal. Section II is an analysis of the distribution and amount of genomic masking in doubly infected plants. A more general problem is the development of methodologies which conclusively prove the phenomenon. Section III is an attempt to investigate the possible significance of genomic masking to the transmission of TMV through seed from doubly infected plants.

LITERATURE REVIEW

1. THE VIRUSES

The two viruses used in this double infection study were barley stripe mosaic virus (BSMV) and tobacco mosaic virus (TMV). Since most properties of these two viruses are different, there is no reason to suppose they are biologically related, although there is a structural similarity between them, both having rigid, tubular rod-shaped particles.

A. Physical Properties

The physical properties of BSMV and TMV are given in table 1 and their appearance is illustrated diagramatically in figure 1. In common with most plant viruses, both BSMV (molecular weight 26×10^6 daltons) and TMV (molecular weight 39×10^6 daltons) are nucleoproteins, each composed of ribonucleic acid (RNA) and protein.

The morphology of the particles of both viruses is that of a short rigid rod, but BSMV has shorter and wider particles than TMV. The dimensions of TMV rods are 300 nm by 18 nm; for BSMV the rod length is variable with three modal lengths of 112 nm, 130 nm and 150 nm (Harrison et al, 1965) and a diameter of 20 nm. The rod like structure is a consequence of the helical aggregation of a protein subunit with the RNA embedded helically in the protein rod, at a diameter of 8 nm for TMV and 11 nm for BSMV, as illustrated

Table 1. Physical and chemical properties of BSMV[1] and TMV[2]

	BSMV	TMV
Nucleoprotein		
Molecular weight	26×10^6 d	39×10^6 d
Morphology	tubular rigid	rod
Length	130 nm (112 nm) (150 nm)	300 nm
Width	20 nm	18 nm
A-260/A-280	1.05	1.19
$E_{260}^{0.1\%}$	2.6	3.2
Sedimentation coefficient	185 S	187 S
Isoelectric point	pH 4.5	pH 3.4
Buoyant density	1.309 g/cm^3	1.324 g/cm^3
Protein		
Molecular weight	21.5×10^3 d	17.5×10^3 d
Amino acids	187-190	158
RNA		
Percentage	3-4%	5%
Molecular weight	1.4×10^6 1.17×10^6 1.04×10^6	2.05×10^6
Base ratio		
Guanidine	20	24
Adenine	31	28
Cytosine	19	20
Uracil	30	28

1. Data taken from Atabekov, 1966, 1971, Finch, 1965, Jackson and Brakke, 1973.
2. Data taken from Matthews, 1970.

Table 2. Structural orientation of RNA and protein in BSMV and TMV virions.

		BSMV	TMV
Structural parameters			
A. Length		130 nm	300 nm
B. M.Wt., particle		26×10^6 d	39×10^6 d
C. M.Wt., RNA		1.1×10^6 d	2.05×10^6 d
D. M.Wt., protein subunit		21.5×10^3 d	17.5×10^3 d
E. Pitch		2.5 nm	2.3 nm
F. Average M.Wt. of a nucleotide		322 d	322 d
Calculations			
G. Protein, total M.Wt.	(B-C)	24.9×10^6 d	36.95×10^6 d
H. Number of subunits	(G/D)	1,163	2,111
I. Number of turns in helix	(A/E)	52	131
J. Number of subunits/turn of helix	(H/I)	22.3	16.2
K. Number of nucleotides	(C/F)	3,416	6,366
L. Number of nucleotides/subunit	(K/H)	2.94	3.01

in figure 1. From the values given in table 2 it can be calculated that for both BSMV and TMV there are three nucleotides associated with each subunit, and that, whereas there are approximately 16 subunits in each turn of the structural helix of TMV, for BSMV there are approximately 22 subunits per turn.

The rod shaped particle of either virus is in fact tubular, as illustrated in figure 1. This is demonstrated by the ability of negative stain to penetrate the hollow central canal. For both virus particles the diameter of this canal is about 4 nm.

In keeping with other rod shaped viruses, the ratio of absorbance of u.v. light at 260 nm to that at 280 nm is low; 1.05 for BSMV and 1.19 for TMV. This indicates a low percentage of RNA in the viruses, in fact 3-4% for BSMV and 5% for TMV. When the sedimenting properties of the two particles are compared by analytical ultracentrifugation or by density gradient centrifugation an unfortunate coincidence is observed since both particles co-sediment, the sedimentation coefficients at infinite dilution being 185 S and 187 S respectively for BSMV and TMV. This precludes rate zonal density gradient centrifugation as a direct tool for separating the viruses, a point which will be referred to later. However, there is a difference in buoyant

Figure 1. Structure of BSMV and TMV

1. BSMV
2. TMV

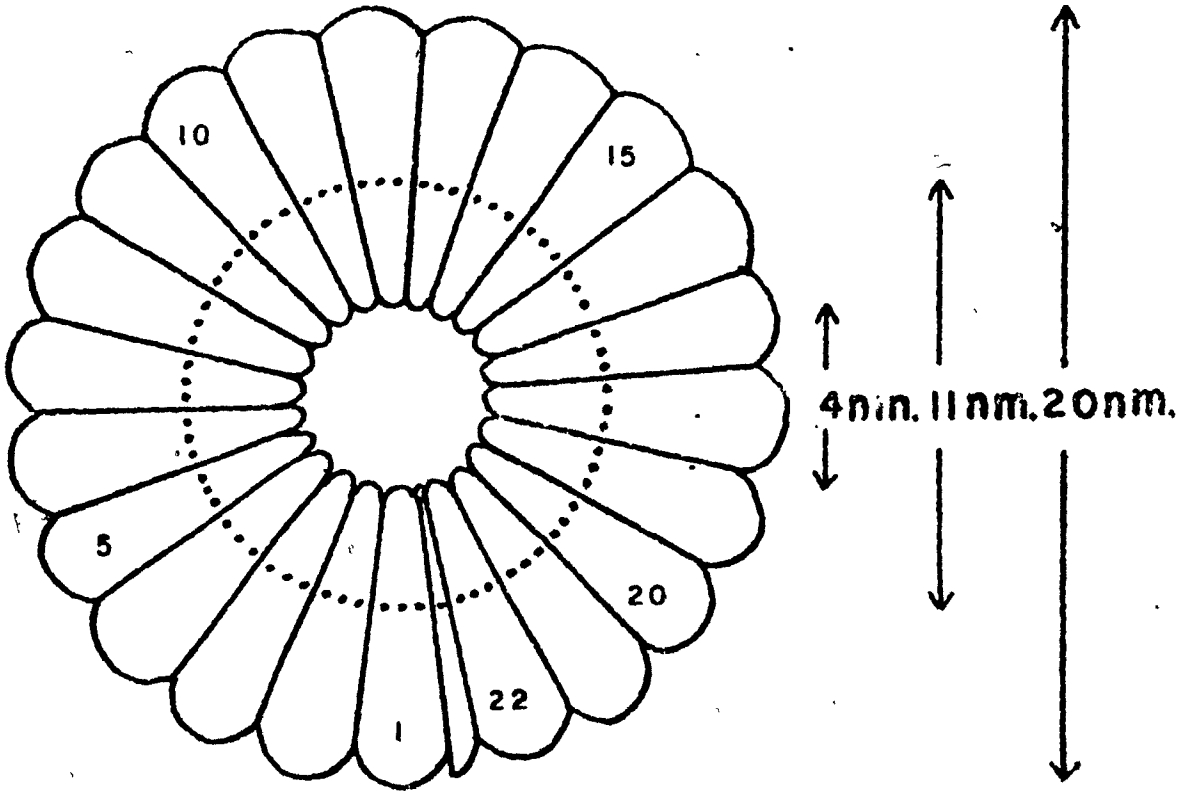
A. Cross section of the tubular rod showing:

   1. the diameter of the central canal, the diameter of the RNA helix, and the outer diameter.
   2. the number of subunits per turn of the protein helix.
   3. the number of nucleotides per subunit (black dots).

B. Face view of part of the tubular rod showing the helical arrangement of the protein subunits. Five (BSMV) and three (TMV) complete turns of the helix separate superimposed subunits.

L BSMV

A.



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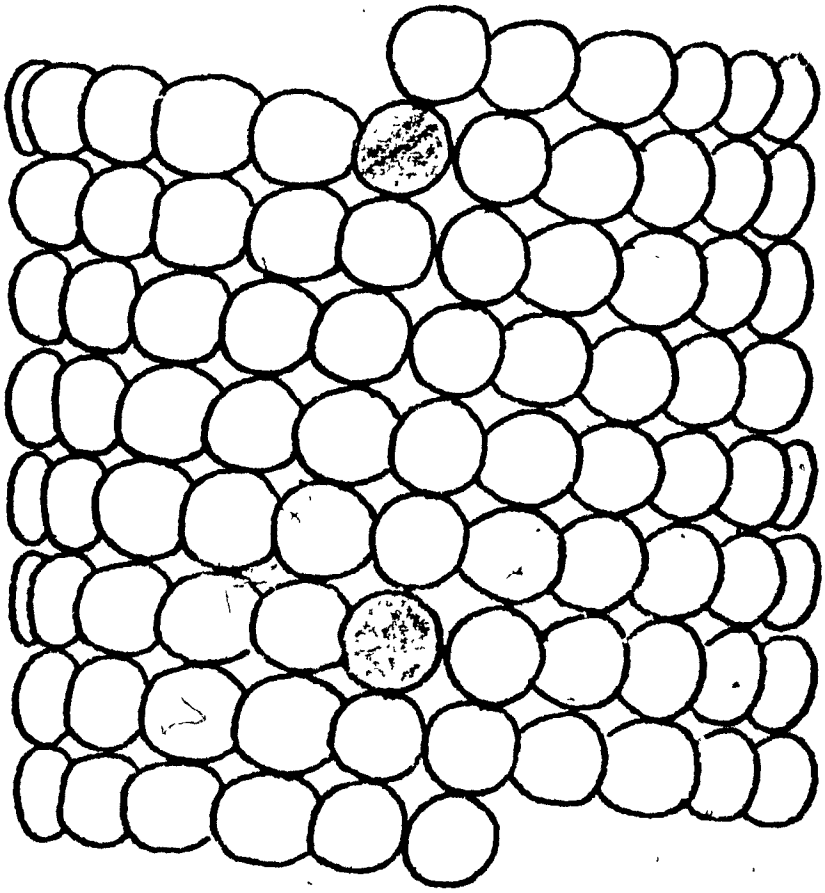
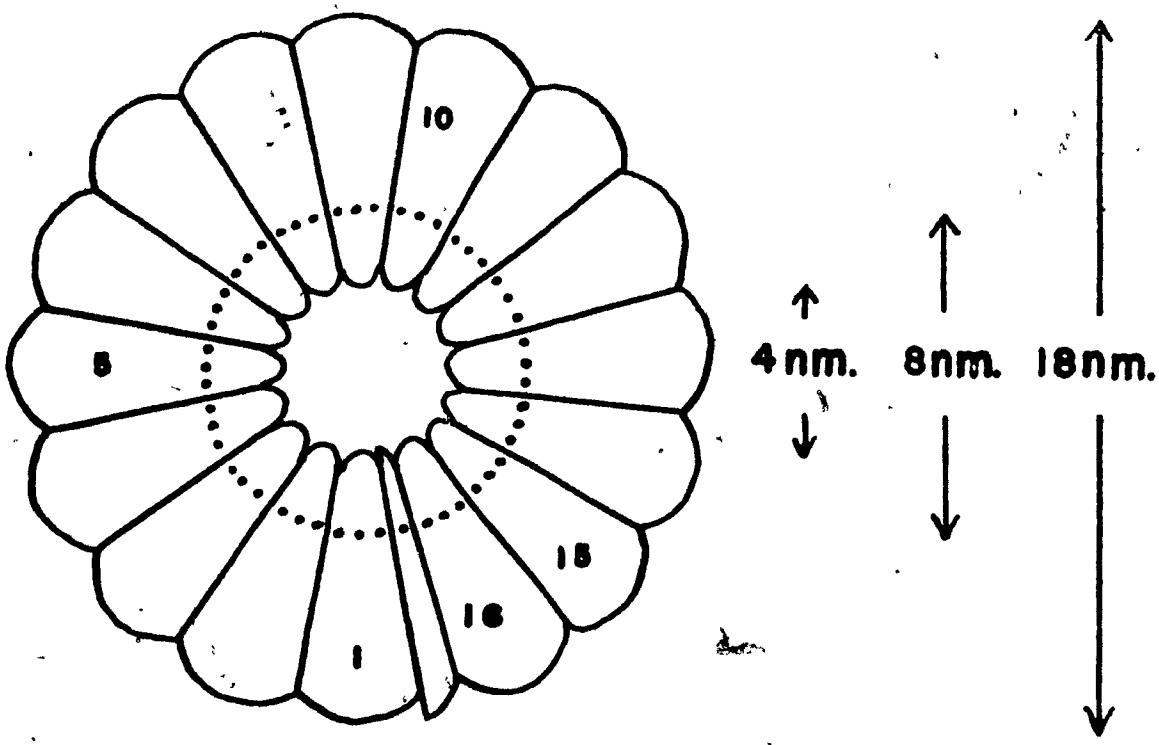
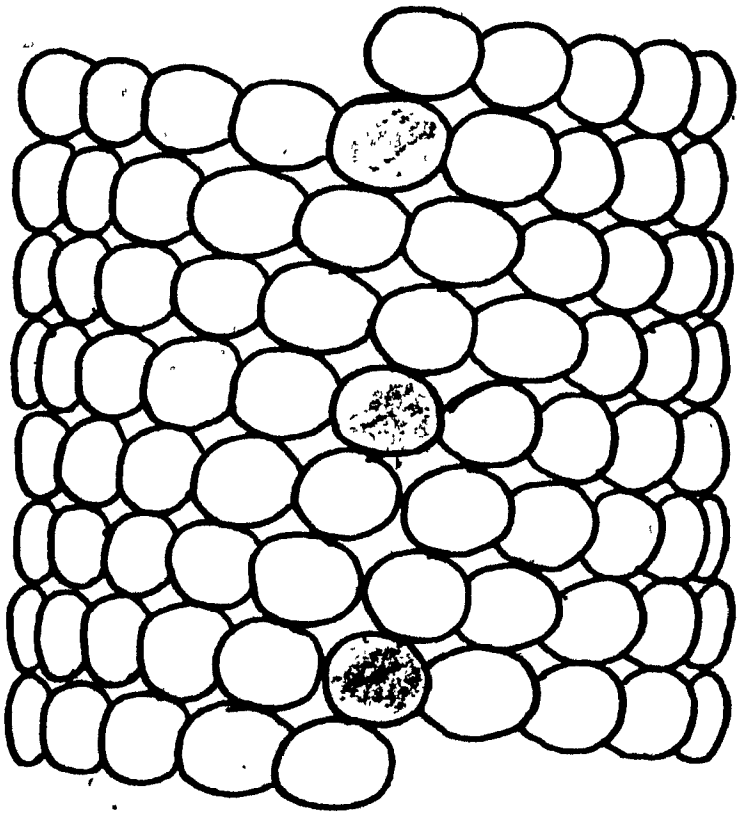


2. TMV

A.



B.



density between these two viruses (1.309 g/ml for BSMV, 1.324 g/ml for TMV), which means that they can be separated by equilibrium density gradient centrifugation. They also have different electrophoretic mobilities, BSMV being more electro-negative than TMV at neutral pH, so they can be separated by electrophoresis.

Both viruses have only one chemical protein subunit, the BSMV molecule (21,500 daltons) being heavier than the TMV molecule (17,500 daltons). The larger size of the BSMV subunit is reflected in the higher number of amino acid residues (187-190) from which it is composed, compared to the TMV protein (158 amino acid residues). The biological unrelatedness of BSMV and TMV is enforced by a lack of serological relatedness between them.

Each TMV rod contains a single molecule of RNA of molecular weight 2.05×10^6 daltons. BSMV, on the other hand, has populations of virus particles containing different sizes of RNA, with molecular weights of approximately 1.0, 1.2 and 1.4×10^6 daltons. The base ratios of TMV-RNA and BSMV-RNA are different.

B. Biological properties

The natural hosts of BSMV are barley and wheat, but the virus infects many other Graminae experimentally. Outside of the Graminae the only experimental hosts are species of Chenopodium, Beta and Spinacea.

Symptoms are dependent on the barley variety and BSMV isolate, but in the combination used in this study two stages, acute and chronic, were recognised (McKinney and Greeley, 1955). The acute symptoms were observed on the leaves that were maturing at the time they were infected and on the inoculated mature leaves below them. Complete chlorosis and subsequent necrosis were typical acute symptoms. Chronic symptoms developed on leaves that were leaf initials or meristems when infected. These developed alternate dark green, light green and white stripes along their length. Infected barley plants were also badly stunted. Yields of BSMV from infected barley are quite high, up to milligram quantities per gram of tissue.

An important property of BSMV is its ability to be seed transmitted, and also pollen born (Bennett, 1969). Rates of transmission as high as 90% are not uncommon, and the mechanism involves direct infection of the embryo (Carroll, 1972). No natural vector is known for the virus (Chiko, 1973).

Many strains of TMV are recognised on the basis of the divergent hosts from which viruses with the general properties of TMV have been isolated. Apart from solanaceous hosts like tobacco, tomato, pepper and potato, TMV-like viruses have been isolated from cucurbits, legumes, grapes, ash, apple and pear trees, orchids, cacti and plantain, but not from cereals. The experimental host

range of TMV is enormous, taking in at least 150 genera, and includes cereals (Hamilton and Dodds, 1970).

The most common symptom in tobacco is stunting and the appearance of mottled dark green and light green areas on leaves which became systemically infected. The dark green areas often have a blistered appearance. Yields of TMV from tobacco are very high, several milligrams per gram of leaf tissue, but there are also hosts in which subliminal infections occur (Cheo and Gerrard, 1971). Barley is one of these (Dodds, 1972).

Unlike BSMV, TMV is not, as a rule, seed transmitted, although low rates of transmission have been detected from apple, pear, grape and tomato seed (Bennett, 1969). It is uncertain whether embryo infection takes place in any of these examples. It is not clear what the natural vectors of TMV might be, if any, but inefficient transmission has been achieved with grasshoppers and aphids. In both cases the mechanism is probably quite mechanical (Matthews, 1970).

2. DOUBLE INFECTIONS WITH PLANT VIRUSES

The usual approaches to double infections of viruses in plant hosts have been to study the effects of double infections on the host compared to either single infection, and the effects of either virus on the concentration or distribution of the other. Results of these kinds of experiments for the double infection of BSMV and TMV in barley were presented in an earlier thesis (Dodds, 1972), where

the pertinent literature was reviewed in detail. In this thesis, it will be sufficient to mention four studies which illustrate some of the possible effects of double infections; potato virus Y (PVY) and potato virus X (PVX) in tobacco (Rochow and Ross, 1955), cowpea chlorotic mottle virus (CCMV) and southern bean mosaic virus (SBMV) in cowpea (Kuhn and Dawson, 1973), barley stripe mosaic (BSMV) and brome mosaic virus (BMV) in barley (Peterson and Brakke, 1973) and BSMV and TMV in barley (Dodds, 1972; Dodds and Hamilton, 1972).

In the first three interactions, there was a synergistic response in the host, but this was not correlated with changes in virus concentration: PVY infection enhanced PVX synthesis up to 10 fold, CCMV infection depressed SBMV synthesis by half, and the synthesis of both BSMV and BMV was depressed in double infections.

The fourth system, also the one used in the present study, is more extreme than the others, because TMV replicates very poorly in singly infected barley plants. In those leaves of barley plants doubly infected with BSMV and TMV that showed acute symptoms (the inoculated leaf and the next youngest leaf), no enhancement of TMV synthesis was detected. In chronically infected leaves, however, there was up to 7 times as much TMV synthesised (7 mg/g) as BSMV (1 mg/g). This represents a massive enhancement compared to the amount of TMV synthesised in equivalent singly infected plants (0.01 - 0.1 mg/g), which appeared healthy. These results can be compared to the PVY/PVX system. In that system, because PVX replicates well in singly infected

plants, the enhancement in the double infection was not as great as the enhancement of TMV synthesis by BSMV infection. Also, in that system enhancement was greatest in tobacco leaves that were rapidly elongating when infected, and less in those infected when they were leaf initials, the reverse pattern being true for TMV enhancement in barley. The massive synthesis of TMV in doubly infected barley did not alter the symptoms of BSMV infection. This enforces the implication made earlier that changes in virus concentrations in response to double infections are not necessarily reflected in the reaction of the host.

Single plants can obviously support the synthesis of two unrelated viruses, and most cells of such plants are probably also doubly infected. Alternatively, the replication of the two viruses could be separated inter-cellularly. Evidence for the former situation will be reviewed since it is pertinent to the first section of this thesis.

Using an optical microscope, McWhorter and Price (1949) observed cytoplasmic crystalline inclusions, associated with TMV infection, together with nuclear crystalline inclusions, characteristic of tobacco etch virus (TEV) infection, in single cells of doubly infected tobacco plants. Fujisawa et al (1967) confirmed this observation at the ultrastructural level. TMV crystalline inclusions were intimately mixed with the pinwheel inclusions characteristic of TEV infection in

the cytoplasm of the majority of cells from doubly infected tobacco plants. Large crystalline inclusions of TMV particles not disrupted by pin-wheel inclusions were also seen alongside areas of mixed inclusions.

The ultrastructural effects seen in tobacco cells doubly infected with TMV and cucumber mosaic virus (CMV) have been studied by Honda and Matsui (1968, 1969, 1971). They showed that the chloroplast abnormalities associated with CMV infections were much more frequent in doubly infected plants. In addition, CMV crystallised in the cytoplasm and the cell vacuole in doubly infected cells, a feature not seen in singly infected plants. A noticeable consequence of this double infection was the frequent location of TMV particles within the nucleus, especially in cells from etiolated leaves. TMV was rarely found in the nuclei of singly infected tobacco cells. Again in this study, the majority of cells showed evidence of being doubly infected.

Another system investigated by the Japanese team (Kamei et al, 1969) was the double infection of turnip mosaic virus (TuMV) and cauliflower mosaic virus (CaMV), in Brassica pervidis. The majority of cells in doubly infected leaves contained pin-wheel inclusions characteristic of TuMV infection together with granular inclusion bodies and associated virions characteristic of CaMV infection.

This study is especially interesting because it demonstrated that an RNA virus and a DNA virus can replicate together in a single cell.

These three systems provide clear evidence that single cells can support the co-replication of two unrelated viruses, indeed, in systemically infected leaves the majority of cells are doubly infected. The results described were, however, for cells which became infected with both viruses at about the same time. The authors also showed that if leaves already infected with one virus were challenge inoculated with the second virus, few cells, if any, became doubly infected.

Another approach to this general problem has been to isolate protoplasts from doubly infected plants, then label them with fluorescein conjugated antibody specific to one or the other virus involved in the double infection. Using this technique, Goodman and Ross (1973) showed that the majority of cells from plants singly infected with PVX, were infected. The frequency of PVX infection was equally high in cells from plants doubly infected with PVX and PVY. Therefore all the cells also infected with PVY were doubly infected. An ultrastructural analysis also indicated that the majority of cells were doubly infected.

Since viruses can establish double infections in cells as well as in plants, it is not surprising that one virus can affect the biological properties of the other. Changes in either the amount

of virus synthesised or the site of intracellular accumulation of particles have already been described. Another important effect recently reviewed by Rochow (1973) concerns several examples of transmission of non-vectored viruses from plants also infected with a virus normally transmitted by the aphid. Two of the mechanisms proposed to explain this phenomenon have been examined experimentally. Kassanis and Govier (1971) showed that potato aucuba virus was unexpectedly transmitted from plants also infected with PVY. In addition, if aphids were first fed on plants singly infected with PVY, then on plants singly infected with potato aucuba virus, the latter was again unexpectedly transmitted, since potato aucuba virus was not transmitted from singly infected plants. The authors suggest that the prior interaction of PVY with aphid stylets allow the stylets to adsorb potato aucuba virus, which they normally do not do.

The second mechanism involves structural interaction between two isolates of barley yellow dwarf virus in doubly infected plants (Rochow, 1971). A small amount of the genome of the non-transmitted isolate became encapsulated in the shell of the transmitted virus. The hybrid particle was then acquired by the vector. Rochow (1973) has also shown that sequential feeding of aphids on plants singly infected with one or other virus is not sufficient to break down the vector specificity of barley yellow dwarf virus.

In addition to the above effects on transmission, a less extreme consequence of double infection can be the alteration of rates of transmission either by aphids (Hampton and Sylvester, 1969) or by seed (Kuhn and Dawson, 1973).

The intimate association of different virus particles in the same cell introduces the possibility of genetic interaction between them, particularly if the two viruses are related strains. Hull and Plaskitt (1970) have illustrated that alfalfa mosaic virus strains can occur together in a single cell. A property of several groups of viruses, of which alfalfa mosaic virus is one, is that the viral genomes are divided amongst several particle classes (van Kammen, 1972). By mixing particle classes from different strains in vitro. infectious viruses have been produced with new or shared biological and physical properties. Such genetic reassortment could easily occur in doubly infected cells, and vectors feeding on such cells could acquire and transmit new strains. Genetic recombination between strains of animal viruses with a single undivided RNA genome has been adequately documented (Fenner, 1970), but this has not been detected using plant viruses.

The final consequence of viruses interacting in doubly infected cells, and the one with which this thesis is concerned, is the possible modification of virus structure. The normal physical and structural properties of BSMV and TMV were reviewed in some detail for this reason. Structural interaction between viruses in doubly infected plants is the subject of the next section.

3. STRUCTURAL INTERACTIONS BETWEEN VIRUSES

The production of single stranded RNA viruses involves the synthesis of single stranded viral RNA from a double stranded RNA replicative form under the direction of a virus specific replicase enzyme (Matthews, 1970). The single stranded RNA product can then function as messenger RNA in the synthesis of virus specific proteins including coat protein, or it can assemble with coat protein to form mature virus particles. In doubly infected cells, two populations of both viral RNA and viral protein are synthesised. Unless virus maturation is a specific event, hybrids might be formed in such cells, containing the RNA of one virus in a shell made up wholly or partly of the protein of the other virus.

A. In vitro reconstitution

In vitro studies have given some insight into the ideal conditions required and the specificities involved in the reassembly of viral nucleic acid and protein. The model systems include the spherical viruses brome mosaic virus, bacteriophage MS_2 and bacteriophage fr and the rod shaped viruses bacteriophage fd, tobacco rattle virus and TMV (Fraenkel-Conrat, 1970). The latter will be reviewed. The original discovery was the ideal in vitro conditions (0.1 M pyrophosphate, neutral pH) in which TMV protein assembled with TMV-RNA to form infectious, stable helical rods undistinguishable from TMV (Fraenkel-Conrat and Williams, 1955).

This landmark study has since been refined in three important ways. The first of these is the observation that reconstitution is polar, protein first associating with the 5′ - hydroxyl end of the TMV-RNA (Stussi et al, 1969, Ohno et al, 1971, Thouvenal et al, 1971). The second is that thirty four protein subunits aggregate into 20 S stacked discs at the optimum condition for reconstitution (neutral pH and ionic strengths up to 0.4) (Durham et al, 1971). Thirdly, it is these discs, rather than much smaller aggregates, which first associate with the 5′ - hydroxyl end of TMV-RNA, and this initial association is a biospecific event (Butler and King, 1971; Klug, 1972; Richards and Williams, 1972; Rodionova et al, 1973). According to Klug (1972) there is a sequence of about 50 nucleotides at the 5′ - hydroxyl end of TMV-RNA which recognises a binding site on the surface of the 20 S protein disc. In this partially reconstituted initiation complex the protein subunits are re-arranged into a helical state under the influence of the RNA. Elongation then occurs by the addition of more protein, either in the form of 20 S discs (Butler and Klug, 1972) or small aggregates (Richards and Williams, 1972). Rodionova et al (1971, 1973) have also provided evidence that TMV reconstitution involves two different steps, initiation and elongation, and they have defined conditions which prevent elongation beyond the initiation step. In addition,

they have indicated that 8 S heptamer protein aggregates, as well as the larger 20 S stacked discs, can associate with TMV-RNA to form an initiation complex. This would suggest that the number of nucleotides involved in recognition may be fewer than the 50 suggested by Klug (1972).

The specific interaction of RNA and protein as a first step in reassembly should be an important control mechanism to ensure homologous assembly and to prevent encapsulation of host nucleic acids, and this has been demonstrated for TMV (Fritsch et al, 1973). The observation that protein of BSMV (Atabekov et al, 1968, Gumpf and Hamilton, 1968) and tobacco rattle virus (Morris and Semancick, 1973) can also be assembled into stacked discs indicates that a similar specific mechanism is involved in the assembly of these rod shaped viruses.

Despite the prediction that assembly of rod shaped viruses should be specific, there are many examples in the literature of heterologous assemblies. Several of these involve the assembly of structural hybrids from type TMV protein and RNA of other TMV strains (Fraenkel-Conrat and Singer, 1957; Holoubek, 1962; Kado and Knight, 1970; Atabekov et al, 1970; Rodionova et al, 1973). Alternatively, proteins from strains of TMV, or related viruses, have been assembled around type TMV-RNA (Atabekov et al, 1970; Okada et al, 1970; Onda, 1972). Rods have also been assembled

from TMV protein and PVX-RNA, BSMV-RNA (from rod shaped viruses) or BMV-RNA (from a spherical virus) (Breck and Gordon, 1970; Atabekov et al, 1970). All these heterologous assemblies were done in conditions ideal for homologous TMV reconstitution, but in the absence of competitive homologous RNA or protein. Infectivity of the reassembled product, when tested, was generally resistant to ribonuclease.

TMV protein has also been assembled into rods with RNA from the spherical virus TYMV (Matthews, 1966) and from the spherical phage MS 2 (Sugiyama, 1966) but the RNA within these assembled products was susceptible to ribonuclease. Yeast RNA (Hart and Smith, 1956) and the polynucleotides poly (I) and poly (A) (Fraenkel-Conrat and Singer, 1964) have also been assembled into rods with TMV protein. Stable spherical particles have been assembled from proteins of the bromovirus group (all spherical viruses) and TMV-RNA (Verduin and Bancroft, 1969; Grouse et al, 1970). Finally, rods formed by the reconstitution of BSMV protein and TMV-RNA have been illustrated by Atabekov et al (1970). These particles are particularly significant for this thesis. From all these studies, a general conclusion is that heterologous reconstitution is possible but the process is not as efficient as homologous reconstitution. Efficiency is

loosely correlated to the degree of relatedness between the two interacting viruses.

A second aspect of heterologous assembly is the degree to which it occurs in the presence of an homologous interaction e.g. A-RNA, A protein and B protein or A-RNA, B-RNA and A protein. The first of these possibilities has been examined using type TMV-RNA, type TMV protein and cucumber green mottle virus (CGMV) protein (Okada et al, 1970) or bean form TMV protein (Onda, 1972). CGMV is a virus related to TMV. Okada et al first formed a heterologous partially reconstituted particle (the initiation step described earlier) with type TMV-RNA and CGMV protein. This complex would only elongate into stable, infectious particles with the protein already attached, CGMV protein, even in the presence of type TMV protein, the homologous protein. No elongation of the heterologous partially reconstituted product occurred with type TMV protein alone. Contrary to these results, Onda demonstrated that the heterologous, partially reconstituted product of type TMV-RNA and bean form TMV protein would elongate efficiently with type TMV protein. This particle, with one segment of type TMV-RNA coated in bean form TMV protein and the remainder in type TMV protein, was infectious and as resistant to ribonuclease activity as homologous particles. The protein from distantly related spherical viruses have also been assembled into mixed capsids (Wagner and Bancroft, 1968, 1971).

The second competitive situation outlined above has been examined by Fritsch et al (1973). They first demonstrated that RNA from the spherical virus TYMV would reconstitute with TMV protein into infectious rods in conditions (0.1 M pyrophosphate, pH 7.2) ideal for homologous TMV reconstitution, particularly when TYMV-RNA was in excess. Efficiency was not as high as in homologous reconstitution. The yield was increased to a maximum 13% as the ratio of TYMV-RNA to TMV protein was increased from 1:22 to 10:22. The infectivity of the product was resistant to ribonuclease. In 0.1 M pyrophosphate, pH 6.0, heterologous and homologous reconstitution were both efficient, but full length rods were not formed. When a mixture of TMV-RNA, TYMV-RNA and TMV protein in the ratio 1:10:22 was reconstituted at pH 7.0, the heterologous assembly was not depressed, and consequently the yield of homologous product was less than it would have been (32% instead of 47%). In effect, about 40% of the reconstituted product contained TYMV-RNA and 60% contained TMV-RNA. In similar experiments done at pH 6.0, which results in efficient homologous and heterologous partial assembly, the corresponding values were 90% for TYMV-RNA and 10% for TMV-RNA, indicating that, at this pH, homologous specificity was effectively absent when the heterologous RNA was present in excess.

In summary, reconstitution of rod shaped viruses probably involves two steps, an initiation stage, which involves specific recognition of RNA and protein, and an elongation stage which

may be less specific. The specificity does not, however, prevent heterologous reconstitution between related and also unrelated viruses, even in conditions ideal for homologous assembly and presumably homologous specificity. Heterologous assembly is less efficient than homologous assembly in conditions ideal for homologous assembly. In conditions less favourable for homologous reconstitution, specificity is lower, since heterologous assembly can be much more efficient than in ideal conditions. In conditions ideal for homologous assembly, and even more so in less favourable conditions, heterologous and homologous assembly can occur together, particularly when the heterologous RNA is in excess.

B. In vivo genomic masking and phenotypic mixing

Since heterologous assembly can occur in vitro, even in the presence of homologous components, it is not surprising that similar interactions also occur in vivo in doubly infected cells. Several kinds of interactions can be envisaged. In singly infected cells the possibility exists that host nucleic acid may be encapsulated in viral protein shells, or capsids. Transduction of bacterial genetic information by temperate phages is the classical example of this phenomenon. Similar encapsulation of host DNA or RNA has been detected with some animal viruses and with TMV (see Siegel, 1971). No genetic function equivalent to transduction has been assigned to these pseudovirions, but this possibility

cannot be ruled out because phenomena resembling transduction have been reported in systems involving animal cells (Merril, Geier and Petricciani, 1971), plant cells (Doy, Gresshoff and Rolfe, 1973) and whole plants (Hess, 1973).

A second type of interaction, comparable to heterologous assembly in vitro, is the encapsulation of the genome of one virus in the capsid of a different virus. This phenomenon is known as transcapsidation (Rapp and Melnick, 1966; Rochow, 1972) or, more commonly, genomic masking (Yamamoto and Anderson, 1961) because the genome of one virus is physically masked in the protein capsid of the interacting virus.

An alternate phenomenon is the encapsulation of the genome of one virus in a capsid assembled from the protein of both the interacting viruses, a phenomenon known as phenotypic mixing (Streisinger, 1956) because the protein capsid, the phenotype of a virus, is mixed. In this review, the term genomic masking is used to describe papers in which the term phenotypic mixing was used loosely to described the interaction defined above as genomic masking.

One experimental approach to genomic masking has been to interact pairs of viruses, one of which is unable to produce mature virions in single infections. Examples involve either double stranded DNA lambdoid phages (Kayajanian, 1971), single

stranded RNA enteroviruses such as poliovirus and coxsackie B_1 virus (Holland and Cords, 1964; Wecker and Lederhilger, 1964) or single stranded RNA plant viruses e.g. strains of TMV (Atabekov et al, 1970; Sarkar, 1969; Kassanis and Bastow, 1971 a,b; Kassanis and Conti, 1971).

Under non-permissive conditions in single infection, if any coat protein is synthesised by the defective virus, it is unable to assemble with viral RNA to form virus particles, and extracts from singly infected hosts are poorly infectious. In double infections with a related competent virus, the RNA of the defective virus matures into complete particles utilising the protein of the "helper" virus. This genomic masking leads to the rescue of the infectivity of the defective virus in extracts from doubly infected hosts.

A similar experimental situation involves the separated components of strains of viruses with a divided genome (Van Kammen, 1972), which can be mixed to produce new virus strains. In experiments with strains of tobacco rattle virus (Sanger, 1969) bean pod mottle virus (Moore and Scott, 1971) alfalfa mosaic virus (Van Vloten Doting et al, 1968) or pea enation mosaic virus (Hull and Lane, 1973) and with the related brome mosaic and cowpea chlorotic mottle viruses (Bancroft, 1972), the new strains made <u>in vitro</u> produced virions with coat protein

determined by only one of the parental strains. This shell enclosed the RNA pieces that made up the genome of the new strain. Since these were derived from the heterologous strain as well as the homologous strain, the RNA from the heterologous strain was in effect genomically masked, although this terminology has not been used in this situation.

Two natural examples can be compared to the experimental systems described above. The first involves Rous sarcoma virus which is a naturally defective virus because, although it can replicate its single stranded RNA in single infections, no membrane bound particles are produced. In double infections with other competent membrane bound leucoviruses, Rous sarcoma virus is enclosed in the membrane specified by the helper virus (Hanafasu et al, 1964). The second example involves the satellite virus of tobacco ringspot virus (Schneider, 1971). The low molecular RNA fragments of the defective satellite virus can only multiply in plants also infected with tobacco ringspot virus. Multiple strands of the satellite virus RNA become encapsulated in tobacco ringspot virus capsid, the satellite virus having no apparent capsid protein of its own.

All the experimental examples of genomic masking described above involved interactions between related viruses, one of which was defective. In this situation only one protein is available

to coat both viral genomes, and because of this, the heterologous encapsulation of nucleic acid takes place in the absence of any competitive protein.

Phenotypic mixing of both viral genomes, however, is the most frequent structural interaction between structurally similar, related viruses when neither of them is defective. Examples include pairs of single-stranded RNA, spherical bacteriophages (Miyake and Shiba, 1971) or animal polioviruses (Ledinko and Hirst, 1961; Sprunt et al, 1955), single-stranded RNA membrane bound animal myxoviruses (Hirst and Gotlieb, 1953), paramyxoviruses (Granoff, 1959) and leucoviruses (Vogt, 1967), single-stranded DNA, spherical bacteriophages (Hutchinson et al, 1967), double-stranded DNA tailed bacteriophages (Streisinger, 1956) or spherical animal adenoviruses (Alstein and Dodonova, 1968; Norrby and Gollmar, 1971).

In some studies it is difficult to distinguish between phenotypic mixing and genomic masking (Burge and Pfefferkorn, 1966; Novick and Szilard, 1951; Valentine and Zinder, 1964; Appleyard et al, 1956). In others, genomic masking as well as phenotypic mixing, was detected (Holland and Cords, 1964; Hutchinson et al, 1967; Miyake and Shiba, 1971; Wecker and Lederhilger, 1964).

In some studies between pairs of fully competent related viruses, which, however, were unrelated serologically, only genomic masking was detected. These included pairs of double-stranded DNA tailed bacteriophages (Ikokuchi and Ozeki, 1970;

Uetake and Hagiwara, 1972; Yamamoto and Anderson, 1961), single stranded RNA spherical animal enteroviruses (Trautman and Sutmoller, 1971) or isolates of barley yellow dwarf virus (Rochow, 1970). In these studies, because the pairs of viruses were not serologically related, it is possible that their coat proteins were too dissimilar to assemble into mixed shells. It would be unwise to equate serological differences between viruses with the inability of their proteins to form phenotypically mixed particles, however, for these were readily formed between pairs of serologically unrelated polioviruses (Ledinko and Hirst, 1961; Sprunt et al, 1955) or leucoviruses (Vogt, 1967).

It can be generalised, however, that if the proteins of two interacting viruses are able to assemble into phenotypically mixed particles, genomic masking represents the extreme example of a series of possible capsids. The shell can be 100% homologous protein (normal virus), varying percentages of both homologous and heterologous protein (phenotypic mixing) or 100% heterologous protein (genomic masking). The result in a given system will depend upon the availability of viral RNA and protein components in the pool from which these components are drawn when viruses are assembled. This is particularly true in systems in which one of the viruses is at a disadvantage, either because one virus replicates slower than the other, or because the inoculum potential

of one was less than that of the other, or because one virus was a defective mutant. As demonstrated and discussed by Niyake and Shiba (1971), if one of a pair of related interacting viruses is disadvantaged, its genome is more likely to be involved in structural interactions and more likely to become genomically masked than in a system where both viruses are produced equally well, which favours equal amounts of phenotypic mixing. This point is emphasised by the interaction between the temperate bacteriophage λ and φ80 (Ikokuchi and Ozeki, 1970). Phenotypic mixing can be demonstrated in vitro, but only genomically masked particles are produced in doubly infected cells, and then only unilaterally with φ80 DNA. In doubly infected cells, phage λ matures faster than φ80 and lysis occurs when little φ80 protein has been produced. The lack of φ80 protein probably explain the above result. In other systems, however, genomic masking may be the only possible interaction if the proteins of the two viruses are unable to assemble into mixed capsids. In this case genomic masking is not an extreme of phenotypic mixing, since phenotypic mixing is impossible.

When two viruses are structurally dissimilar and unrelated this last situation is almost certain to be the case, and there are some reports of genomic masking, but not phenotypic mixing, between such viruses. These include interactions between the single stranded DNA bacteriophages fd, a rod shaped virus, and

ϕX174, a spherical virus (Knippers and Hoffman-Berling, 1966); between the double stranded DNA animal viruses SV-40, a 45 nm spherical virus, and adenovirus, a 70 nm spherical virus (Easton and Hiatt, 1965; Rapp and Melnick, 1966); and between the single stranded RNA plant viruses BSMV, a rod shaped virus and brome mosaic virus, a spherical virus (Peterson and Brakke, 1973). The system studied in this thesis falls into this category since it involves BSMV and TMV, two unrelated rod shaped viruses with different dimensions.

None of the structurally dissimilar unrelated viruses just described are enclosed in a membrane, but when this is the case both genomic masking and phenotypic mixing can be detected in certain double infections (Choppin and Compans, 1970; Zavada et al, 1971). This would suggest that the protein determinants from two unrelated viruses are more readily incorporated into a single membrane than into a single protein capsid.

In summary, in addition to the formation of two interacting viruses in double infections, phenotypic mixing and/or genomic masking can occur. Phenotypic mixing is the more common phenomenon between fully competent related viruses, but it can occur together with genomic masking, or genomic masking can occur without phenotypic mixing. Some of the possible situations which could lead to these alternatives have been described. The amount of structural interaction between related viruses can be as high as 100% phenotypic mixing (Ledinko and Hirst, 1961),

with little production of normal virus of either kind, or as low as no interaction at all. Lack of interaction has been detected between pairs of structurally similar bacterial (Ling et al, 1971), animal (Pringle, 1969) and fungal (Bozarth et al, 1971) viruses, even though, in the first example, heterologous assembly was possible in vitro. Genomic masking is the only interaction detected between structurally dissimilar unrelated viruses which lack a membrane. Such particles form a small proportion of the total virus produced. Some studies have failed to detect structural interaction between unrelated viruses. (Burge and Pfefferkorn, 1966; Kassanis and Conti, 1971; Kayajanian, 1971; Morris, 1971).

Structural interactions are significant wherever the phenotype of a virus determines its properties. Thus Rochow (1970, 1972) has shown that genomic masking of the RNA of one isolate of barley yellow dwarf virus in the shell of another isolate leads to the transmission of the first isolate by the aphid vector normally specific for the other isolate. This indicates that the basis for vector specificity lies in the viral shell of barley yellow dwarf virus. Similarly, genomic masking of poliovirus-RNA in the shell of coxsackie B_1 virus allows poliovirus to infect mice, which it normally cannot do (Cords and Holland, 1964). Since coxsackie B_1 virus, and also poliovirus-RNA, can infect mice, the basis for host specificity must lie in the recognition of the viral shell by mice cells.

Unstable forms of plant viruses can be isolated from naturally infected plants along with stable forms. It has been suggested (Kassanis, 1968; Kassanis and Bastow, 1971) that these unstable forms survive in double infections with a competent strain, from which they were probably derived by mutation, because they are encapsulated by the shell of the competent strain. Other examples in which structural interaction was the mechanism whereby a dependent virus relied on a helper virus for its survival were described earlier.

Another theoretical significance is the possible use of structural interactions to detect relationships between viruses (Miyake and Shiba, 1971). It has been established that genomic masking occurs between unrelated viruses, so it is an unreliable guide to relatedness. Phenotypic mixing, however, is largely restricted to related viruses, even if they are not serologically related.

The detection of genomically masked and phenotypically mixed particles is normally based on their expected properties. Either type of particle is first detected following a physical separation of the two interacting viruses A and B, produced in doubly infected cells. Techniques which separate the infectivity of virus A and virus B from <u>in vitro</u> mixtures of the viruses fail to do so from mixtures derived from doubly infected cells.

The methods which have been used to separate virus A from virus B include rate zonal density gradient centrifugation in sucrose (Peterson and Brakke, 1973), equilibrium density gradient centrifugation in cesium chloride (Ikokuchi and Ozeki, 1970; Knippers and Hoffman-Berling, 1966), electrophoresis in various media (Hutchinson et al, 1967; Miyake and Shiba, 1971), selective inactivation of virus A or B by pH (Trautman and Sutmoller, 1971), temperature (Burge and Pfefferkorn, 1966), specific antiserum (Valentine and Zinder, 1964) or ribonuclease (Atabekov et al, 1970; Kassanis and Bastow, 1971, a, b; Kassanis and Conti, 1971; Sarkar, 1969), or biological agents which selectively adsorb virus A or B such as vectors (Rochow, 1970) or specific hosts (Novick and Szilard, 1951; Uetake and Hagiwara, 1972).

In the case of genomic masking, particles with virus A infectivity cannot be separated from the population of virions that should be only virus B. In the case of phenotypic mixing, where the separatory tool is usually electrophoresis, particles with the infectivity of virus A or B have an electrophoretic mobility between those of the two interacting viruses.

To confirm genomic masking or phenotypic mixing, the following techniques have been used. Genomically masked particles will infect specific hosts of the coat donor, but are unable to infect specific hosts of the genome donor (Cords and Holland, 1964; Uetake and Hagiwara, 1972). Phenotypically mixed particles

can infect the specific hosts of either virus A or B (Vogt, 1967). These tests cannot be used for plant viruses because the capsid does not contribute to host specificity.

The infectivity of genomically masked particles is neutralised by antibody specific for the capsid donor, but not by antibody specific for the genome donor. The infectivity of phenotypically mixed particles is neutralised by antibody specific to both the capsid donor and the genome donor. This neutralisation test has been used in most studies.

Norrby (1970) was able to visualise phenotypically mixed adenoviruses. Both short fibres from strain A and long fibres from strain B were seen at the vertices of single phenotypically mixed particles.

C. A model experiment to detect genomic masking

An ideal experimental system to detect genomic masking should have the following features. Two serologically unrelated viruses A and B should be used. The two viruses should have specific vectors. Methods should be available to separate the two virus particles, the two viral nucleic acids and the two viral proteins from each other. There should be a propagative host in which both virus A and B will replicate efficiently in double infections, each producing viral RNA, viral protein and virus particles. Double infection of single cells should be demonstrated to occur at a high frequency. There should be specific assay hosts for each virus and the assay of one virus

should not be affected by the presence of the other virus. If a common host is used to assay both viruses, interference could be a problem since genomically masked nucleic acid is usually only a small proportion of the total inoculum dose (Peterson and Brakke, 1973).

The infectivity of virus A should be completely separated from the infectivity of virus B following a physical separation of virus A and virus B from an in vitro mixture. On the other hand, if genomic masking of virus A nucleic acid in virus B protein has occurred, the population of virus B-like particles, separated from the mixture derived from doubly infected cells, should infect not only the host specific for virus B, but also the host specific for virus A. It should be demonstrated that the virus A infectivity associated with the virus B population is neutralised by antibody specific for virus B, but not by antibody specific for virus A. The virus B population with the infectivity of both virus A and virus B should contain virus B nucleic acid and virus A nucleic acid, but only virus B protein. The specific vector of virus B should transmit both virus B and virus A from doubly infected plants, if the basis for vector specificity is the nature of the capsid.

Finally, upon inoculation of the genomically masked genome of virus A to a host susceptible to virus A, under conditions of single infection, the biological and physical properties of the

virus produced should be indistinguishable from normal virus A. This final test distinguishes structural interactions from genetic recombination, and has been used in nearly all the studies described. Another desirable experiment would be the demonstration that the genomically masked particles detected *in vivo* could also be assembled *in vitro* from the heterologous components. This ideal experimental system was the basis for the experiments described in section II of this thesis.

SECTION 1. ULTRASTRUCTURE OF BARLEY LEAVES DOUBLY INFECTED WITH BSMV AND TMV

INTRODUCTION

Because both BSMV and TMV occur in high concentrations in doubly infected barley leaves showing chronic BSMV symptoms, it is likely that many individual cells of these leaves contain both viruses. If the alternative cellular distribution occurred, the exclusion of one virus by the other, there would be little chance for structural interaction between BSMV and TMV. An analysis of thin sections by electron microscopy was therefore undertaken to determine the cellular distribution of BSMV and TMV in chronically infected barley leaves.

An immediate problem was the possible difficulty of distinguishing between two different rigid rod-shaped viruses in thin sections, without using ferritin-conjugated antibody or some other labelling technique. The morphology of TMV in tobacco was first ascertained by analysis of leaves doubly infected with brome mosaic virus (BMV) and TMV. Since BMV is a spherical virus, the elongated particles of TMV were easily recognised. The dimensions of TMV, particularly in cross section, were then determined and compared to those of the elongate particles found in cells of leaves doubly infected with BSMV and TMV. The appearance of organelles and virus-specific inclusions and the morphology and distribution of virions in doubly infected cells are compared with those in singly infected cells. Descriptions of ultrastructure in single infections were taken from Paliwal (1970) for BMV in barley, Shalla (1966), Gardner (1967) and Carroll (1970) for BSMV in barley, and Esau (1968) and Matthews (1970) for TMV in tobacco.

MATERIALS AND METHODS

Barley plants (Hordeum vulgare var. Black Hulless) were inoculated at the one leaf stage with BSMV or BMV alone or together with TMV, then grown to the fourth leaf stage at 30° (see section II). Leaves at the fourth leaf stage were cut into small pieces 1 mm x 5 mm. Tissue pieces were fixed and embedded according to the following scheme:

1. Fixation was in 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 60 min.
2. Washing was in two changes of 0.01 M phosphate buffer for 30 min.
3. Post-fixation was in 1% osmium tetroxide in Palade's buffer (Palade, 1952) for 90 min.
4. Dehydration was in the following graded alcohol series:

   50% - 15 min

   70% - 15 min

   95% - 15 min, twice

   100% - 15 min, twice

5. Final dehydration was 1:1 propylene oxide/ 100% alcohol, and then in propylene oxide, 30 minutes each.
6. Infiltration was in 1:1 propylene oxide/ Epon overnight and final embedding was in Epon which was cured for 24-36 hr at 60°. The Epon mixture was as follows; equal parts Epon A (100 parts Epon 812 + 62 parts dodecyl succinic anhydride), and Epon B (100 parts Epon 812 + 89 parts methyl nadic anhydride), 1.5% DMP (tridimethylaminomethyl phenol).

The Epon-embedded leaf pieces were sectioned with glass knives on a Reichert OM U2 ultramicrotome. Thin sections were picked from the water bath with a clean slot grid and placed on 100 or 200 mesh grids coated with carbon-supported collodion films. The sections were first stained with 5% uranyl acetate in 50% methanol for 20 min, washed with water, then stained with lead citrate (Reynolds, 1963), diluted 1:5 with 0.01 M NaOH for 10 min, then rewashed. Stained sections were examined with a Philips EM 200 electron microscope operated at 60 KV. Electron micrographs were recorded on 35 mm film.

RESULTS

The electron micrographs selected for presentation are collected together in 18 figures at the end of this section. Only parenchyma cells from fourth leaves were examined.

The appearance of healthy cells is shown in figure 2. Oval chloroplasts were distributed around the cell periphery within a thin layer of cytoplasm. Cytoplasmic inclusions were occasionally observed within them. Mitochondria were often found next to chloroplasts.

In cells doubly infected with BMV and TMV chloroplasts and mitochondria were normal. The layer of cytoplasm next to the cell wall was somewhat thicker than in healthy cells and this was because of the large accumulation of BMV-like particles in the cytoplasm. Large "bulges", bounded by the tonoplast and containing massive aggregates of TMV in semi-crystalline array, were common. Two of these are shown in figure 3.

An approximately longitudinal orientation of TMV is shown in figure 3-A, and a cross-sectional view in figure 3-B. Also found in the cytoplasm were amorphous inclusion bodies, or X-bodies, which, along with the crystalline aggregates of TMV particles, are characteristic of TMV infections in several hosts. The inclusion bodies will be described later.

The sharpness of the boundary between the TMV crystalline aggregate and the cytoplasm is shown at higher magnification in figure 4-A. The cytoplasm was full of spherical particles about 24 nm in diameter with a prominent central cavity. These particles filled the entire cytoplasmic layer around the cells and were assumed to be BMV virions rather than ribosomes, which did not occur at such a high concentration in healthy cells, nor did they have a central cavity. Failure of the BMV particles to crystallise, even though they were in high concentration, was also observed by Paliwal (1970) in barley, but crystallisation did occur in oats.

In cells which did not have massive TMV aggregates, small aggregates were commonly found next to the rounded ends of oval chloroplasts. Two such aggregates are shown in figure 4-B, -C, with the particles in longitudinal and cross-sectional view respectively.

The length and diameter of TMV particles in barley also infected with BMV is shown at high magnification in figure 5. The particles in the clearly resolved middle file in figure 5-A had an average length of 295 nm, very close to the actual value of 300 nm. Two parallel dark lines, the

RNA core, can be seen in some particles, embedded in a more translucent protein matrix. In cross section (figure 5-B), the RNA core was revealed as a darkly stained ring with a poorly stained circumference of protein external to it. The diameter of the particles in cross section was 14-16 nm, less than the actual value for TMV of 18 nm.

Because BSMV protein stained better than TMV protein, the cross-sectional view of BSMV particles from singly infected barley (figure 5-C) showed more detail external to the darkly stained RNA core. The diameter of BSMV particles in thin sections was 20-22 nm, similar to the actual value of 20 nm. Therefore, in stained thin sections, the 2 nm difference in rod diameter between BSMV and TMV was accentuated to 4 nm or more because TMV protein stained poorly, whereas BSMV protein stained well. This fortuitous difference made the recognition of BSMV and TMV particles in doubly infected cells much easier. Although not always illustrated, evidence for double infection was found in the sections of all the cells about to be described.

Abnormal chloroplasts were very common in cells from leaves doubly infected with BSMV and TMV (figure 6-A). They were frequently rounded rather than oval and appeared amoeboid, in that cytoplasmic areas became encircled by projecting arms, which could be sufficiently distant from the bulk of the chloroplast to appear disconnected. These projections did not always rejoin the chloroplast (figure 6-C). Cytoplasmic pockets

were also commonly found in invaginations within the chloroplasts. The chloroplast limiting double membrane formed a boundary around either kind of cytoplasmic entrapment. BSMV virions were commonly found in these cytoplasmic areas.

In cells doubly infected with BSMV and TMV, another chloroplast abnormality was the presence of vesicles 60 nm wide, bounded by a unit membrane and containing fibrillar material, in swollen areas of the periplastidial space. These are illustrated in figure 7. One to many vesicles could be found in such separations of the chloroplast limiting double membrane, and the vesicles were commonly attached by a thin neck to the outer of the two chloroplast membranes. The association of the vesicles with the outer membrane is clearly shown in figure 7, B-E, which are alternate serial sections of a single rounded chloroplast containing several starch grains. In two places the outer membrane, with many vesicles attached to it, has blistered away from the chloroplast. The membrane of each blister can be traced to the point where it is a normal component of the chloroplast and no longer has vesicles attached to it.

Virions of BSMV were consistently associated with chloroplasts, often forming a nearly continuous layer around them. The virions were perpendicular to the chloroplast limiting membrane, with one end touching the outer membrane (figure 8). The length of these particles was commonly 120-160 nm, the expected range for BSMV. One of the best examples found

of the attachment of virions to the chloroplast outer membrane is shown at high magnification in figure 8-C. The particles had the typical width of BSMV (21 nm) but were longer (300 nm) than the expected range. Such particles will be discussed later. TMV- like virions were not found attached perpendicularly to chloroplasts.

The three main features relating to chloroplasts, amoeboid behaviour, vesiculation in the periplastidial space and attachment of BSMV virions perpendicular to the outer membrane, are all characteristic effects of BSMV in single infections, and appear unaltered in double infections with TMV. Another characteristic of BSMV infections is the location of virions in the nucleus, and this was also very common in doubly infected cells (figure 9-A). The virions usually appeared in areas of the nucleus devoid of chromatin. The cross-sectional diameter of these particles (figure 9-B) was 20 nm, confirming that the virions were BSMV. No TMV virions were detected in nuclei in doubly infected cells.

In addition to the signs of BSMV infection, many individual cells of leaves doubly infected with BSMV and TMV also contained inclusion bodies typical of TMV infections (X-bodies) (figure 10), often with small aggregates of TMV at their edges. These bodies were assemblages of endoplasmic reticulum, ribosomes, darkly stained wide bands, and TMV particles. Similar bodies were not found in leaves infected with only BSMV.

Large cytoplasmic bulges into the vacuole, bounded by the tonoplast, were a common feature in cells of leaves doubly infected with BSMV and TMV. Six examples are shown in figure 11. Figure 11-A indicates the general size of these bulges in relation to a whole cell, and the other micrographs show that these bulges were filled with virus-like particles. Two of these micrographs (figure 11-C,-E) are shown at higher magnification to illustrate the distribution of BSMV and TMV in these areas (figures 12, 13). The particles in these two bulges are mostly in longitudinal view.

In figure 12-A, next to the cell wall the cytoplasmic area around and between the abnormal chloroplasts was full of short, thick BSMV particles, often surrounding the chloroplasts. The enlarged cytoplasmic area above the chloroplasts was also filled with rod-shaped virus particles (figure 12-B). In the central region, the majority of these were thin TMV particles in very long aggregates. Around the periphery, however, there was a substantial accumulation of BSMV and some TMV particles, neither in crystalline array. This mixing is illustrated in the inset (figure 12-C), in which the different diameter of the two particles was clearly revealed in cross section.

In figure 13 a similar distribution of BSMV and TMV was observed. In the area beneath and to either side of the cytoplasmic bulge (figure 13-B), there were mostly BSMV particles arranged in short files packed together at various angles. A mixture of loosely organised BSMV and TMV particles was distributed around the perimeter of the cytoplasmic bulge

(figure 13-A). Within the perimeter, there was a large and small aggregate of TMV in longitudinal view separated by an aggregate of particles in cross-sectional view. This latter aggregate is shown at higher magnification in figure 14-A, which indicated that the centre of the bulge contained a swirl of particles 20 nm in diameter (BSMV) surrounded by particles 16 nm in diameter (TMV). A part of the cytoplasmic bulge shown earlier in figure 11-D is also shown at higher magnification in figure 14-B. Most of the particles were seen in cross section and towards the centre the majority were the narrow TMV particles. Around the perimeter there was a mixture of TMV particles and the thicker BSMV particles.

A picture emerges of TMV aggregates expanding into large bulges surrounded by, and sometimes penetrated by, cytoplasm in which BSMV has accumulated. Mixing of particles occurred where the two viruses adjoined.

In other cells, massive accumulation of both viruses occurred with far more mixing than described above. One such area is shown in figure 15, where it is very difficult to delimit the two viruses.

In figure 16, a part of a cell is shown in which the majority of particles were BSMV which had crystallised into files, one particle wide. In the bottom right hand corner of this micrograph, however, there are five files of TMV particles stacked together. They are shown at higher magnification in figure 17-A. The orientation of the shorter BSMV files seems to have been influenced by the arrangement of the TMV

files, in that each had a common base with the TMV file to which it was joined laterally. Another small aggregate of TMV particles in the midst of BSMV particles is shown in figure 17-B. The three superimposed files to the left of this aggregate are interesting since the upper two files were comprised predominantly of TMV particles, but the lower file contained wider particles which nevertheless had the length of TMV. Similar unusual particles can be found elsewhere on this micrograph and also in figure 17-A at the points of fusion between the BSMV files and TMV files.

Unusually long particles with BSMV width were in fact common in doubly infected tissue, particularly in massive mixed aggregates like the one shown in figure 18, where, however, their length was difficult to deduce. In this figure, there is a change from TMV particles to BSMV particles between the upper left and lower right diagonal in the centre of the micrograph. Many of the BSMV-like particles in the centre of this diagonal are longer than the normal length BSMV particles either to the right of them or below the chloroplast in the upper part of the micrograph. A similar comparison between a file of TMV particles below a file of long BSMV particles can also be seen in the lower right hand corner of the micrograph.

Unusually long particles were not observed in leaves infected with BSMV alone, although particles of variable length, all far longer than those described here, have been described by Gardner (1967) in singly infected tissue.

In the final micrograph (figure 19) supporting evidence for the long particles is presented. In three cases they are shown attached to the chloroplast outer membrane. In each case, normal length BSMV particles are shown next to the long particles. Features of these particles are their near uniform length of about 300 nm, a width of 20 nm, a detectable darkly staining core (RNA) and no evidence for any mid length disjunction. All these features are best illustrated in figure 8-C shown earlier.

CONCLUSIONS

Evidence for both BSMV and TMV infections were found in the majority of cells from fourth leaves of doubly infected barley plants grown at 30°. Chloroplast abnormalities, described below, and the presence of virions in the nucleus, which are characteristic of single infections with BSMV, were found in cells which contained amorphous inclusion bodies (X bodies) characteristic of TMV infections. The signs of one virus infection did not seem to be altered by co-infection with the other virus.

The chloroplast abnormalities were as follows; cytoplasmic areas were found within rounded amoeboid chloroplasts, which also sent out protrusions into the cytoplasm; there were areas on the surface of chloroplasts where unit membrane bound vesicles were attached by narrow necks to the inner surface of the outer of the two limiting chloroplast membranes; files of BSMV virions were attached perpendicularly to the chloroplast outer membrane.

TMV particles were not difficult to distinguish from BSMV particles because TMV protein stained poorly, resulting in a minimum 4 nm difference in width between the two particles.

TMV particles were commonly found as massive crystalline inclusions which formed bulges protruding into the cell vacuole. These inclusions were surrounded by an irregular layer of BSMV particles which sometimes penetrated the TMV crystalline aggregate. Large BSMV aggregates, associated with abnormal chloroplasts, together with small TMV aggregates, were normally found in the cytoplasm between the bulges and the cell wall.

Alternatively, the two viruses crystallised together and large cytoplasmic areas were filled with files of both viruses. The presence of files of one virus could affect the orientation of files of the other virus.

Unusually long particles with the width of BSMV were commonly observed where TMV files merged laterally with BSMV files. Similar particles were also found attached to chloroplast membranes where they could be more easily measured. They were 300 nm long and 20 nm wide, had a darkly stained RNA core, and did not appear disjointed at their midpoint.

Figures 2-19

ABBREVIATIONS

B	BSMV
BM	BMV
CH	Chloroplast
D	Dictyosome
I-MB	Chloroplast inner limiting membrane
M	Mitochondrion
N	Nucleus
O-MB	Chloroplast outer limiting membrane
ST	Starch grain
T	TMV
TO	Tonoplast
VA	Cell vacuole
VE	Vesicle
W	Cell wall
X	X-body, TMV amorphous inclusion body

In low magnification micrographs (x 17,000 or less), a bar representing 1 micron is shown.

In high magnification micrographs (x 19,000 or more), a bar representing 300 nm (the length of a TMV particle) is shown.

NOTES ON TECHNIQUE

Figures 2-19 are all electron microscope micrographs. They represent thin sections of parenchyma cells from barley leaves sampled 20 days after inoculation. Tissue was fixed in glutaraldehyde-osmium tetroxide and sections were stained with uranyl acetate and lead citrate.

Figure 2. Healthy barley cells.

A. Oval chloroplasts are embedded in a thin parietal layer of cytoplasm. The cell vacuole is large. Mitochondria are associated with the chloroplasts. x 11,250.

B. As in A. Some chloroplasts contain small cytoplasmic inclusions (arrowed). x 5,250.

The bar represents 1 micron.

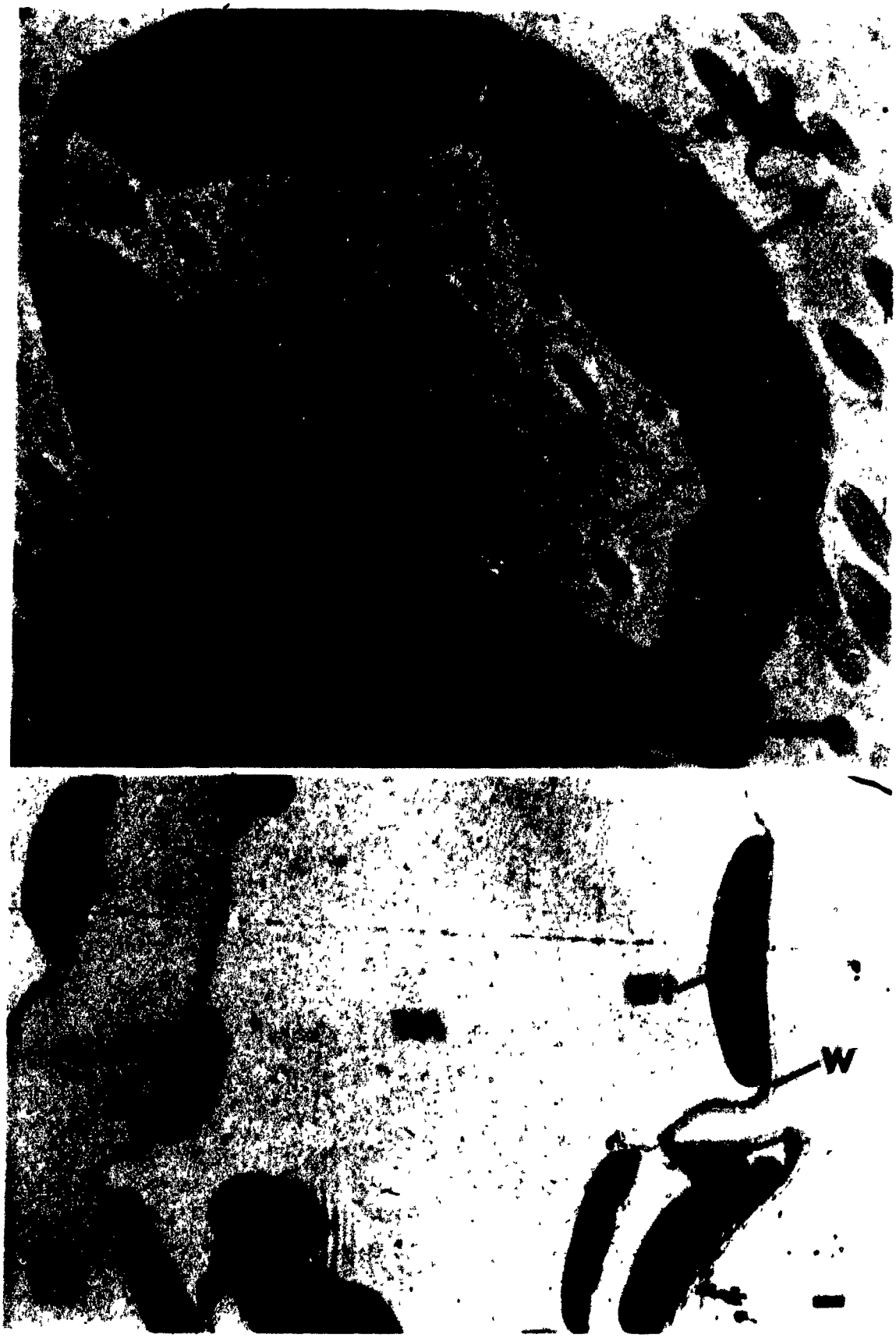

W

Figure 3. Cells doubly infected with BMV and TMV, I.

Massive crystalline aggregates of TMV particles bulge into the cell vacuole. The bulges are bounded by the tonoplast.

A. TMV particles are in longitudinal array. A TMV amorphous inclusion body (X-body) is present. The parietal cytoplasmic layer is thicker than in healthy cells. The junction between the TMV crystalline aggregate and the cytoplasm is shown in figure 4-A. x 7,050.

B. TMV particles are in cross-sectional view, and are shown at higher magnification in figure 5-B. This crystalline aggregate fused with the cytoplasm in subsequent sections. x 7,050.

The bar represents 1 micron.

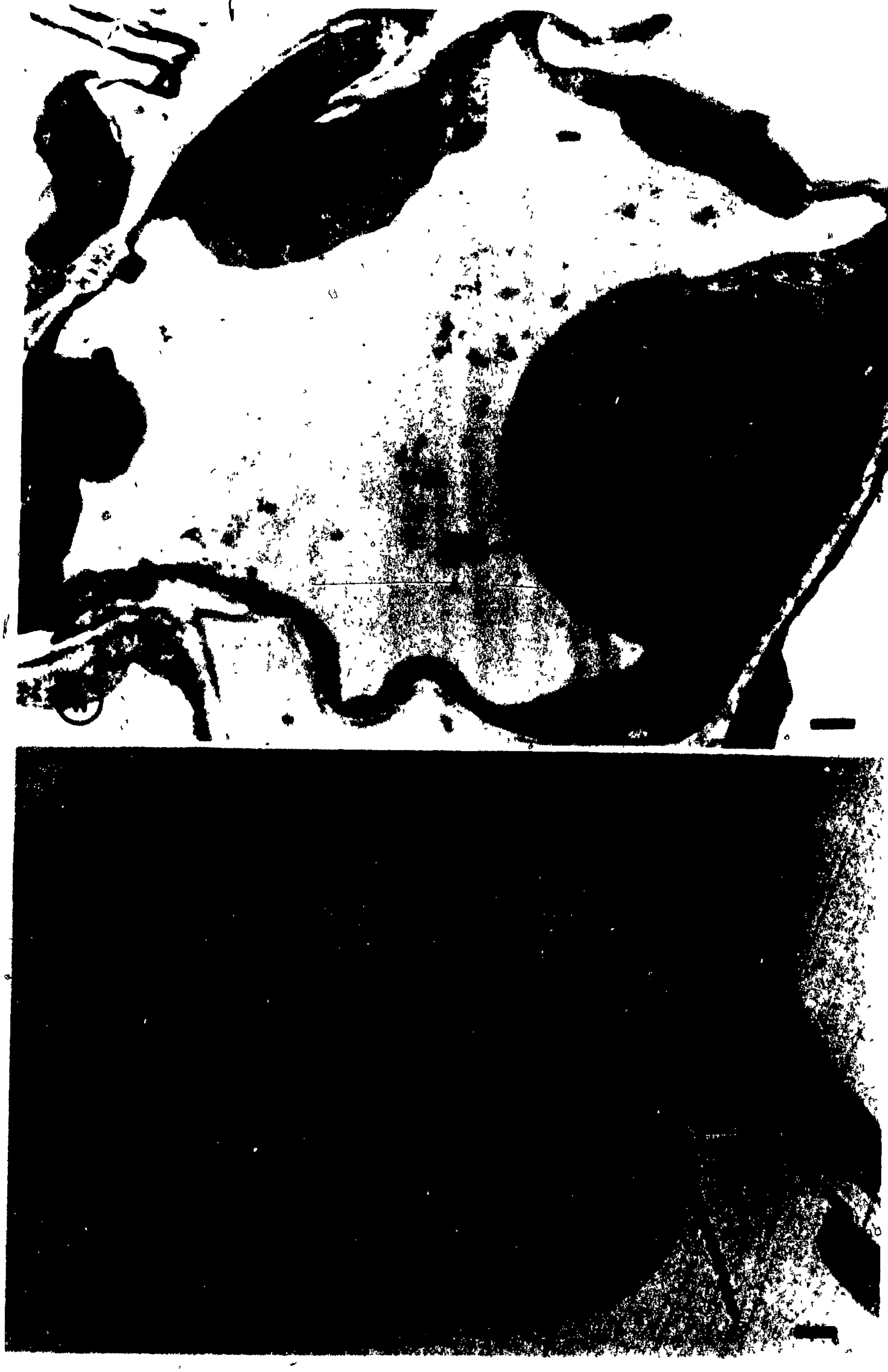


Figure 4. Cells doubly infected with BMV and TMV, II.

A. A clear demarcation between the TMV crystalline aggregate and the cytoplasm is evident. The cytoplasm is packed with 22-24 nm spherical particles with a poorly stained core. These were assumed to be BMV particles. x 55,850.

B. A small aggregate of TMV particles arranged longitudinally, next to a chloroplast. x 50,100.

C. A small aggregate of TMV particles, some in cross-sectional view, next to a chloroplast. x 50,100.

The bar represents 300 nm.

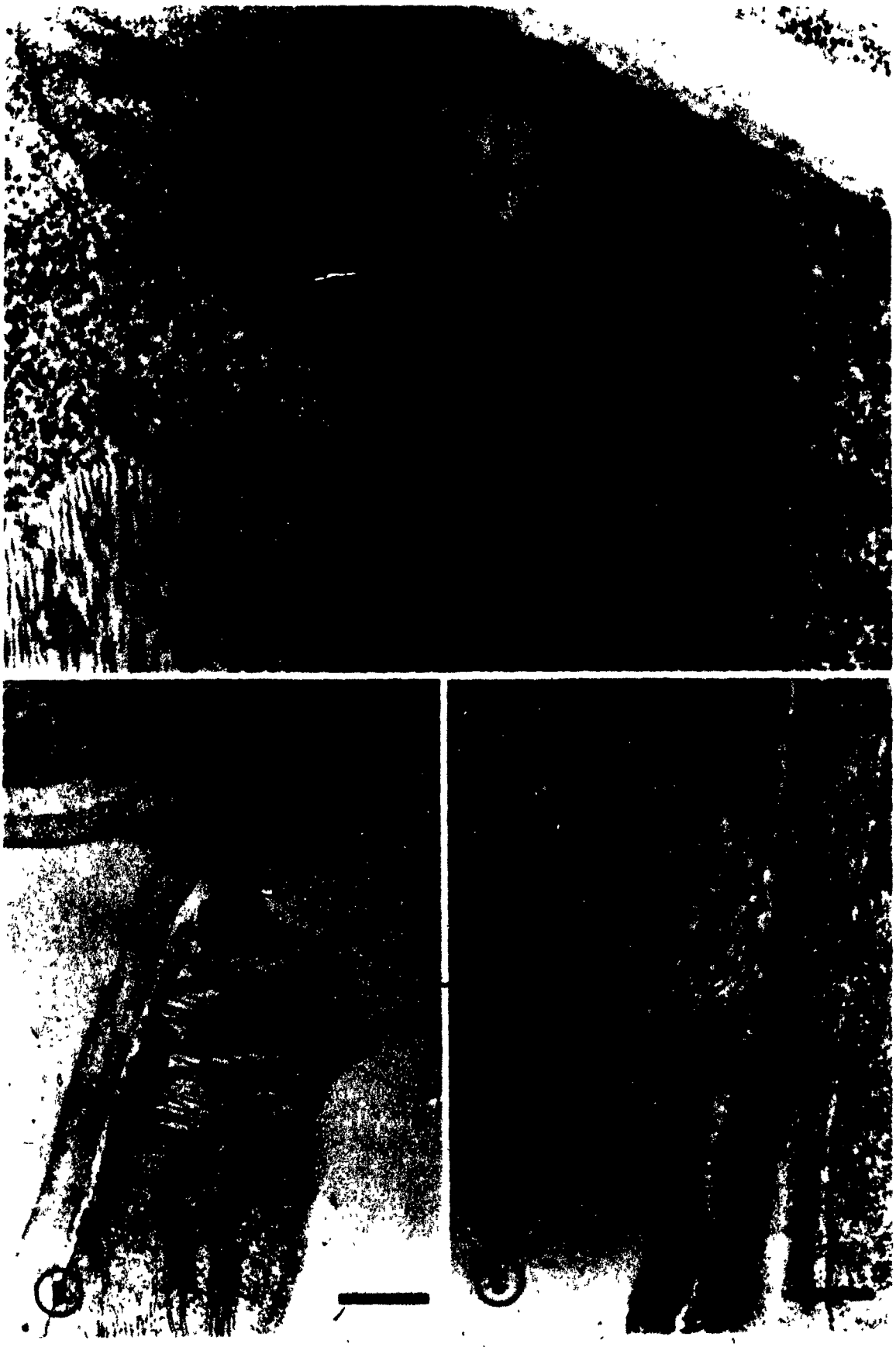


Figure 5. Cells doubly infected with BMV and TMV, III (A,B). Cells singly infected with BSMV (C).

Particle morphology of TMV and BSMV.

A. Three files of TMV particles next to a chloroplast. Some particles can be resolved as two darkly stained lines in a lighter matrix. Particle length is about 300 nm. x 164,150.

B. TMV particles in cross section. A darkly stained ring around an empty core is surrounded externally by a lightly stained circumference. Particle diameter is 14-16 nm. x 164,150.

C. BSMV particles in cross section. The material external to the central ring has stained better than in the TMV particles. Particle diameter is 20-22 nm. x 164,150.

The bar represents 300 nm.

Figure 6. Cells doubly infected with BSMV and TMV, I.

Chloroplast abnormalities: deformation.

A. Rounded chloroplasts appear amoeboid, and large pockets of cytoplasm are occluded within them. Arms projecting from the chloroplasts can appear free in the cytoplasm (arrowed). x 5,250.

B. A pocket of cytoplasm near the periphery of a chloroplast. The occluded cytoplasm is separated from the stroma by a normal chloroplast double membrane (arrowed). BSMV particles are present in the occluded cytoplasm. x 38,250.

C. A projecting arm has failed to fuse with the body of the chloroplast (arrowed). x 38,250.

The bar represents 1 micron (A) or 300 nm (B and C).

Figure 7. Cells doubly infected with BSMV and TMV, II.

Chloroplast abnormalities: vesiculation.

A. Vesicles within the periplastidial space are joined by narrow necks to the outer of the two membranes which surrounds the chloroplast. The vesicles contain fibrillar material. x 94,800.

B-E Alternate serial sections of a rounded chloroplast full of starch grains. The small letters "o" and "x" mark two areas on the surface of the chloroplast where the outer membrane, lined with vesicles, has blistered away from the body of the chloroplast. x 16,600.

The bar represents 300 nm (A) or 1 micron (B-E).

Figure 8. Cells doubly infected with BSMV and TMV, III.

Attachment of BSMV particles to the chloroplast.

A. A chloroplast almost completely surrounded by a single layer of BSMV particles arranged perpendicular to its surface. x 27,400.

B. The attachment of many particles to the outer chloroplast membrane can be seen. x 56,050.

C. Unusually long BSMV-like particles are attached to the outer chloroplast membrane. x 166,600.

The bar represents 300 nm.

C-MB

Figure 9. Cells doubly infected with BSMV and TMV, IV.

BSMV particles within the nucleus.

A. Virus particles are present in an area of the nucleus free of chromatin. x 19,550.

B. The same particles at higher magnification. Particle diameter is at least 20 nm. x 164,150.

Figure B can be located in figure A by superimposing the abbreviation "B" in each figure.

The bar represents 300 nm.

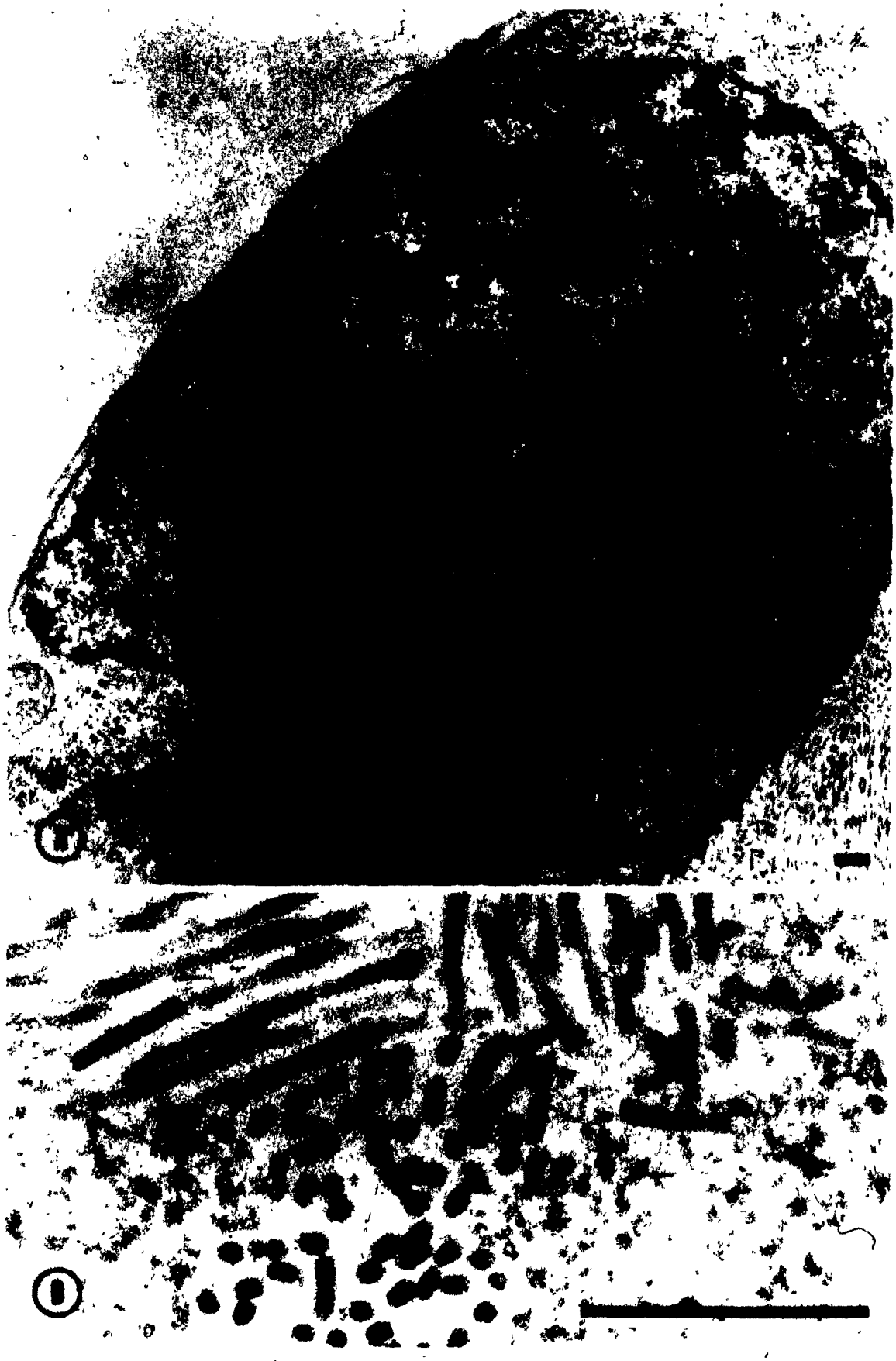


Figure 10. Cells doubly infected with BSMV and TMV, V.

TMV amorphous inclusion bodies (X-bodies).

A. X-body next to a nucleus. Dark bands, ribosomes and membranes make up the bulk of the inclusion body. x 19,500.

B. TMV particles adjoin an X-body. x 19,500.

The bar represents 300 nm.

Figure 11. Cells doubly infected with BSMV and TMV, VI.

Six examples of crystalline aggregates bulging into the cell vacuole.

A. Relationship of a bulge to the whole cell. x 2,400.

B. Higher magnification of the bulge shown in A. x 7,412.

C. This bulge and the adjoining cytoplasm are shown at higher magnification in figure 13. x 5,800.

D. Particles in this crystalline aggregate are in cross-sectional view, and are shown at higher magnification in figure 14-A. x 9,750.

E. Another cell selected for more detailed analysis in figure 12. x 5,800.

F. A TMV amorphous inclusion body next to a crystalline aggregate. x 16,350.

The bar represents 1 micron.

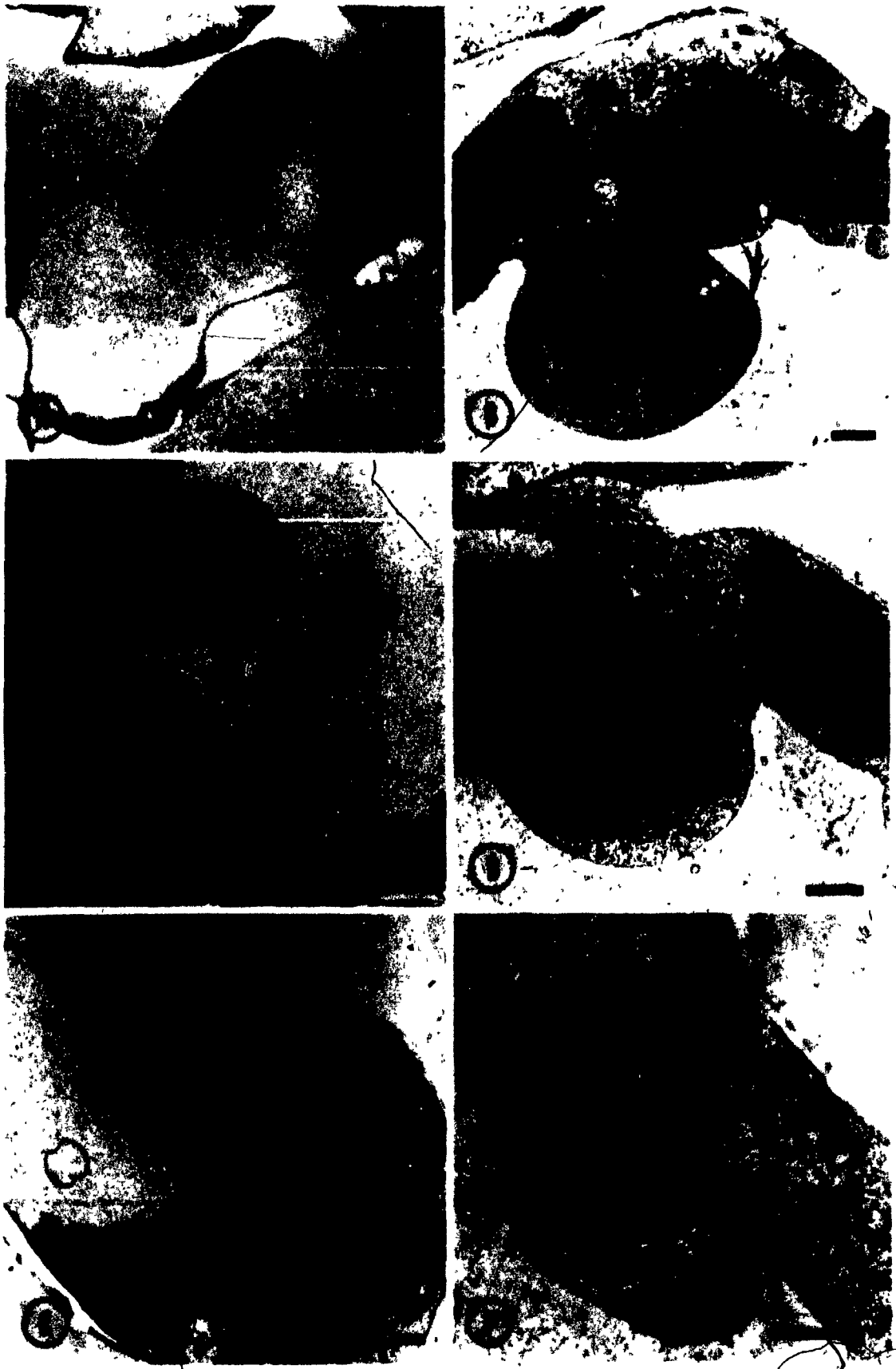


Figure 12. Cells doubly infected with BSMV and TMV, VII.

Distribution of BSMV and TMV.

A. The cytoplasm around and between the vesiculated chloroplasts contains BSMV particles, the majority of which are associated with the chloroplasts. x 21,600.

B. The crystalline aggregate above the chloroplasts is comprised of TMV particles. It is surrounded by unorganised BSMV particles, mixed with TMV particles in some areas. x 21,600.

C. At higher magnification, the particles seen in cross section within the inset in B are shown to be both BSMV and TMV. x 56,050.

Figure A and B can be joined by superimposing the abbreviation "CH" in each figure.

The bar represents 300 nm.

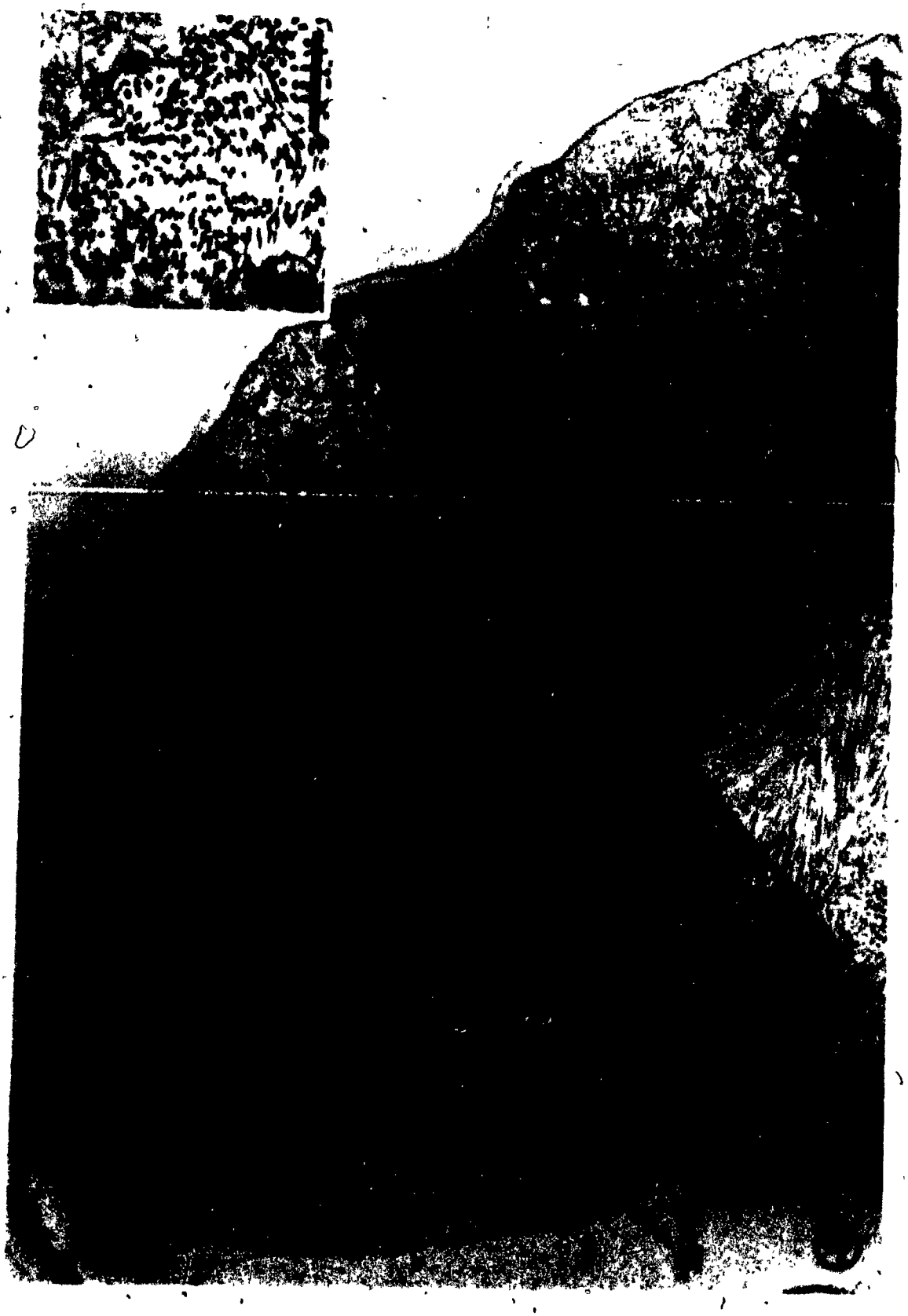


Figure 13. Cells doubly infected with BSMV and TMV, VIII.

Distribution of BSMV and TMV.

A. Crystalline aggregates of TMV are enclosed within a bulge, which is bounded by the tonoplast. Unorganised BSMV and TMV particles surround the crystalline aggregate. The particles in cross-sectional view between the large and small longitudinal array of TMV particles are shown at higher magnification in figure 14. x 19,550.

B. BSMV particles are found between the chloroplasts near to the crystalline aggregate in the bulge. A small group of TMV particles is also present. x 19,550.

Figure A and B can be joined by superimposing the abbreviation "CH" in each figure.

The bar represents 300 nm.

TO

Figure 14. Cells doubly infected with BSMV and TMV, IX.

Distribution of BSMV and TMV.

A. Wide particles (BSMV) at the edge of the bulge shown in figure 11-D, surround thinner particles (TMV). x 164,150.

B. The area between the two aggregates of TMV shown in figure 13-A is comprised of a swirl of wide particles (BSMV) surrounded by thinner particles (TMV). x 164,150.

The bar represents 300 nm.

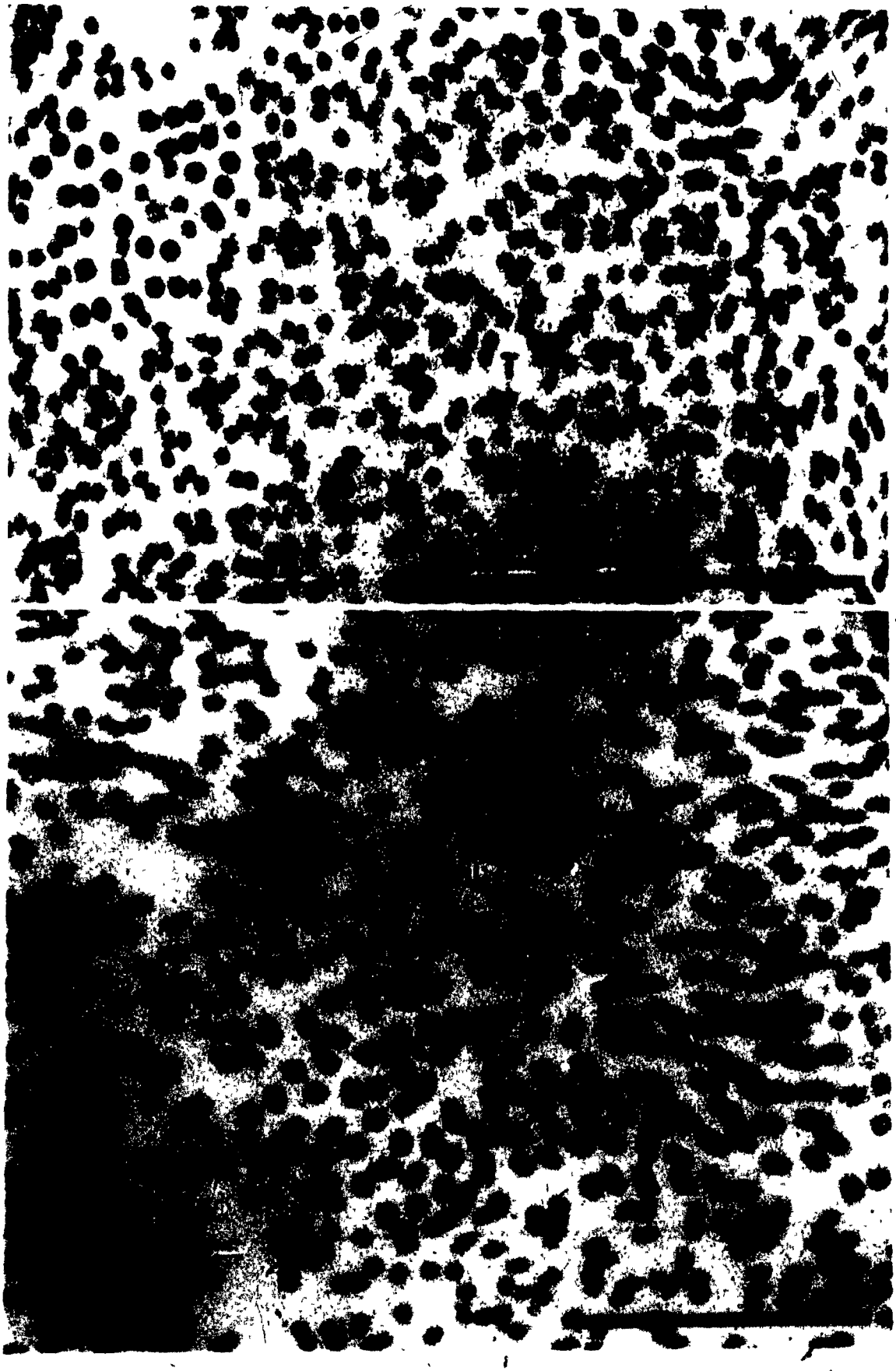


Figure 15. Cells doubly infected with BSMV and TMV, X.

Distribution of BSMV and TMV.

A thick layer of cytoplasm is packed with rod-shaped virus particles. In places, both BSMV and TMV aggregates can be recognised but there is no clear separation of one from the other. In most areas it is difficult to distinguish between BSMV and TMV. x 23,850.

The bar represents 300 nm.

Figure 16. Cells doubly infected with BSMV and TMV, XI.

Distribution of BSMV and TMV.

The cytoplasm around the chloroplast contains BSMV particles in long files. In one area (bottom right) a small aggregate of TMV particles is present. This aggregate is shown at higher magnification in figure 17. x 21,050.

The bar represents 300 nm.

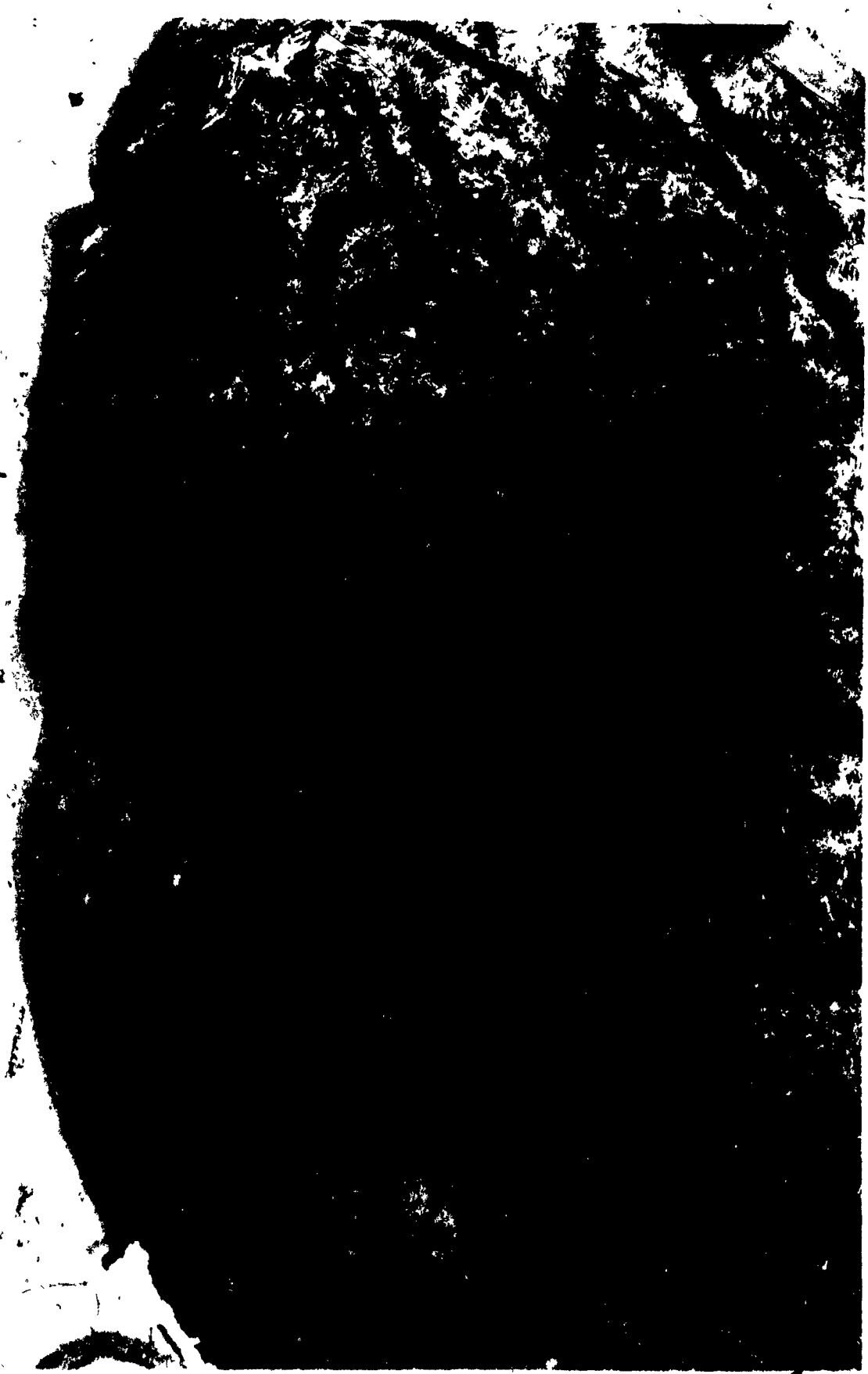


Figure 17. Cells doubly infected with BSMV and TMV, XII.

Distribution of BSMV and TMV.

A. Five files of TMV particles packed side by side are surrounded by files of shorter BSMV particles. Some particles (arrowed) at the edge of the TMV files appear as long as TMV but as wide as BSMV particles. x 66,500.

B. Another small aggregate of TMV particles surrounded by BSMV particles. Again unusually long particles (arrowed) with BSMV particle width are present. x 60,350.

The bar represents 300 nm.

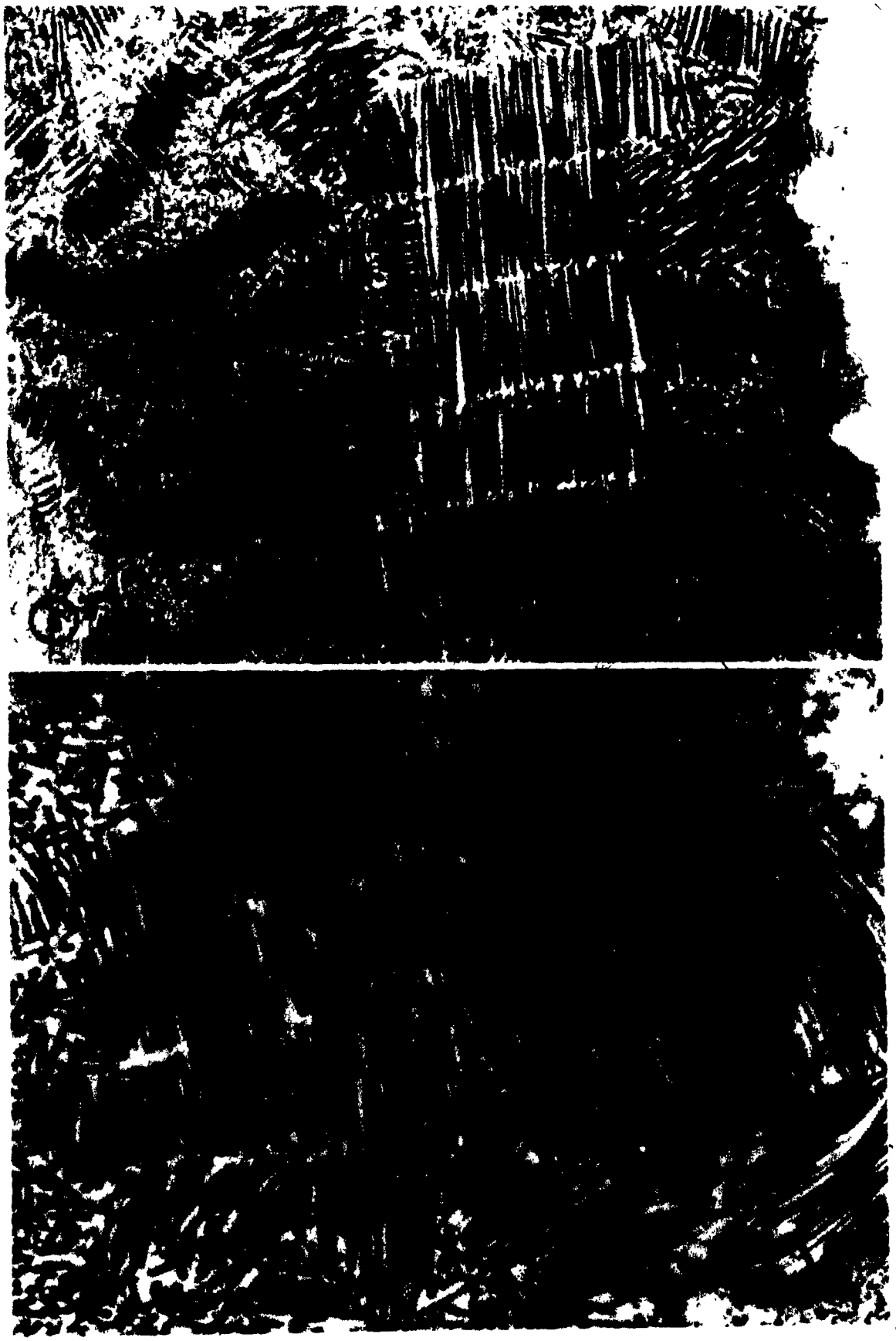


Figure 18. Cells doubly infected with BSMV and TMV, XIII.

Distribution of BSMV and TMV.

Unusually long particles (arrowed) with the width characteristic of BSMV are found in the area between an aggregate of TMV particles and normal BSMV particles. x 56,050.

The bar represents 300 nm.

Figure 19. Cells doubly infected with BSMV and TMV, XIV.

Unusual particles approximately 300 nm long and 20 nm wide.

A-C. Unusual particles (arrowed) are seen attached to chloroplasts along-side normal length BSMV particles. x 75,400.

D. Unusual particles (arrowed) in the cytoplasm. x 75,400.

The bar represents 300 nm.

1
2
3

SECTION 11. GENOMIC MASKING BETWEEN BSMV AND TMV IN DOUBLY INFECTED BARLEY PLANTS

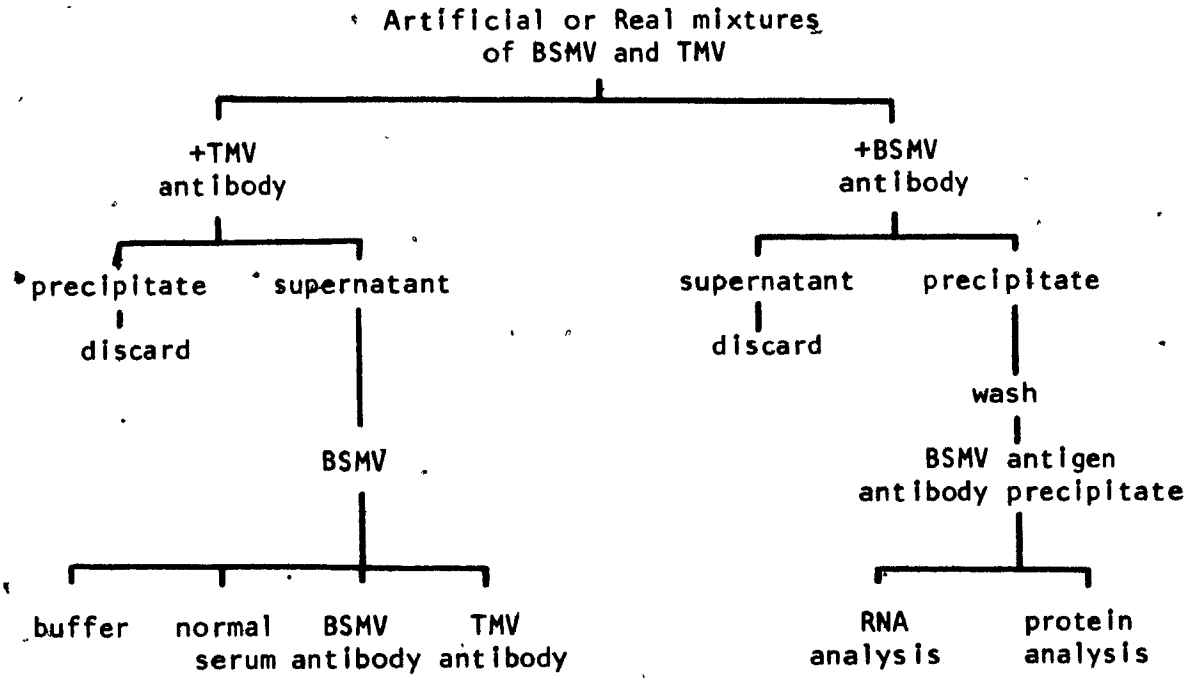
INTRODUCTION

In the literature review, an ideal experimental model for the detection of genomic masking was described. In this section, the results of those kinds of experiments will be described for the BSMV/TMV system in barley. Essentially there were two experiments, which are outlined in figure 20.

The first experimental approach was originally described in an earlier thesis (Dodds, 1972) but is re-presented here so that all experiments on genomic masking between BSMV and TMV can be found in a single thesis. This approach was to determine whether TMV infectivity could be separated from BSMV infectivity following a physical separation of BSMV and TMV antigen from real and artificial mixtures of the two viruses. A real mixture is defined as a mixture of BSMV and TMV prepared from doubly infected plants. An artificial mixture is prepared by mixing the two viruses *in vitro* so as to be quantitatively equivalent to a real mixture with which it is being compared. Separation in this case involved retention of BSMV left in suspension following precipitation of TMV from mixtures with TMV antibody. This was coupled with an analysis of the susceptibility to neutralisation by either BSMV antibody or TMV antibody of any residual infectivity in the BSMV population retained.

The second experimental approach was to determine whether TMV-RNA or protein could be detected in BSMV antigen-antibody precipitates collected from mixtures of BSMV and TMV.

Figure 20. Outline of experiments to detect genomic masking between BSMV and TMV in doubly infected barley plants.



Finally, since genomic masking of TMV-RNA in BSMV protein was detected, the biological properties of the genomically masked TMV-RNA isolated by the second experiment were examined.

MATERIALS AND METHODS

1. The viruses

The barley stripe mosaic virus (BSMV) used for this work was derived from the V-1 isolate (Gumpf and Hamilton, 1968) which, however, no longer produced local lesions on Chenopodium amaranticolor. The tobacco mosaic virus (TMV) used was originally derived from the U-1 strain and did produce local lesions on C. amaranticolor, and, like the U-1 strain, induced a systemic infection in Nicotiana sylvestris (Siegel and Wildman, 1954).

Both viruses were maintained in stock plants grown under greenhouse conditions; barley, Hordeum vulgare var. Black Hulless was the propagative host for BSMV, and tobacco, Nicotiana tobacum var. Samsun, or var. Haranova, for TMV. Viruses were transferred by mechanical inoculation at 2 to 3 week intervals from infected plants to young healthy plants.

2. Antisera

Throughout this thesis the term antibody is used loosely to mean antiserum. Normal rabbit serum, BSMV antibody and TMV antibody were provided by Dr. R.I. Hamilton, Department of Plant Pathology, Macdonald College, Quebec. Both the BSMV antibody and the TMV antibody had a reciprocal titre in the microprecipitin test (described on p. 73) of

between 512 and 1024, when titred against specific antigen at 0.5 mg/ml. At a 10% antibody concentration, complete precipitation of specific antigen at a concentration of up to 2.0 mg/ml was achieved (Dodds, 1972). This was indicated by the absence of sedimenting virus when, following removal of antigen-antibody precipitates, 0.2 ml of the supernatant was analysed by sucrose density gradient centrifugation (10-40% sucrose gradients in 0.01 M phosphate buffer pH 7.2, 0.1% Igepon, 40,000 rpm for 90 min in a Beckman SW 40 rotor). Centrifuged gradients were scanned with an ISCO UA-2 absorbance monitor in the range 0-0.25 absorbance units at 254 nm.

3. Inoculation and Maintenance of Experimental Plants

The experimental host for these double infection studies was barley, Hordeum vulgare var. Black Hulless. Seeds were sown in pasteurised soil in 9 cm pots, and allowed to germinate at room temperature. Plants emerged three days after seeding and were then transferred to controlled environment cabinets maintained at 30° with an 18 hour photoperiod. Seven days after seeding, when the second leaf was beginning to emerge, the number of seedlings in each pot was trimmed to ten or less, selected for uniformity. These were either singly inoculated with BSMV or doubly inoculated with BSMV and TMV and then returned to 30°, at which temperature they remained for the duration of the experiment. Usually about 100 seedlings were inoculated with each treatment.

For the propagation of TMV for purification, young Samsun or Haranova tobacco plants were inoculated with TMV, then maintained in the greenhouse for 14 days, at which time infected leaves were harvested, passed through a meat grinder, and the pulp frozen until required.

The following inocula were used:

i. BSMV inoculum. Young barley leaves recently infected with BSMV were removed from stock plants maintained in the greenhouse. These were ground in 2 ml of 0.01 M phosphate buffer pH 7.2 for each 1 g of leaves, with a mortar and pestle. The liquid extract was strained into a tube and Celite was added as an abrasive to facilitate inoculation.

ii. TMV inoculum. Young tobacco leaves recently infected with TMV were ground in 4 ml of 0.01 M phosphate buffer pH 7.2 for each 1 g of leaves, then Celite was added.

iii. Mixed inoculation. TMV infected tobacco tissue (1 g) and BSMV infected barley tissue (2 g) were mixed, then ground in 4 ml of 0.01 M phosphate buffer pH 7.2 and then Celite was added.

Barley plants were inoculated by first soaking a sterilised gauze in inoculum, containing Celite, then drawing the first leaf of a plant between thumb and finger wrapped in the soaked gauze. The whole of the first leaf was evenly inoculated by two such motions.

4. Selection of infected plants

Except in those experiments where selected leaf positions were examined, singly and doubly infected barley plants were harvested 20 days after inoculation, by which time the fourth leaf was fully elongated. The plants were cut from the pots at soil level and the dehydrated first and second leaves removed. The remaining tissue was used as a source of virus for purification.

When separate analyses of first, second, third and fourth leaves were done, the sampling dates were 7, 10, 14, and 20 days after inoculation respectively.

An assay for the presence of TMV in doubly infected plants was adopted as a routine procedure. This involved sampling the basal quarter of the fourth leaf of 20 of the 100 doubly infected plants. Each piece of tissue was extracted in 1 ml of 0.02 M K_2HPO_4 and the liquid extract was heated at 40° for 1 hour, then centrifuged at 5,000 g for 20 minutes. The supernatant or "heat clarified sap" from each leaf sample was then used as a test antigen and reacted with normal serum, BSMV antibody and TMV antibody in the microprecipitin test (van Slogteren, 1954). This test was performed by first depositing a small drop of antiserum onto the surface of a plastic petri dish, then an equally small drop of test antigen (heat clarified sap) was placed on top of the antiserum drop. This was repeated for all samples and then the petri dish was flooded with mineral oil until the drops were covered. This prevented evaporation from the drops during the subsequent 1 hour incubation at 40° in an oven. The drops were then examined for visible precipitates with a stereo microscope. A precipitate indicated a positive reaction while the absence of a precipitate was interpreted as a negative reaction. TMV could easily be detected with TMV antibody in extracts prepared from small parts of individual leaves from doubly infected plants.

5. Virus purifications

A. Preparation of clarified sap from barley

Singly or doubly infected tissue was weighed. A mortar and pestle was used to grind samples less than 10 g, but for larger weights the tissue was passed through a meat grinder into twice its weight (1 g = 1 ml) of 0.02 M K_2HPO_4. This mixture was then blended for a few minutes at top speed in a Waring Blendor. The extract, after being strained through two layers of cheesecloth, was heated for 1 hour at 40°. Following this incubation, the extract was centrifuged for 20 minutes at 5,000 g. The supernatant solution, or "heat clarified sap", was collected and the green pellet discarded.

For the second experimental approach described in the introduction, the heat clarified sap from the BSMV infected plants was the starting material for the construction of an artificial mixture of BSMV and TMV. For the first approach, the heat clarified sap from singly or doubly infected tissue was subjected to one cycle of differential centrifugation (next section, steps 1-3) before the artificial mixture was made.

B. Further purification of viruses from barley

The steps involved are outlined below:

1. The heat clarified extract was centrifuged for 1.5 hr at 30,000 rpm in the Beckman Type 30 rotor (approximately 80,000 g) to pellet the virus. After centrifugation the supernatant was discarded.

2. The pellets were covered with 1-2 ml of 0.02 M tris (hydroxymethyl) amino methane -0.0064 M citrate buffer (0.02 M Tris-citrate buffer) pH 6.5, then left overnight to soften and resuspend.
3. Resuspension of the pellet was completed by stirring the suspension and then Igepon T-73 was added to a concentration of 0.1%. This anionic detergent helps to prevent and reverse BSMV particle aggregation (Brakke, M.K., 1959). The solution was then centrifuged for 20 minutes at 5,000 g and any pellet obtained was discarded.
4. 2 ml of the supernatant virus suspension were layered over 5 ml of 45% sucrose, dissolved in 0.02 M Tris-citrate buffer pH 6.5, 0.01% Igepon T-73, in a cellulose nitrate tube designed for the buckets of the Beckman SW 25 or SW 27 rotor. The tube was then filled with mineral oil to prevent it from collapsing during centrifugation.
5. The prepared tubes were centrifuged for 5 hrs at 25,000 rpm (27,000 rpm) in a Beckman SW 25 (SW 27) rotor.
6. After centrifugation the mineral oil and sucrose were discarded and the virus pellet was resuspended in 0.02 M Tris-citrate buffer pH 6.5, 0.1% Igepon T-73 as in steps 2 and 3.
7. The partially purified virus was stored at 4° with a drop of chloroform added to prevent microbial growth.

C. Purification of TMV from tobacco

1. Frozen pulp from infected plants was thawed in twice its weight (1 g=1 ml) of 0.02 M K_2HPO_4 containing 1% mercaptoethanol. The mixture was blended for a few minutes at high speed in a Waring blender, then the extract was squeezed through two layers of cheesecloth.
2. 10 g of activated charcoal (Norite A) were added to the extract for every 100 g of original tissue, and the extract was stirred for 30 minutes.
3. The extract was clarified by two consecutive low speed centrifugations for 20 minutes at 7,700 g, discarding the black pellet each time.
4. The clear supernatant was centrifuged for 1.5 hrs at 30,000 rpm in the Beckman Type 30 rotor (approximately 80,000 g) to pellet the virus.
5. After centrifugation the supernatant was discarded and the virus pellets covered with 0.02 M Tris-citrate buffer pH 6.5, and left overnight at 4°.
6. The pellets were resuspended by stirring, then centrifuged at low speed to remove any remaining charcoal, as in step 3.
7. The virus was further purified by centrifugation through a cushion of 45% sucrose as described in steps 4, 5 and 6 of the purification scheme for viruses from barley.
8. The virus pellet was resuspended in Tris-citrate buffer pH 6.5 and stored at 4° with a drop of chloroform added.

The concentration of partially purified virus preparations was determined by spectrophotometery at 260 nm. Suspensions (1 mg/ml, 0.1%) of either BSMV, TMV or mixtures of the two were assumed to absorb 3.0 units of light at a wavelength of 260 nm ($OD_{260}^{0.1\%} = 3.0$). Spectrophotometers used included a Beckman DU and a Unicam SP 800A.

6. Construction of artificial and real mixtures of BSMV and TMV

Either the heat clarified sap from doubly infected plants (2nd approach), or the suspension after one cycle of differential centrifugation (1st approach) contained both viruses, BSMV and TMV, at an unknown concentration and in an unknown ratio. To quantify these mixtures the following method was used. It is predisposed upon the fact that both viruses co-sediment during sucrose density gradient centrifugation. The validity of the method was established in an earlier thesis (Dodds, 1972).

From the total volume (usually in excess of 50 ml) of clarified sap from doubly infected plants, 3 ml were removed. This was divided into three equal portions and to each was added 0.1 ml of either normal serum, BSMV antibody or TMV antibody. After a 1 hour incubation at room temperature, the three samples were stored at 4° overnight. Any antigen-antibody precipitates which formed were removed by low speed centrifugation. A small volume (0.2 ml) of sample was floated onto the surface of a 10-40% sucrose density gradient in 0.01 M potassium phosphate buffer pH 7.2, 0.1% Igepon, preformed in a Beckman SW40 or SW41 cellulose nitrate centrifuge tube. An equal volume (0.2 ml) of purified TMV at a known concentration (0.5 mg/ml) was floated onto the surface

of a fourth gradient. The four gradients were centrifuged at 40,000 rpm for 90 minutes (SW40) or 45 minutes (SW41) in a Beckman SW40 or SW41 rotor. After centrifugation each gradient was pumped from the centrifuge tube with 45% sucrose through the ISCO UA-2 or UA-4 absorbance monitor coupled to a chart recorder. This combination graphically recorded absorbance at 254 nm through the length of the centrifuged gradient column. The position of any virus band in the gradient was indicated by a peak on the chart. The amount of virus in the gradient was a function of the area under the peak. The four results obtained for the sap from doubly infected plants were; the total amount of BSMV and TMV together (sap + normal serum), the amount of BSMV after removal of TMV (sap + TMV antibody), the amount of TMV after removal of BSMV (sap + BSMV antibody), and the peak area from a known concentration of TMV (0.5 mg/ml). If these four values were 200, 50, 150 and 100 arbitrary area units (calculated by counting graph paper squares) respectively, then the combined concentration of BSMV and TMV in the mixture was calculated as:

$\frac{0.5 \text{ mg/ml}}{100} \times 200 = 1.0$ mg/ml, of which

$\frac{0.5 \text{ mg/ml}}{100} \times 50 = 0.25$ mg/ml was BSMV and

$\frac{0.5 \text{ mg/ml}}{100} \times 150 = 0.75$ mg/ml was TMV.

A fifth sample, 0.2 ml of the clarified sap from BSMV infected plants, was analysed concurrently with the others by the above method, to estimate the concentration of BSMV in the sap from singly infected plants. Sufficient

partially purified TMV, from a 20 mg/ml stock, was then added to the clarified sap from BSMV infected plants to make an artificial mixture in which BSMV and TMV were in the same amount and ratio as in the real mixture to which it was being compared. When partially purified virus, instead of clarified sap, was the starting material, the same procedure was adopted as above, except that a suitable dilution of a small sample was used for antibody incubation followed by quantitative sucrose density gradient centrifugation.

An alternative method of constructing an artificial mixture was used in some experiments. This method was based on the observation that the concentration of BSMV in singly and doubly infected plants is approximately the same and that the concentration of TMV in fourth leaves of doubly infected plants rarely exceeds 5 mg/g of tissue, third leaves containing less (Dodds, 1972). The starting material for these experiments was usually pooled third and fourth leaves and so, in some experiments, 5 mg of TMV for every 1 g of BSMV infected tissue was added to the clarified sap from singly infected plants. This ensured that there was at least as high, if not a higher, ratio of TMV to BSMV in the artificial mixture compared to the real mixture. The usual aim was to remove TMV from BSMV, so if the separatory method was successful for such an artificial mixture, it would certainly be successful for the real mixture. This alternative method for preparing an artificial mixture was only adopted for some experiments using the second experimental approach.

7. Separation of components from mixtures

A. First approach: removal of TMV with TMV antibody

Equivalent artificial and real mixtures (5-10 ml of partially purified virus at 7.0 mg/ml) were made 10% with TMV antibody and 0.8% with NaCl and incubated for 1 hour at room temperature on a mechanical shaker. The incubated mixtures then stood overnight at 4°. The TMV antigen-antibody precipitate which formed was removed by low speed centrifugation and the supernatant was reincubated with TMV antibody twice more, removing the precipitate each time. The final supernatant was centrifuged through a cushion of 45% sucrose at 27,000 rpm in a Beckman SW27 rotor for 5 hours. The virus pellet was resuspended in 0.02 M Tris-citrate buffer pH 6.5, 0.8% NaCl, and re-incubated with TMV antibody until no more pellet, indicative of an antigen-antibody precipitate, was obtained upon low speed centrifugation. One incubation was usually sufficient. The virus in the supernatant was again centrifuged through 45% sucrose and the final pellet was resuspended in 0.02 M Tris-citrate pH 6.5. The concentration of virus was adjusted to 0.5 mg/ml. The above procedure was designed to fully exploit the capacity of high titred TMV antibody to remove TMV from suspension. The final resuspended pellets should have been essentially BSMV, one suspension derived from an artificial mixture and one from a real mixture of BSMV and TMV.

B. Second approach: precipitation of selected antigen with specific antibody

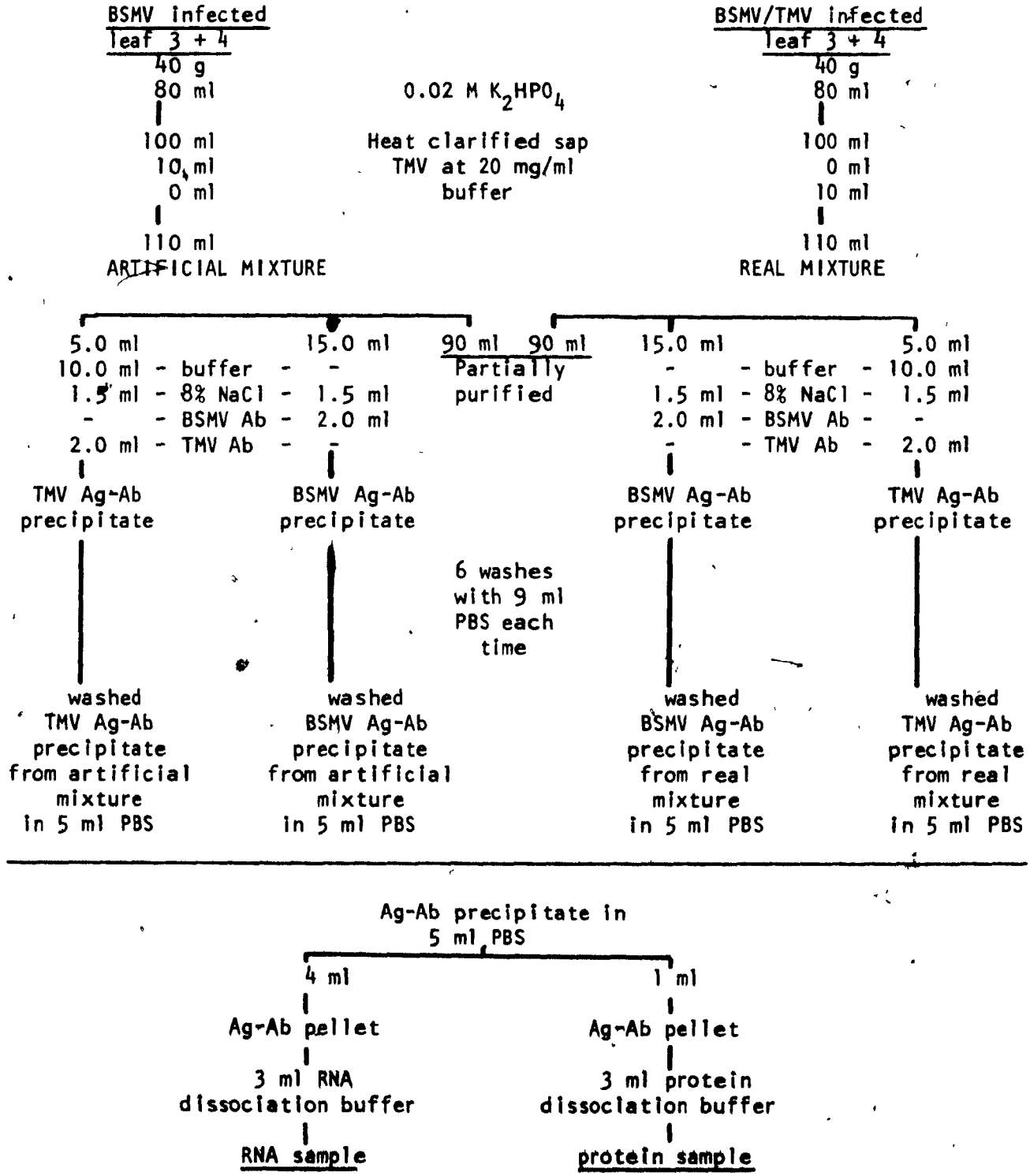
Stated simply, BSMV was collected from clarified sap by precipitation with its specific antibody, i.e. BSMV antibody. The

BSMV antigen-antibody precipitate was washed free of contaminating TMV particles with 0.01 M phosphate buffer pH 7.2, 0.8% NaCl (phosphate buffered saline, PBS). TMV was similarly collected free of BSMV by precipitation with TMV antibody. The RNA and protein components of the washed antigen-antibody precipitates were then analysed.

The quantitative details in the preparation of the washed antigen-antibody precipitates are described in greater detail below. The details are for one of several experiments but can be taken as typical. The steps are outlined in table 3.

The real mixture was made by extracting 40 g of doubly infected tissue in 80 ml of 0.02 M K_2HPO_4 resulting in 100 ml of heat clarified sap. The artificial mixture was made by adding 200 mg of TMV to the 100 ml of clarified sap from 40 g of BSMV infected leaves (equivalent to 5 mg TMV/g of tissue). The expected recovery of BSMV from both singly and doubly infected tissue is 1 mg/g of tissue, so there would be 40 mg of BSMV in 100 ml of extract or 0.4 mg BSMV/ml. The maximum expected recovery of TMV is 5 mg/g, so there would be a maximum of 200 mg of TMV in 100 ml of the artificial or real mixture, or 2.0 mg/ml. Five ml of either mixture (10 mg of TMV) was diluted to 15 ml with 0.01 M phosphate buffer pH 7.2 (0.65 mg TMV/ml), and was made 10% with TMV antibody and 0.8% NaCl. Upon standing, the 10 mg of TMV became complexed in the form of a TMV antigen-antibody precipitate. Similarly, 15 ml of either mixture (6 mg of BSMV) was made 10% with BSMV antibody and 0.8% NaCl, whereupon the 6 mg of BSMV became complexed

Table 3. Collection of washed antigen-antibody precipitates from artificial and real mixtures.



in the form of a BSMV antigen-antibody precipitate. The remaining 80 ml of both mixtures was subjected to the purificiation scheme outlined on p. 74 and the partially purified virus was retained for possible future use.

After an overnight incubation at 4° the four antigen-antibody precipitates were collected by low speed centrifugation (5,000 g for 20 minutes). Taking the BSMV antigen-antibody precipitates as an example, the majority of the TMV, which was originally mixed with the BSMV, was discarded with the supernatant. Within the pelleted BSMV antigen-antibody precipitate, however, there would still have been some non-specific occlusion of TMV particles. To rid the precipitate of this TMV, it was resuspended in 9 ml of PBS using a pasteur pipette. This resulted in the trapped TMV particles being released from the pelleted precipitate and going into suspension. The BSMV, however, stayed in the form of an antigen-antibody precipitate, now dispersed. On standing at 4° this precipitate settled out again and was collected by low speed centrifugation. The TMV left in suspension was discarded. Each precipitate was washed at least five times using 9 ml of PBS each time. After each resuspension, a minimum period of five hours was allowed to elapse to ensure that complete binding of BSMV and BSMV antibody re-occurred. It was noted that quicker wash cycles resulted in a rapid reduction in the size of the antigen-antibody pellets. TMV antigen-antibody pellets were similarly washed with PBS to free them of BSMV.

At the end of the treatment, there were four washed antigen-antibody precipitates dispersed in 5 ml of PBS and containing a) BSMV from the real mixture, b) TMV from the real mixture, c) BSMV from the artificial mixture and d) TMV from the artificial mixture. Between 6 and 10 mg of virus was trapped in each precipitate, assuming no loss during the washing procedure. The method by which the precipitates were analysed is described in the second part of the next section.

8. Analysis of separated components

A. First approach: neutralisation of infectivity by specific antibody

BSMV, at 0.5 mg/ml, derived from artificial or real mixtures was split into four 1 ml aliquots. To each of these was added 0.1 ml of either 0.02 M Tris-citrate buffer pH 6.5, normal serum, BSMV antibody or TMV antibody. After an overnight incubation at 4°, any antigen-antibody precipitates which formed were removed by low speed centrifugation. Celite was added to the supernatants which were then inoculated to assay hosts.

Purified TMV at 0.1 mg/ml was similarly treated and acted as a control to establish how normal TMV infectivity responded to the different antibody treatments.

The assay hosts specific for TMV were Chenopodium amaranticolor and Nicotiana glutinosa, both of which develop local lesions when inoculated with TMV but remain symptomless when inoculated with the BSMV isolate used in these experiments. The number of leaves was trimmed to four per plant and each sample was inoculated to each of the four leaf positions spread over different plants. The assay host

specific for BSMV was Black Hulless barley which develops a systemic chlorosis upon inoculation with BSMV but remains symptomless when inoculated with TMV. Each sample was inoculated to 25 barley seedlings. All assay plants were maintained in a greenhouse. Lesions were counted 7-10 days after inoculation and systemic symptoms were scored 14 days after inoculation.

B. Second approach: RNA and protein analysis of washed antigen-antibody precipitates

1. Sample preparation

Several single phase buffers have been described which will dissociate viruses into their native RNA or protein components. There is no reason to expect that BSMV or TMV, held in the form of an antigen-antibody precipitate, cannot be similarly dissociated.

The "RNA dissociation buffer" used was essentially that which has been described by Brakke and his co-workers (Brakke and van Pelt, 1970b, Pring, 1971). It consists of 0.02 M Tris pH 9.0, 1% sodium dodecyl sulphate (SDS), 0.001 M disodium ethylenediaminetetraacetate (EDTA) and bentonite at 0.5 mg/ml. The bentonite was graded and treated with EDTA as described by Fraenkel-Conrat (1961).

Antigen-antibody precipitates were collected by low speed centrifugation. The supernatant was discarded and the pellet was drained. Between 0.5 ml and 1.0 ml of RNA dissociation

buffer was then added for every 1 mg of virus trapped in the pelleted antigen-antibody precipitate. The pellet was resuspended in the RNA dissociation buffer with a pasteur pipette and allowed to stand first at room temperature for 1 hour and then overnight at 4° in order to release the RNA.

To prepare a protein sample for analysis, pelleted, drained antigen-antibody precipitates were suspended in "protein dissociation buffer"; 0.1 M sodium phosphate buffer pH 7.2, 24% urea, 1% SDS, 1% mercaptoethanol. After suspension, the sample was placed in a boiling water bath for 90 seconds to release the protein.

Both BSMV and TMV are mostly protein and only 5% RNA or less, so this was a factor in preparing these samples. As a standard approach, the washed antigen-antibody precipitates were finally resuspended in 5 ml of PBS, and this was split into two aliquots, one of 4 ml and one of 1 ml. The precipitate collected from the larger aliquot was resuspended in RNA dissociation buffer and that collected from the smaller aliquot in an equal volume of protein dissociation buffer. The volume of either extraction buffer used was qualitatively assessed. Ideally, if 2 mg of virus was thought to be trapped in the antigen-antibody precipitate before it was split into a 4:1 ratio, then 1 ml of dissociation buffer was used. This had to

be balanced against the possible loss of virus during the washing procedure and so the size of the final antigen-antibody pellet was taken into account. The procedure described above resulted in four times as much virus, in the form of an antigen-antibody precipitate, being used to prepare an RNA sample as was used to prepare a protein sample.

Purified virus was also used as a source of RNA and protein for use as standards. For this purpose, BSMV and TMV were diluted from a 20 mg/ml stock to 2.0 mg/ml with RNA dissociation buffer and to 0.5 mg/ml with protein dissociation buffer; the same 4:1 ratio described above for the division of the antigen-antibody precipitates. The 4 RNA molecules of brome mosaic virus with molecular weights of 1.09×10^6d, 0.99×10^6d, 0.75×10^6d and 0.28×10^6d (Lane and Kaesberg, 1971) were used as RNA standards for molecular weight determinations. The proteins used as molecular weight standards were bovine serum albumin (62,000 d), ovalbumin (46,000 d), alcohol dehydrogenase (37,000 d), carbonic anhydrase (29,000 d) and myoglobin (17,500 d) (Dunker and Rueckert, 1969, Weber and Osborn, 1969). RNA and protein molecular weight standards were provided by Shirley Blevings, technician, CDA Research Station, 6660 N.W. Marine Drive, Vancouver, B.C.

2. Analysis of RNA and protein samples by polyacrylamide gel electrophoresis

A cylindrical gel cast from polyacrylamide provides an ideal matrix for the electrophoresis of macromolecules such as RNA and proteins. At a suitable gel concentration, these molecules can be sieved by electrophoresis according to their molecular size, but only if each different sized molecule has the same charge. RNA molecules have approximately the same net negative charge and in the presence of the anionic detergent sodium dodecyl sulphate (SDS) most protein molecules assume the same net negative charge. Under these conditions, electrophoretic mobility shows a linear relationship with the logarithm of the molecular weight of the migrating molecule. This, of course, is a generalisation and is affected by conformational differences between molecules and the degrees by which the intrinsic charge of different protein molecules can affect mobility.

The use of polyacrylamide gel electrophoresis for the analysis of viral RNA (Adesnik, 1971) and SDS- polyacylamide gel electrophoresis for the analysis of viral proteins (Maizel, 1971) has been reviewed. In this study RNA samples were analysed on 2.4% polyacrylamide gels in 0.036 M Tris, 0.03 M NaH_2PO_4 pH 7.8, 0.1% SDS, 0.001 M EDTA (Loening, 1969). Protein samples were analysed on 5% polyacrylamide gels in 0.1 M sodium phosphate pH 7.2, 0.1% SDS, 0.1% mercaptoethanol (Agrawal and Tremaine, 1972).

The quantities and types of buffers and reagents used for polyacrylamide gel electrophoresis of RNA and protein are given in tables 4 and 5.

Gels were cast by dispensing 1.8 ml of freshly mixed gel solution into glass tubes 12 cm long with an internal diameter of 0.5 cm to produce a gel 9 cm long. Before the gel set, a small volume of water was layered on top of the gel solution to ensure a flat gel surface. The bottom ends of the tubes were sealed with a rubber stopper resembling a small serum bottle stopper.

When the gels were set, (30 minutes) the stoppers were removed and the glass tubes inserted through holes in the base of an upper cylindrincal electrophoresis chamber, made from Perspex. The bottom ends of the tubes were immersed in 1,000 ml of electrophoresis buffer in the lower electrophoresis chamber. The upper chamber was then filled with 1,000 ml of electrophoresis buffer.

Samples were applied to the top of the gel with a lambda pipette attached to a Macrostat dispenser to control the flow. The pipette was lowered through the buffer in the upper chamber and into the gel tube until the tip of the pipette was just above the gel surface. The sample volume (25µl or 50µl) was then carefully layered onto the surface of the gel. To ensure that the sample would not float away, a drop of tracker dye

Table 4. Solutions and buffers for analysis of RNA samples by polyacrylamide gel electrophoresis.

A Electrophoresis buffer. (0.036 M Tris, 0.03 M NaH_2PO_4, pH 7.8, 0.001 M EDTA, 0.1% SDS.)

10 X Stock

	M. Wt.	Concentration	g/l	pH
Tris	121	0.36 M	43.6	7.8
$NaH_2PO_4.H_2O$	138	0.30 M	41.4	
Na_2EDTA	372	0.01 M	3.7	
SDS		1.0%	10.0	
H_2O			to 1,000 ml	

Diluted 200 ml to 2,000 ml for electrophoresis buffer.

B Solutions and volumes for 12 X 2.4% gels.

	Volumes (ml)
1. 20% acrylamide 1% bis-acrylamide	3.0
2. 11.4 ml of 10X electrophoresis buffer, pH 7.8 0.2 ml Temed (N,N,N^1,N^1 - Tetramethyl-ethylenediamine) 88.4 ml H_2O	21.75
3. 10% ammonium persulphate	0.25

Table 5. Solutions and buffers for analysis of protein samples by polyacrylamide gel electrophoresis.

A Electrophoresis buffer. (0.1 M sodium phosphate pH 7.2, 0.1% SDS 0.1% mercaptoethanol)

10 X Stock

	M. Wt.	Concentration	g/l	pH
Na_2HPO_4 $7H_2O$	268	1.0 M	193	7.2
$NaH_2PO_4.H_2O$	138		38.6	
SDS		1%	10	
mercaptoethanol		1%	10 ml	

Diluted 200 ml to 2,000 ml for chamber buffer.

B Solutions and volumes for 12 X 5% gels.

	Volumes (ml)
1. 20% acrylamide 0.6% bis-acrylamide	6.0
2. H_2O	6.0
3. 0.2 M sodium phosphate, pH 7.2 0.2% SDS 0.2% Temed (N,N,N^1,N^1 - Tetramethyl-ethylenediamine)	12.0
4. 10% ammonium persulphate	0.12

(1% bromophenol blue in 10% glycerol) was added to each sample before it was pipetted. Electrodes were placed in the upper and lower chamber and the current was applied. Twelve gels could be electrophoresed concurrently. RNA gels were pre-electrophoresed at 6 mA/gel for 30 minutes before layering the samples. For 12 RNA gels, electrophoresis was at 75 mA and 90 volts until the tracker dye reached the end of the gel (1.5 hr). For 12 protein gels, electrophoresis was at 75 mA and 40 volts until the tracker dye migrated 3/4 of the length of the gel (3.5 hr).

After electrophoresis, the gels were removed from the glass tubes by rimming them with a long hypodermic needle from which water flowed steadily. RNA gels were stained overnight in 0.03% toluidine blue in 40% ethylene glycol mono-methyl ether (methyl cellosolve). They were then destained and stored in water. Destaining took several days and several changes of water. Protein gels were first placed in 20% trichloroacetic acid (TCA) for 30 minutes in order to precipitate the proteins in the gels. They were stained overnight in 0.075% coomassie brilliant blue in 12.5% TCA, then destained for 24 hours in 10% TCA and then stored in 7.5% acetic acid. Stained gels were stored and photographed in plastic tubes.

As an alternative or in addition to staining gels, gels were cast in quartz tubes of 3 in length. These could be scanned at 254 nm, after electrophoresis, with an ISCO UA-4 absorbance monitor coupled to a linear transport device and a chart recorder.

3. Analysis of RNA samples by centrifugation through linear log sucrose density gradients

Although cylindrical polyacrylamide gels are excellent for resolving size classes of RNA molecules by electrophoresis, as a preparative tool they are less than ideal. Only small quantities can be layered on the gel and recovery of RNA from the gel matrix after electrophoresis is difficult, particularly with such a labile molecule as single stranded viral RNA.

Centrifugation of RNA through linear 0-20% sucrose gradients will separate different sized RNA molecules, but with poor resolution. Resolution can be improved through the use of linear log sucrose density gradients (Brakke and van Pelt, 1970a), but not to the degree achieved with polyacrylamide gel electrophoresis. However, much larger quantities of RNA can be loaded onto sucrose density gradients, the RNA can be readily recovered from the centrifuged gradient by fractionation and the recovered RNA can be directly inoculated to an assay plant. A clear separation of TMV-RNA and BSMV-RNA was obtained after centrifugation on linear log sucrose density gradients and so these were used to recover genomically masked TMV-RNA in an infectious form. They were also used analytically in experiments carried out at the start of this project (see section III).

Linear log sucrose density gradients were constructed manually by layering different volumes of various concentrations of ribonuclease-free sucrose one on top of the other, beginning

with the most dense. The exact volumes and concentrations for SW40 and SW41 cellulose nitrate centrifuge tubes are shown in figure 21. Sucrose was dissolved in 0.015 M sodium citrate pH 7.0, 0.15 M sodium chloride (SSC). The concentration gradient was established by allowing the sucrose to diffuse overnight at 10°. The top 0.5 ml of a gradient was carefully removed and replaced with 0.5 ml of RNA sample. Gradients were then centrifuged at 40,000 rpm for 5 hr at 14°. Temperature was important since at 5° the SDS in the sample crystallised and spoiled the run.

The centrifuged gradient was pumped out of the centrifuge tube and through an ISCO UA-2 or UA-4 absorbance monitor coupled to a chart recorder and a fraction collector. The absorbance profile of the centrifuged gradient was traced on the chart paper, along with witness marks indicating the 1.0 ml samples collected by the faction collector. Alternatively, at the same time that an absorbance peak registered on the chart, the corresponding fraction of the gradient was collected manually.

9. Methods used to investigate the biological properties of genomically masked TMV-RNA

The aim here was to establish infections in Chenopodium amaranticolor by inoculation with genomically masked TMV-RNA. The resulting lesions were transferred singly to tobacco plants. The virus produced in the tobacco plants was purified, quantified and characterised by serology,

Figure 21. Volumes and concentrations of sucrose for linear-log gradients[a].

SW40 rotor		SW41 rotor	
ml	mg/ml sucrose	ml	mg/ml sucrose
1.5	0	1.4	0
1.4	80	1.2	100
1.9	160	1.5	160
2.4	210	2.3	210
3.7	270	3.4	270
1.9	325	1.8	325

[a]Jackson et al (1973)

sucrose density gradient centrifugation and its ability to infect barley in double infections with BSMV.

RNA samples from washed BSMV antigen-antibody precipitates from real mixtures were analysed and fractionated on linear log sucrose density gradients as described in the last section. Celite and a drop of bentonite (35 mg/ml) were added to each 1 ml gradient fraction. Each fraction was inoculated to four leaves of Chenopodium amaranticolor. Ten days later, single lesions which resulted from infection with genomically masked TMV-RNA were excised with a #1 cork borer. The borer was flamed in ethanol between cuts. Each excised lesion was placed in a depression of a porcelain spot plate with three drops of water. The rounded end of a sterile glass tube was then used to grind the excised lesion.

Certain of the lesion extracts were used to prepare negative stained grids for examination in the electron microscope to determine whether TMV particles could be detected in the extract. A drop of extract was placed on a collodion coated carbon backed grid for 10 minutes and then dried off with filter paper pieces. A drop of 2% uranyl acetate was then placed on the grid for 10 minutes, then removed by absorption with filter paper pieces. The grids were then examined in a Philips EM-200 electron microscope.

Other lesion extracts were inoculated to single tobacco plants and to two leaves of Nicotiana glutinosa. Assay plants were dusted with carborundum before inoculation. The end of the glass tube used for grinding was also used for inoculation.

Lesions produced on Chenopodium amaranticolor by inoculation with TMV-RNA from normal TMV, and tissue excised from uninoculated Chenopodium amaranticolor were also treated as above.

Two weeks later, there were 15 infected tobacco plants for comparison. Ten of these were infected with isolates derived from genomically masked TMV-RNA, and five were from isolates derived from normal TMV-RNA.

Each plant was harvested, weighed, frozen and extracted later in twice its weight of 0.02 M K_2HPO_4, 1% mercaptoethanol, using a Waring blender. Since there were so many samples, the virus in the extract was purified with polyethlene glycol (PEG) mol. wt. 6,000. The liquid extract was strained through cheesecloth, then heated at 40° for 60 minutes. The incubated extract was centrifuged at 5,000 g for 20 minutes and the supernatant was made 0.1 M with sodium chloride and 4% with PEG. After a low speed centrifugation (5,000 g for 20 minutes), the virus-containing pellet was retained and resuspended in water. The virus in suspension was given a low speed centrifugation, and then reprecipitated from the supernatant with 4% PEG, 0.1 M NaCl. After low speed centrifugation, the virus pellet was resuspended in water, given a final low speed centrifugation and the virus-containing supernatant stored at 4° with a drop of chlorobutanol added. The volume of water used in the final resuspension was half the original tissue weight.

The concentration of virus in each sample and the yield of virus from each gram of tissue was estimated by recording the absorbance at 260 nm of suitable dilutions, usually 1:20.

The sedimentation characteristics of selected preparations were determined by layering 0.2 ml of diluted (10^{-1}) sample on 10-40% linear sucrose gradients in 0.01 M potassium phosphate pH 7.2. Gradients were centrifuged for 45 minutes at 40,000 rpm in a Beckman SW41 rotor. The absorbance profile, at 254 nm, of centrifuged gradients was recorded with an ISCO UA-4 absorbance monitor coupled to a chart recorder.

The virus preparations were also used undiluted as test antigen in Ouchterlony double diffusion tests in agar against TMV antibody, BSMV antibody and normal serum. Because TMV was the test antigen, the test required the preparation of 1% agar in 0.01 M potassium phosphate buffer pH 7.2 with no sodium chloride (Wetter, 1967). Agar was solidified in plastic petri-dishes and then wells were cut with a #2 cork borer in the patterns shown in figure 36. Certain wells were filled with test antigen and others with antibody and the gel between the wells observed for the development of precipitation lines. When lines were fully developed, the plates were photographed.

The infectivity in barley of the partially purified TMV derived from genomically masked TMV-RNA was assayed by co-inoculation of each sample with an extract from BSMV-infected barley. The plants were grown at 30° until they reached the fourth leaf stage. Heat clarified extracts from fourth leaves were tested with TMV antibody in the microprecipitin test (p 73).

RESULTS

1. First approach: antibody neutralisation of infectivity associated with BSMV derived from artificial or real mixtures

The infectivity associated with BSMV separated from artificial or real mixtures was assayed on Chenopodium amaranticolor, Nicotiana glutinosa and Hordeum vulgare and the results are shown in table 6. Three sets of results are given for two separations, A and B. The BSMV obtained from the second separation was assayed on two separate occasions, B_1 and B_2. The antibody incubations in B_2 were separate from those in B_1 and were done after the results of B_1 were known. They were carefully monitored to ensure that no mistakes were made.

In all three sets of results, the BSMV at 0.5 mg/ml separated from artificial mixtures of BSMV and TMV produced no lesions or an insignificant number (one) after inoculation to Chenopodium amaranticolor or Nicotiana glutinosa, assay hosts for TMV. This indicated that the use of TMV antibody to remove TMV from BSMV had been successful for the artificial mixture.

The same BSMV was infectious on barley, which developed the normal symptoms of a BSMV infection. Incubation with BSMV antibody, followed by removal of the BSMV antigen-antibody precipitate which formed, abolished the infectivity on barley, an expected result. Incubation with normal serum or TMV antibody did not abolish BSMV infectivity, but both sera did lower the specific infectivity of BSMV. Non specific inhibition of virus infectivity by normal serum has been reported (Rappaport and Siegel, 1955).

Table 6. Infectivity of BSMV derived from real and artificial mixtures of BSMV and TMV. Viruses were incubated with different antisera before inoculation to test plants.

Inoculum	Antibody incubation before inoculation	Chenopodium amaranticolor			Nicotiana glutinosa	Hordeum vulgare	
		A	B_1	B_2	B_2	A	B_2
TMV (0.1 mg/ml)	Buffer	212[a]	-[b]	-	-	0	-
	NS	60	-	-	-	0	-
	BSMV-Ab	55	-	-	-	-	-
	TMV-Ab	0	-	-	-	-	-
BSMV - artificial (0.5 mg/ml)	Buffer	0	1	0	0[a]	95[c]	43
	NS	0	0	0	0	72	35
	BSMV-Ab	0	0	0	0	0	0
	TMV-Ab	0	0	0	0	14	22
BSMV - real (0.5 mg/ml)	Buffer	125	70	254	76	74	41
	NS	5	40	121	52	35	23
	BSMV-Ab	0	0	0	0	0	0
	TMV-Ab	2	38	129	12	12	29

[a]Average number of lesions on 1 inoculated leaf (average of 3).

[b]Not tested.

[c]Percentage of inoculated plants that were infected (average of 25).

In sharp contrast to the above results, the BSMV separated from real mixtures of BSMV and TMV produced local lesions on both Chenopodium amaranticolor and Nicotiana glutinosa. This demonstrated that TMV infectivity was still associated with BSMV derived from real mixtures, even after removal of TMV antigen with TMV antibody. This TMV infectivity, however, was abolished by treatment with BSMV antibody, the same treatment which, at the same time, abolished BSMV infectivity on barley. The TMV infectivity was inhibited equally, but not abolished, by treatment with TMV antibody or normal serum. Therefore, the BSMV separated from real mixtures of BSMV and TMV had two kinds of infectivity associated with it, normal BSMV infectivity in that it was neutralised by BSMV antibody and abnormal TMV infectivity in that it was also neutralised by BSMV antibody but not by TMV antibody. The pattern of neutralisation of infectivity associated with normal TMV given in figure 6 was the reverse of that for the TMV infectivity associated with BSMV from real mixtures. As expected, infectivity was neutralised by TMV antibody and inhibited non-specifically by BSMV antibody and normal serum.

The significance of these results will be discussed in the conclusions section of this part of the thesis. However, it is clear that genomic masking is the simplest explanation for the TMV infectivity associated with the BSMV from the real mixtures. Particles consisting of TMV-RNA in BSMV protein would not have separated from BSMV particles using TMV antibody. They would impart TMV infectivity to the BSMV population and they would be susceptible to BSMV antibody, but not TMV antibody.

2. Second approach: Validity of the preparative and analytical methods

A. Equivalence of RNA or protein from virus and from antigen-antibody precipitates

It was hoped that TMV or BSMV, in the form of antigen-antibody precipitates, would be dissociated with either RNA dissociation buffer or protein dissociation buffer into the same RNA or protein components that result from direct dissociation of virus. The dissociated antigen-antibody precipitates were not expected to contain any additional RNA components to those within the trapped antigen (virus). They would, however, contain antibody proteins in addition to antigen (viral) proteins.

The following samples were prepared. Two were made by diluting BSMV or TMV from 20 mg/ml to 1 mg/ml in RNA dissociation buffer. Two were made by diluting BSMV or TMV to 0.25 mg/ml in protein dissociation buffer. These were the RNA and protein samples from virus. For comparison, 0.2 ml of specific antibody (BSMV antibody or TMV antibody) were added to 2 ml of BSMV or TMV at 0.5 mg/ml. The resulting antigen-antibody precipitate from 1.6 ml of each sample was collected and dissociated in 0.8 ml of RNA dissociation buffer. The antigen-antibody precipitate from the remaining 0.4 ml of each sample was dissociated in 0.8 ml of protein dissociation buffer.

For RNA analysis, 50µl of dissociated BSMV, TMV, BSMV antigen-antibody precipitate and TMV antigen-antibody precipitate were co-electrophoresed on 2.4% polyacrylamide gels. The stained gels are shown in figure 22-A. The four RNA components of BMV were also analysed for comparison.

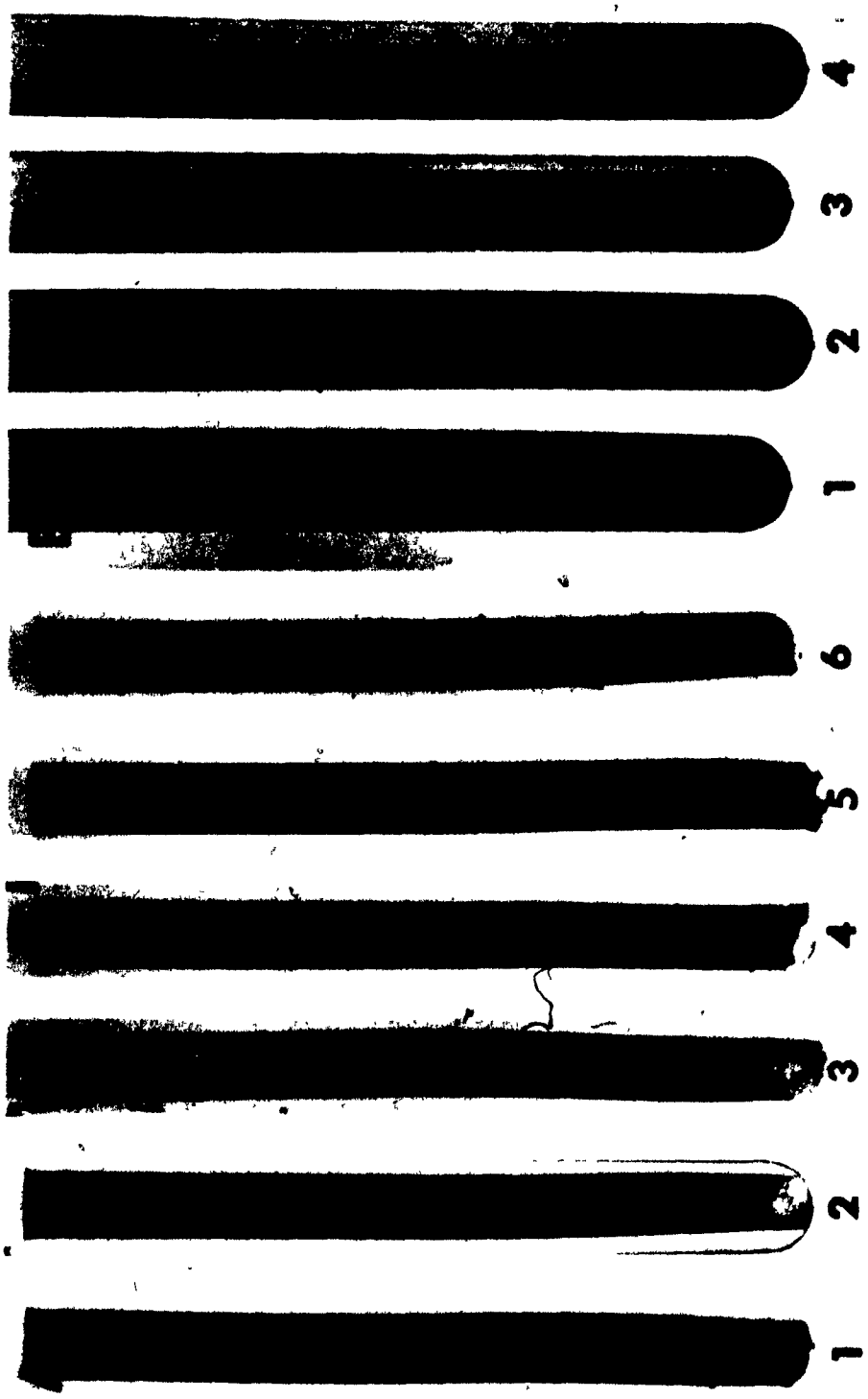
Figure 22. Equivalence of RNA or protein from virus and from antigen-antibody precipitates.

A. Stained 2.4% polyacrylamide gels after electrophoresis of virus or antigen-antibody precipitates resuspended in RNA dissociation buffer.

1. RNA from BMV
2. RNA from BMV
3. RNA from BSMV
4. RNA from BSMV antigen-antibody precipitate
5. RNA from TMV
6. RNA from TMV antigen-antibody precipitate

B. Stained 5% polyacrylamide gels after electrophoresis of virus or antigen-antibody precipitates resuspended in protein dissociation buffer.

1. Protein from TMV
2. Protein from TMV antigen-antibody precipitate
3. Protein from BSMV
4. Protein from BSMV antigen-antibody precipitate


4
3
2
1
6
5
4
3
2
1

It is clear that the stained bands resulting from electrophoresis of RNA samples from virus or antigen-antibody precipitates are indistinguishable for both BSMV and TMV.

In this experiment, the TMV-RNA resolved into a single, quite diffuse component which migrated only a short distance into the gel. In experiments with fresher TMV than was used here, TMV-RNA resolved as two bands close together. The BSMV-RNA resolved as two components one of which migrated about 1/3 the length of the gel and a minor component which migrated a shorter distance but further than TMV-RNA. It is apparent that TMV-RNA is readily distinguishable from BSMV-RNA after electrophoresis on polyacrylamide gel.

As was mentioned in the materials and methods section, it is possible to estimate the molecular weight of RNA components by using a suitable set of standards, such as the four BMV-RNA components. A graph shown in figure 23 indicates how this is done. Two sets of values were used, taken from figure 22-A and figure 30 (table 7). The log mol. wt. of each of the four BMV-RNA components (1.09, 0.99, 0.75, 0.28 x 10^6) is plotted against the distance they migrated. The straight line drawn through these four points was extrapolated and used to obtain a figure for the log mol. wt. of the BSMV-RNA components and the TMV-RNA components. These gave values of 2.2 and 2.4 x 10^6 for the TMV-RNA components. The resolution of TMV-RNA into two components of similar molecular weights has been noted by others (Fowlks and Young, 1970). The two components are probably the consequence of two alternative conformational states for the single

Table 7. Values used to estimate the molecular weight of the RNA components of BSMV and TMV by polyacrylamide gel electrophoresis.

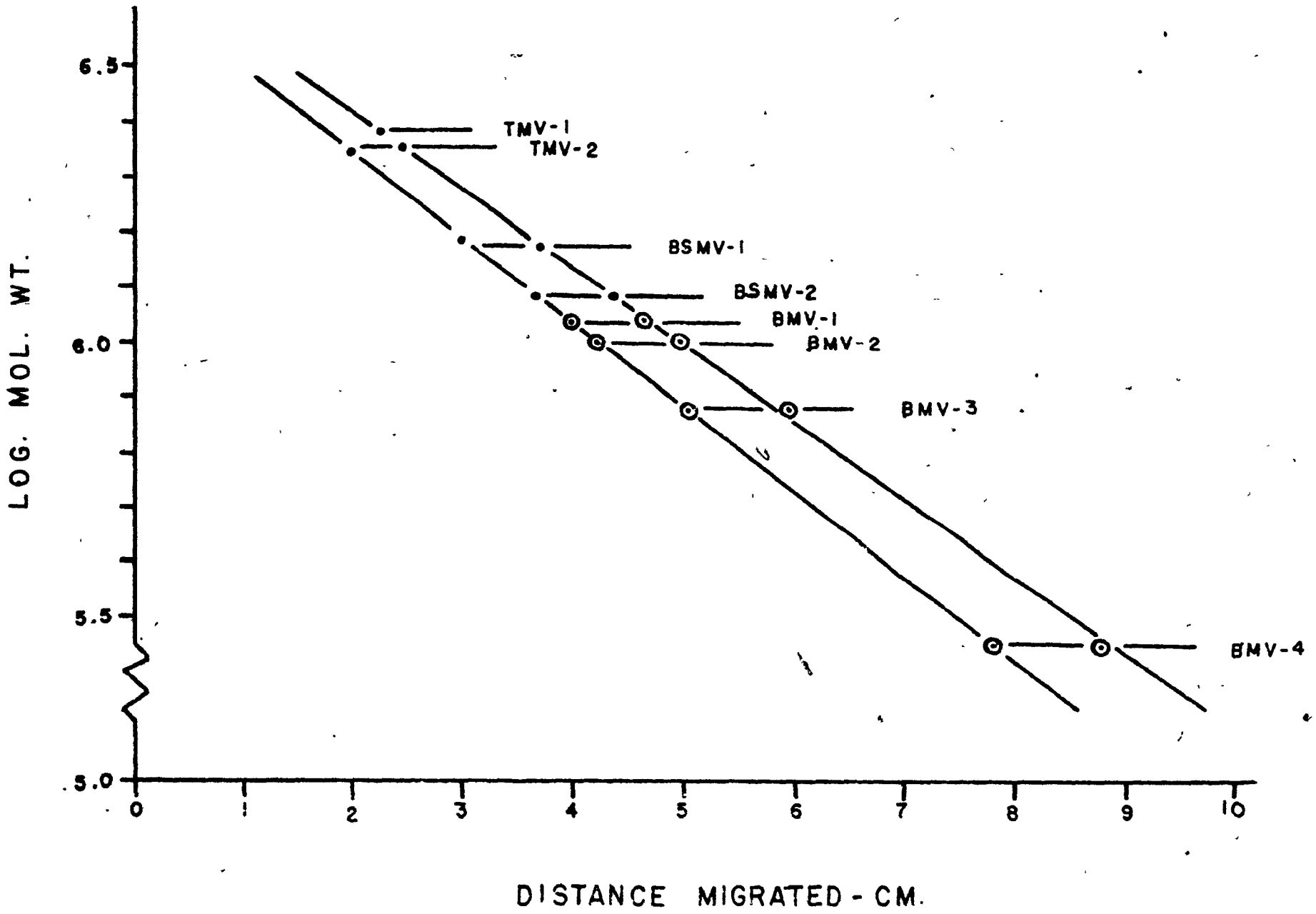
Expt		RNA components	Distance migrated (cm)	Log Mol. Wt.	Mol. Wt. x 10^6
A[a]	Standards	BMV-1	4.65[b]	6.0334[b]	1.09
		BMV-2	5.00	5.9956	0.99
		BMV-3	6.00	5.8751	0.75
		BMV-4	8.85	5.4472	0.28
	Unknowns	TMV-1	2.25[b]	6.37[c]	2.4
		TMV-2	2.45	6.34	2.2
		BSMV-1	3.70	6.18	1.5
		BSMV-2	4.40	6.08	1.2
B[a]	Standards	BMV-1	4.00[b]	6.0334[b]	1.09
		BMV-2	4.25	5.9956	0.99
		BMV-3	5.10	5.8751	0.75
		BMV-4	7.90	5.4472	0.28
	Unknowns	TMV-1	2.0[b]	6.34[c]	2.2
		BSMV-1	3.0	6.19	1.5
		BSMV-2	3.7	6.08	1.2

[a]Values for experiment A and B were taken from figures 30 and 22-A respectively.

[b]Values plotted in graph shown in figure 23.

[c]Values extrapolated from graph shown in figure 23.

Figure 23. Estimation of the molecular weight of the RNA components of BSMV and TMV by polyacrylamide gel electrophoresis. Mobilities of the unknowns were compared to the mobilities of BMV-RNA components. The values plotted are from table 7.


LOG. MOL. WT.
6.5
6.0
5.5
5.0
0
1
2
3
4
5
6
7
8
9
10
TMV-1
TMV-2
BSMV-1
BSMV-2
BMV-1
BMV-2
BMV-3
BMV-4
DISTANCE MIGRATED - CM.

molecule. The molecular weight values are higher than the 2.05×10^6d value normally given for TMV-RNA, a figure calculated from structural considerations, but, as mentioned, similar to the values calculated by others using this method.

The major and minor BSMV-RNA components had molecular weights of about 1.2×10^6d and 1.5×10^6d respectively, similar values to those calculated by Jackson and Brakke (1973) using this method.

For protein analysis 50µl of dissociated BSMV, TMV, BSMV antigen-antibody precipitate and TMV antigen-antibody precipitate were co-electrophoresed on 5.0% polyacrylamide gels. The stained gels are shown in figure 22-B. The BSMV virus sample produced a single protein band, as did the TMV sample except that the TMV protein had electrophoresed further into the gel than the BSMV protein, the predicted result since it is the smallest molecule.

These same protein bands were detected in the gels representing BSMV antigen-antibody precipitates or TMV antigen-antibody precipitates, indicating that both BSMV and TMV had been dissociated into their native proteins from antigen-antibody precipitates. Three extra bands were common to gels representing both types of antigen-antibody precipitates. These were presumed to be the antibody proteins which, fortunately, were of higher molecular weight than either of the viral proteins and migrated a much shorter distance into the gel. They would not interfere with analyses for the presence or absence of viral proteins.

The several proteins of known molecular weight described in the materials and methods section were used as standards to estimate the molecular weight of BSMV protein and TMV protein. The procedure was the same as that described and illustrated for RNA. The average of six determinations was 24,500 d for BSMV protein and 19,000 d for TMV protein, which can be compared with the figures quoted in the literature review of 21,500 d for BSMV protein and 17,500 d for TMV protein. These latter figures were determined by amino acid analysis.

No real attempt was made in this study to obtain a careful estimate of RNA or protein molecular weights by this method. However, the values obtained were sufficiently close to published values to leave no doubt as to the identity of the components detected.

These experiments demonstrate that BSMV- or TMV-RNA and protein can be released from antigen-antibody precipitates in a native state by the use of suitable dissociation buffers. The RNA and protein samples can be directly analysed by electrophoresis on suitable polyacrylamide gels. The TMV-RNA resolves as two components (mol. wt. 2.2 and 2.4 x 10^6d), which are probably conformational forms of a single molecule. BSMV-RNA resolves as two bands, a major component (mol. wt. 1.2 x 10^6d) and a minor component (mol. wt. 1.5 x 10^6d). Electrophoresis on 2.4% polyacrylamide gels separates BSMV-RNA and TMV-RNA extremely well, so that detection of one is not affected by the presence of the other. BSMV protein and TMV protein are separated from each other by electrophoresis on 5% polyacrylamide gels, and

again, detection of one is not affected by the presence of the other. Antibody proteins migrate only a short distance into the gels and do not interfere with the analysis of viral proteins.

B. Detection of RNA and protein in the same sample.

It was necessary to establish that TMV protein would be detected whenever TMV-RNA was detected in a sample (see part D of this section), using the division of sample adopted, which was to use four times as much virus to produce an RNA sample than was used to produce a protein sample.

As a first demonstration, TMV was diluted to 4.0 mg/ml in RNA dissociation buffer and to 1.0 mg/ml in protein dissociation buffer. The RNA and protein samples were then serially diluted in ten halving dilutions with RNA and protein dissociation buffer respectively. The ten RNA and ten protein samples were then analysed on 2.4% and 5% polyacrylamide gels respectively. The results are illustrated in figure 24. TMV-RNA was readily detected in the first five dilutions, was difficult to detect in the sixth (0.125 mg/ml) and seventh (0.062 mg/ml) dilutions and was not detected at higher dilutions. TMV protein was detected in all the parallel dilutions in which TMV-RNA was detected, that is up to the seventh dilution, and even in the next dilution. This result was duplicated in a second experiment.

Figure 24. Detection of TMV-RNA and protein in the same sample.

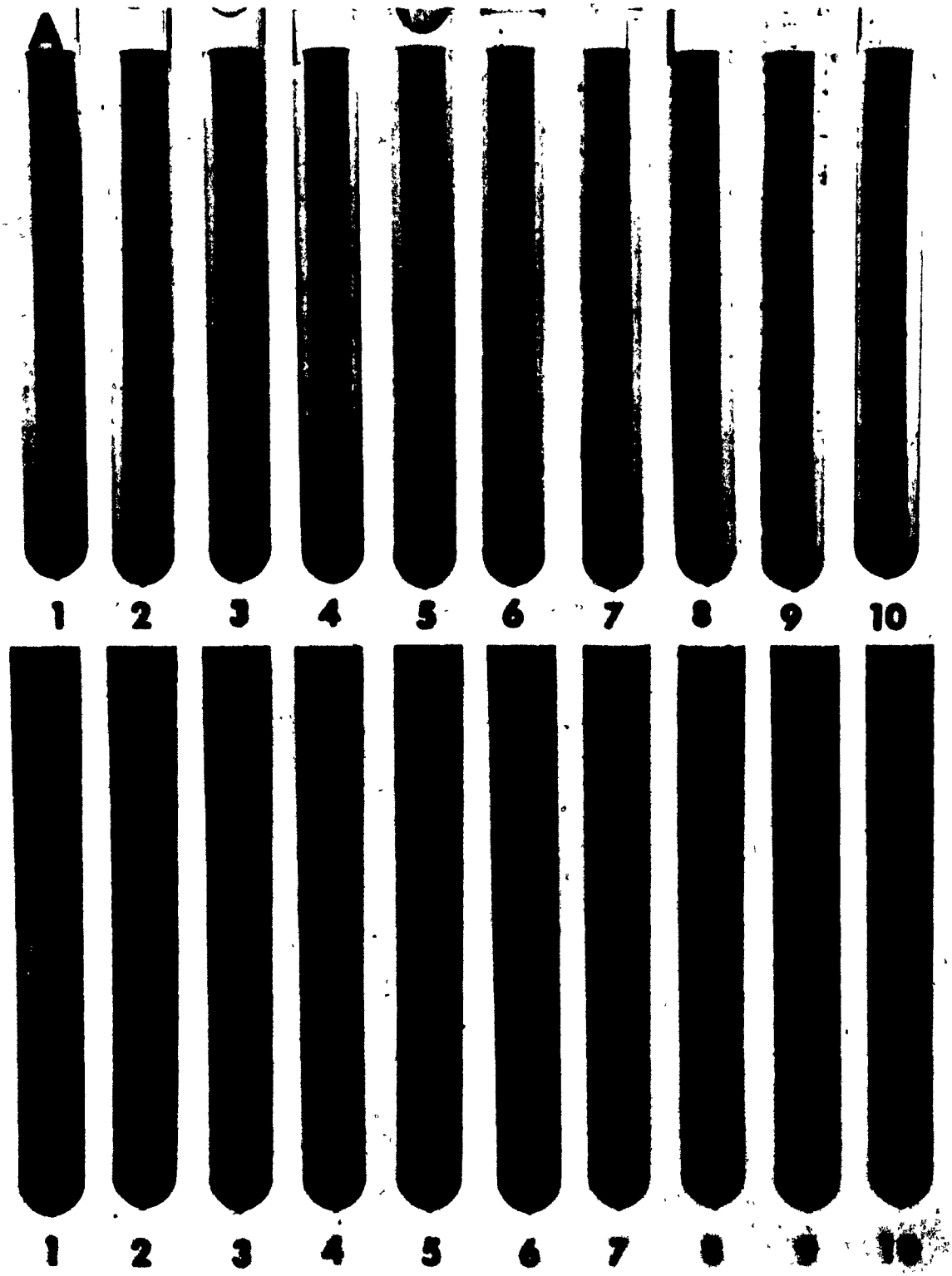
For RNA analysis, TMV was diluted to 4x mg/ml in RNA dissociation buffer.

For protein analysis, TMV was diluted to x mg/ml in protein dissociation buffer.

A. Stained 2.4% polyacrylamide gels after electrophoresis of RNA samples

B. Stained 5% polyacrylamide gels after electrophoresis of protein samples

TMV concentrations were 4 mg/ml in A_1 and 1 mg/ml in B_1. Halving dilutions are represented by 1-12.


1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7

As a second demonstration, six tubes were set up, each containing 1 ml of BSMV at 1 mg/ml. The first tube also contained TMV at 8 mg/ml, the second contained TMV at 4 mg/ml and there were further halving dilutions of TMV in the subsequent tubes. Each tube was made 10% with BSMV antibody and divided into aliquots of 0.8 ml and 0.2 ml. The BSMV antigen-antibody precipitate from each aliquot was collected by low speed centrifugation and resuspended in either 0.8 ml of RNA dissociation buffer (0.8 ml aliquot) or 0.8 ml of protein dissociation buffer (0.2 ml aliquot). The reason for this procedure was to collect six equal sized pellets of BSMV antigen-antibody precipitate which would be contaminated with decreasing amounts of TMV. After the pellets were dissociated there would be TMV-RNA as well as BSMV-RNA in the sample, but at some dilution the contaminating TMV-RNA would be at too low a concentration to detect. Based on the last experiment, TMV protein should also have been detected in all those samples in which TMV-RNA was detected.

The results of one experiment are shown in figure 25. When the BSMV antigen-antibody precipitate was collected in the presence of TMV at 8.0 mg/ml, TMV-RNA was readily detected as a contaminant, but separate from the BSMV-RNA. TMV had to be at a concentration of 2.0 mg/ml or more before it was detected as contaminating TMV-RNA. Similar results were obtained in the protein analysis except that, as before, TMV was detected at an even lower concentration. These results were duplicate in a second parallel experiment.

Figure 25. Detection of TMV-RNA and TMV protein in unwashed BSMV antigen-antibody precipitates contaminated with increasing concentrations of TMV

A. Stained 2.4% polyacrylamide gels after electrophoresis of BSMV antigen-antibody precipitates resuspended in RNA dissociation buffer

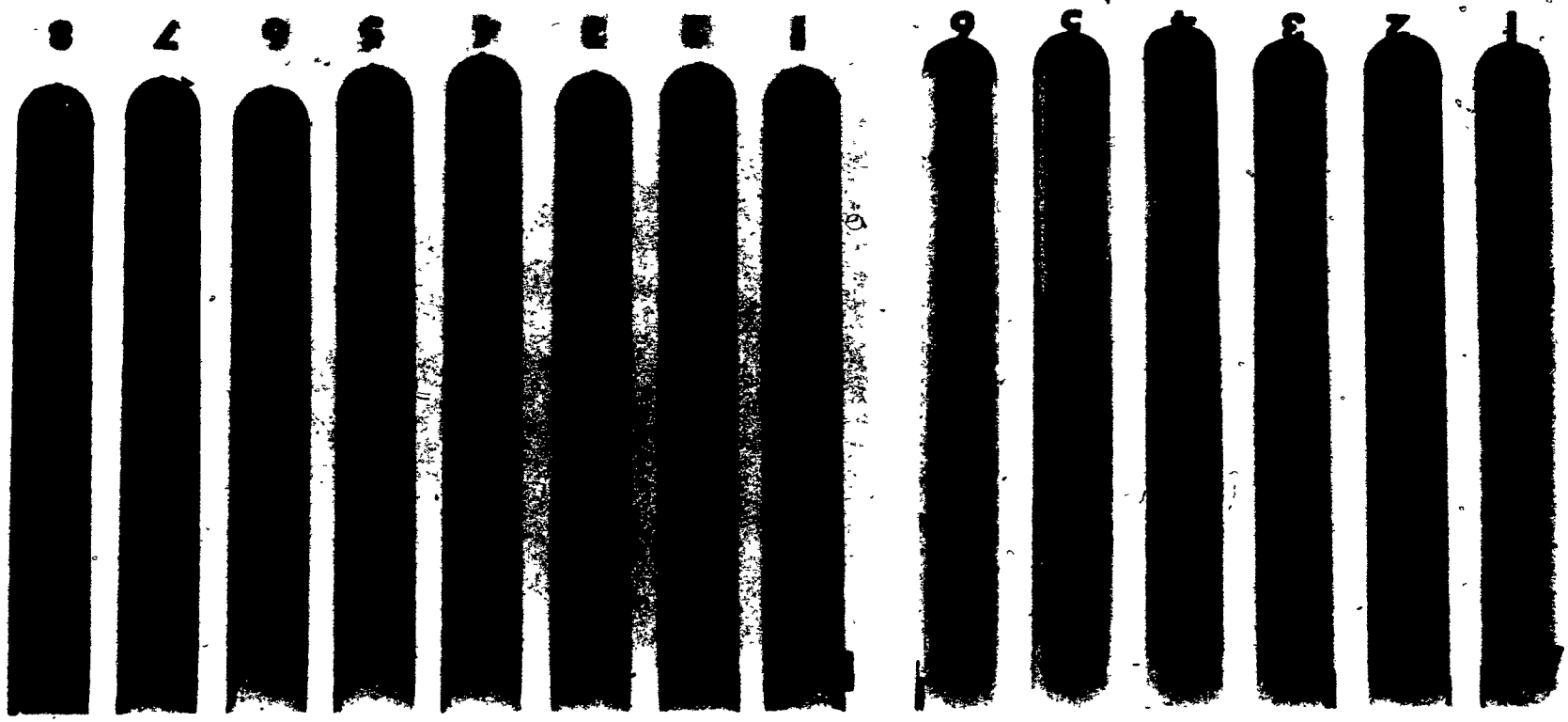
B. Stained 5% polyacrylamide gels after electrophoresis of BSMV antigen-antibody precipitates resuspended in protein dissociation buffer

1-6. BSMV antigen-antibody precipitates were collected from 1 ml solutions containing BSMV at 1 mg/ml and TMV at:

1. 0.25 mg/ml
2. 0.50 mg/ml
3. 1.00 mg/ml
4. 2.00 mg/ml
5. 4.00 mg/ml
6. 8.00 mg/ml

7. TMV protein

8. BSMV protein



In summary, upon dissociation of a BSMV antigen-antibody precipitate collected from a mixture of BSMV and TMV, if TMV-RNA is detected as well as BSMV-RNA, then TMV protein will be readily detected if the source of TMV-RNA is TMV particles contaminating the BSMV antigen-antibody precipitate. In addition, TMV would have had to have been at a concentration of 2.0 mg/ml or more in the mixture from which BSMV was precipitated if TMV-RNA were to be detected as a contaminant.

C. Removal of contaminating TMV from BSMV antigen-antibody precipitates by washing with PBS.

In the preparative experiment described in the materials and methods section, 6 mg of BSMV were precipitated with BSMV antibody. The precipitate was collected as a pellet by low speed centrifugation and washed six times with 9 ml of PBS each time. The experiment presented here is to demonstrate that this method was more than adequate in ridding the BSMV antigen-antibody precipitate of any contaminating TMV particles.

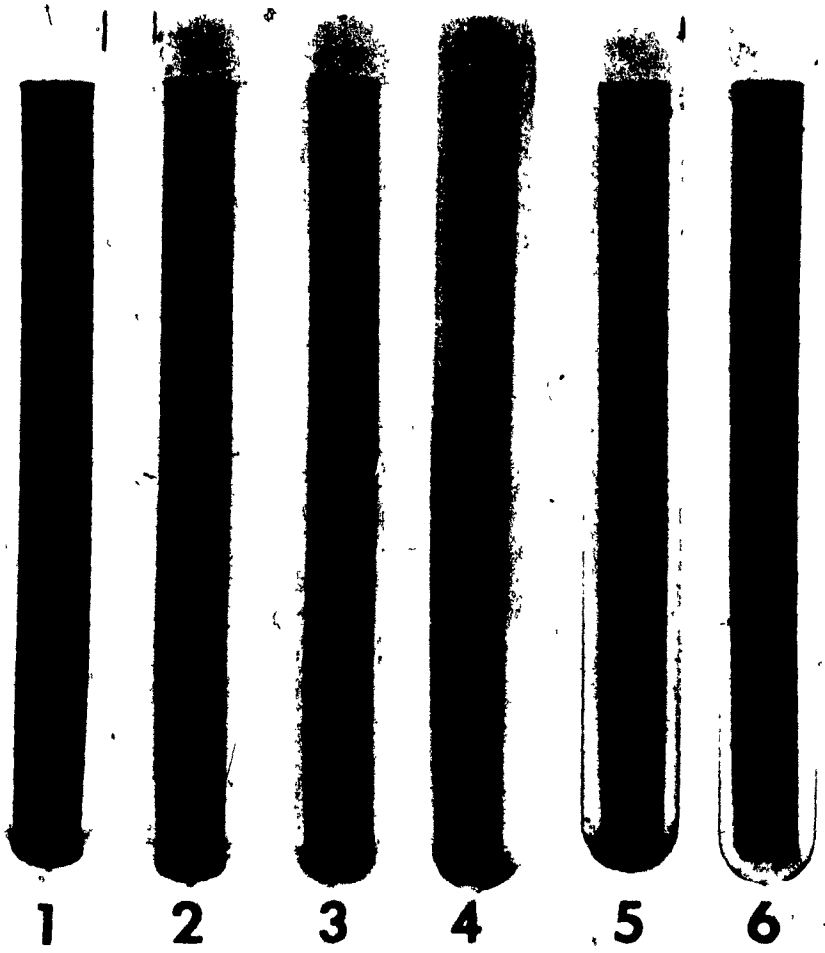
Six duplicate tubes were set up containing 1 ml of PBS, 1 mg BSMV, 8 mg TMV and 0.1 ml of BSMV antibody. The BSMV was collected from each tube as an antigen-antibody precipitate which was either left unwashed, or washed one to five times using 1.5 ml of PBS each time. Normally, precipitates containing 6 mg of BSMV were washed six times with 9 ml of PBS each time. The final precipitates were each resuspended in 1.0 ml of RNA dissociation buffer. The RNA sample was analysed by electrophoresis on 2.4% polyacrylamide gels and the results are shown in figure 26.

Figure 26. Detection of TMV-RNA in BSMV antigen-antibody precipitates washed different numbers of times to remove contaminant TMV.

Stained 2.4% polyacrylamide gels after electrophoresis of BSMV antigen-antibody precipitates resuspended in RNA dissociation buffer.

BSMV antigen-antibody precipitates were collected from 1 ml solutions containing BSMV at 1 mg/ml and TMV at 8.0 mg/ml. The precipitates were washed with 1.5 ml of PBS -

1. five times
2. four times
3. three times
4. twice
5. once
6. unwashed.


1
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6

TMV-RNA, along with BSMV-RNA was detected in the sample prepared from the unwashed BSMV antigen-antibody precipitate and to a lesser extent from the precipitate which had been washed only once. Further washings, however, reduced the contaminating TMV to non-detectable levels. Therefore, it can be assumed that no significant number of TMV particles remain non-specifically held in BSMV antigen-antibody precipitates collected from mixtures of BSMV and TMV and washed 6 times with PBS.

D. Summary of results

In this section it has been established that the RNA and protein components of antigen-antibody precipitates can be identified as native viral RNA and protein by electrophoresis on polyacrylamide gels. What is more, this method adequately separates BSMV-RNA or protein from TMV-RNA or protein. The presence of RNA or protein of one virus does not interfere with the detection of the RNA or protein of the other virus.

To collect BSMV from mixtures of BSMV and TMV, BSMV is precipitated with BSMV antibody and the precipitate is washed several times with PBS. It has been established that the washing procedure is sufficient to rid the BSMV antigen-antibody precipitate of contaminating TMV. On the other hand, after dissociation of such BSMV antigen-antibody precipitates, if TMV-RNA, as well as BSMV-RNA, is detected, the protein analysis will confirm the source of the TMV-RNA. If the source is contaminating TMV particles not washed out of the precipitate, then TMV protein will be readily detected, as well

as BSMV protein. If only BSMV protein is detected then the washed BSMV antigen-antibody precipitate contained both BSMV-RNA and TMV-RNA, but no TMV protein. The significance of that result will be the subject of the next section.

3. Second approach: component analysis of washed antigen-antibody precipitates from artificial or real mixtures

The preparation of washed antigen-antibody precipitates was outlined earlier in table 3. The results of four experiments are summarised in table 8 and illustrated in figures 27-33. With the results of experiment 1 as an example, the explanation of table 3 is as follows. Leaf tissue from pooled third and fourth leaves from BSMV or BSMV-TMV inoculated plants was collected 20 days after inoculation. The percentage of doubly inoculated plants that became doubly infected was 70%, a figure determined by the microprecipitin test. Doubly infected tissue (40 g) was extracted and after an analysis of antibody treated samples by quantitative sucrose density gradient centrifugation, it was estimated that 1 mg of BSMV (40 mg total) and 3 mg of TMV (120 mg total) were extracted from each 1 mg of tissue. An artificial mixture was constructed by adding 200 mg of TMV to the extract prepared from 40 g of BSMV-infected tissue. This provided an artificial mixture with the ratio of BSMV:TMV (1:5) greater than that in the real mixture (1:3). The mixtures were still in the form of clarified sap. BSMV antigen-antibody precipitates and TMV antigen-antibody precipitates were collected from both mixtures. The precipitates were washed with PBS. Aliquots of the washed precipitates

Table 8. RNA and protein components detected in antigen-antibody precipitates from artificial and real mixtures of BSMV and TMV.

Expt.	Leaf	Infection	% DI[a]	Wt. g	mg/g[b] BSMV	Ratio[c] B:T	mg TMV added	Ag-Ab ppt.	RNA[d] BSMV	RNA[d] TMV	Protein[d] BSMV	Protein[d] TMV
1.	3+4	BSMV	-	40	1.0	1:5	200	BSMV	+	-	+	-
								TMV	-	+	-	+
								BSMV[e]	+	-	+	-
								TMV[e]	-	+	-	+
		BSMV/TMV	70	40	1.0	1:3	-	BSMV	+	+	+	-
								TMV	-	+	-	+
								BSMV	+	+	+	-
								TMV	-	+	-	+
2.	1	BSMV	-	12	0.5	5:5	6	BSMV	+	-	+	-
		BSMV/TMV	NT	13	0.5	5:1	-	BSMV	+	-	+	-
	2	BSMV	-	7.5	1.0	4:4	8	BSMV	+	-	+	-
		BSMV/TMV	NT	7.0	1.0	4:1	-	BSMV	+	-	+	-
	3+4	BSMV	-	7.0	0.75	1:3	200	BSMV	+	-	+	-
		BSMV/TMV	50	5.0	0.75	1:2	-	BSMV	+	+	+	-

Table 8. continued

Expt.	Leaf	Infection	% DI[a]	Wt. g	mg/g[b] BSMV	Ratio[c] B:T	mg TMV added	Ag-Ab ppt.	RNA[d] BSMV	RNA[d] TMV	Protein[d] BSMV	Protein[d] TMV
3.	1	BSMV	-	13	0.5	5:5	6	BSMV	+	-	NT	NT
		BSMV/TMV	NT	13	0.5	5:1	-	BSMV	+	-	NT	NT
	2	BSMV	-	5	1.0	3:3	6	BSMV	+	-	NT	NT
		BSMV/TMV	NT	8	1.0	3:1	-	BSMV	+	-	NT	NT
	3	BSMV	-	10	1.0	1:2	20	BSMV	+	-	NT	NT
		BSMV/TMV	100	7	1.0	1:2	-	BSMV	+	+	NT	NT
	4	BSMV	-	11	0.75	1:3	33	BSMV	+	-	NT	NT
		BSMV/TMV	100	11	0.75	1:3	-	BSMV	+	+	NT	NT
4.	3+4	BSMV/TMV	50	41	NT	1:6	-	BSMV	+	+	+	-
								TMV	-	+	-	+
5.	1	BSMV/TMV	50	10.0	0.75	7:1	-	BSMV	+	-	+	-
	2	BSMV/TMV	50	8.5	1.5	2:1	-	BSMV	+	-	+	-
	3	BSMV/TMV	70	8.0	1.5	1:2	-	BSMV	+	+	+	-
	4	BSMV/TMV	90	10.0	1.5	1:3	-	BSMV	+	+	+	-

Table 8. continued

[a] DI - doubly infected

[b] An approximate value determined by quantitative sucrose density gradient centrifugation, or the predicted maximum.

[c] For artificial mixtures, the ratio was adjusted by addition of partially purified TMV until there was an equal amount or excess amount to that in the real mixtures. For real mixtures, the ratio was determined as in b.

[d] Components detected by polyacrylamide gel electrophoresis of dissociated antigen-antibody precipitates.

[e] Antigen-antibody precipitates collected from partially purified mixtures. All other results from clarified sap mixtures.

were dissociated in either RNA or protein dissociation buffer then analysed by polyacrylamide gel electrophoresis. The stained gels are illustrated in figure 27.

Only the major and minor BSMV-RNA components were detected in the BSMV antigen-antibody precipitate from the artificial mixture, which implies that all contaminating TMV had been washed out of the precipitate (figure 27-A_1). In the BSMV antigen-antibody precipitate from the real mixture, however, a small amount of TMV-RNA was detected as well as BSMV-RNA (figure 27-A_2). The protein analysis of the same precipitates indicated the presence of only BSMV protein (figure 27-$B_{1,2}$). Therefore, both BSMV-RNA and TMV-RNA , but only BSMV protein, were present in the BSMV antigen-antibody precipitate from the real mixture. In the TMV antigen-antibody precipitate from both the artificial and real mixtures, only TMV-RNA (figure 27-$A_{3,4}$) and TMV protein (figure 27-$B_{3,4}$) were detected. A single antibody protein was common to all four precipitates. The TMV protein standard was overloaded and resolved as a very broad band of TMV monomer protein and, in addition, what was assumed to be a small dimer band.

The viruses in the artificial and real mixtures were also partially purified and then used to prepare washed antigen-antibody precipitates with BSMV antibody and TMV antibody. The RNA components detected in these four precipitates are shown in figure 28, where they are compared with those detected in equivalent precipitates collected from clarified sap. Clarified sap was the only source of precipitates in subsequent

Figure 27. Experiment 1. RNA and protein components from washed antigen-antibody precipitates from artificial and real mixtures of BSMV and TMV prepared from leaves 3+4.

A. Stained 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the artificial mixture.

2. Washed BSMV antigen-antibody precipitates from the real mixture.

3. Washed TMV antigen-antibody precipitates from the artificial mixture.

4. Washed TMV antigen-antibody precipitates from the real mixture.

5. RNA from BSMV.

6. RNA from TMV.

B. Stained 5% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in protein dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the artificial mixture.

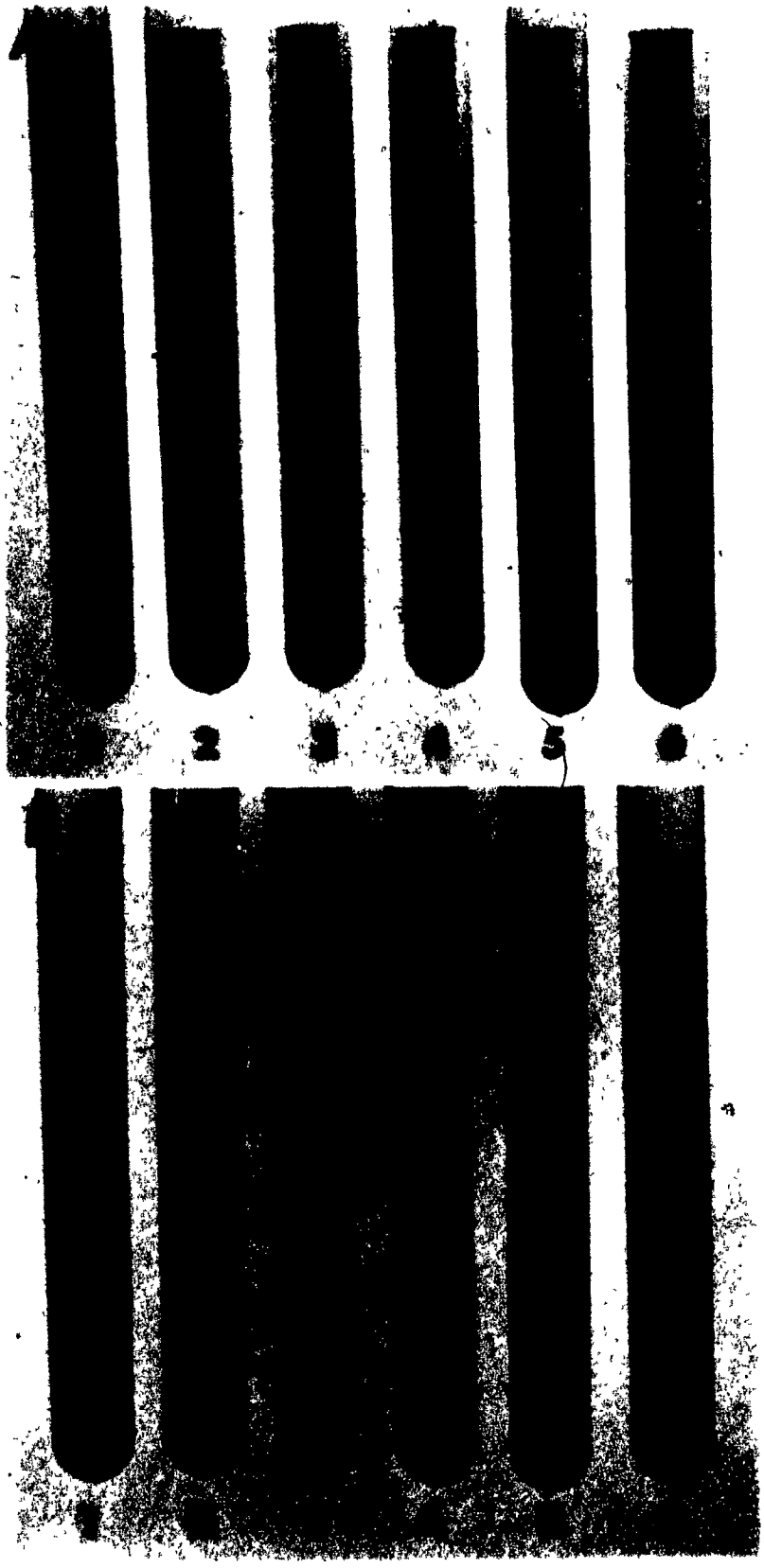
2. Washed BSMV antigen-antibody precipitates from the real mixture.

3. Washed TMV antigen-antibody precipitates from the artificial mixture.

4. Washed TMV antigen-antibody precipitates from the real mixture.

5. Protein from BSMV.

6. Protein from TMV.



The text continues on page 124, which is preceded on page 123 by the legend for figure 28.

Figure 28. Experiment 1. RNA components from washed antigen-antibody precipitates from artificial and real mixtures of BSMV and TMV prepared from leaves 3+4. Clarified sap mixtures compared with partially purified mixtures.

Stained 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer.

1,6 washed BSMV antigen-antibody precipitates from the artificial mixture.

2,7 washed BSMV antigen-antibody precipitates from the real mixture.

3,8 washed TMV antigen-antibody precipitates from the artificial mixture.

4,9 washed TMV antigen-antibody precipitates from the real mixture.

5 RNA from BMV.

1-4 precipitates collected from clarified sap mixtures.

6-8 precipitates collected from partially purified mixtures.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experiments. The results were the same, the important result being the detection of TMV-RNA as well as BSMV-RNA in the washed BSMV antigen-antibody precipitate from the real mixture (figure 28-7). The bands in this particular gel are uneven because the tip of the pipette used to layer the sample penetrated the top of the gel. The RNA from BMV was electrophoresed for comparison. In addition to the four expected components, two thin bands of higher molecular weight components were resolved. Their origin is not known.

The quantitative description of the preparative steps for the other four experiments will not be described but the values can be found in table 8 and substituted in the description of experiment 1.

In experiment 2, in addition to sampling leaves 3 and 4 at 20 days after inoculation, leaves 1 and 2 were also analysed at 7 and 14 days after inoculation respectively. Only small amounts of TMV were detected in leaf 1 and 2 and so correspondingly small quantities of TMV were used in the construction of artificial mixtures. Only BSMV antigen-antibody precipitates were analysed (figure 29). Those prepared from leaves 1 and 2 contained only BSMV-RNA and BSMV protein, whether from artificial or real mixtures. This was also the result for precipitates collected from artificial mixtures prepared from leaves 3 and 4. TMV-RNA in addition to BSMV-RNA, but only BSMV protein, was detected again in washed antigen-antibody precipitates from real mixtures prepared from pooled leaves 3 and 4 (figure 29-A_2,B_2).

Figure 29. Experiment 2. RNA and protein components from washed antigen-antibody precipitates from artificial and real mixtures of BSMV and TMV prepared from leaves 3+4.

A. Stained 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the artificial mixture.

2. Washed BSMV antigen-antibody precipitates from the real mixture.

3. RNA from TMV.

4. RNA from BMV.

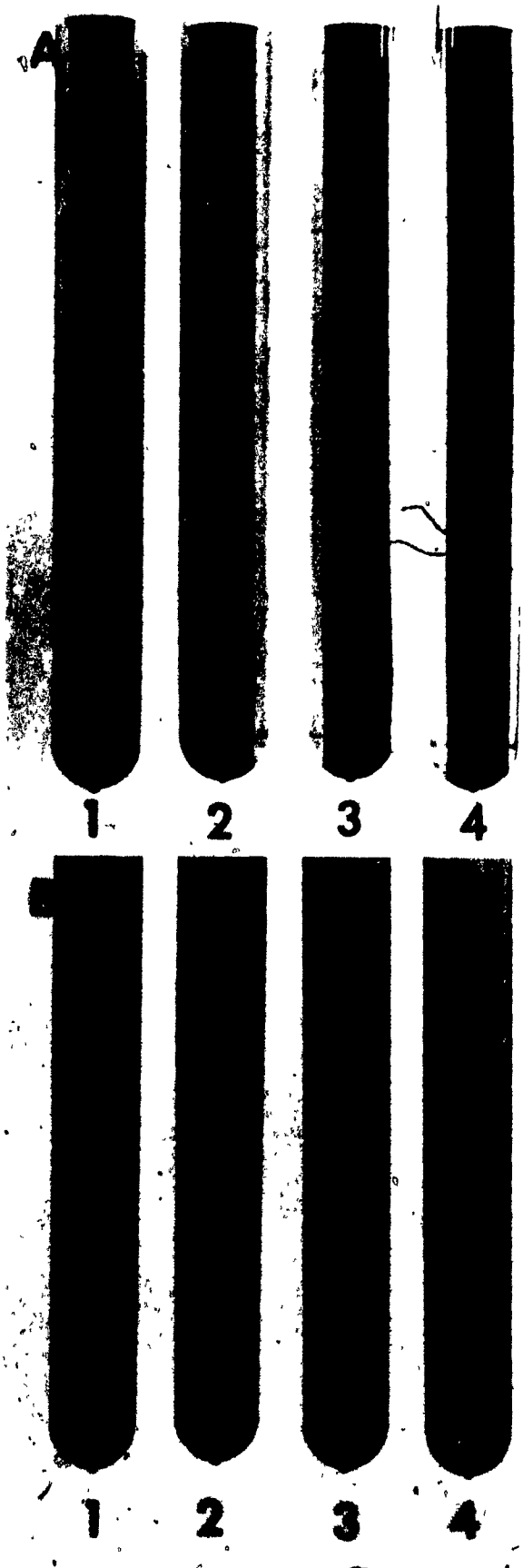
B. Stained 5% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in protein dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the artificial mixture.

2. Washed BSMV antigen-antibody precipitates from the real mixture.

3. Protein from BSMV.

4. Protein from TMV.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In experiment 3, all four leaf positions were tested. Only the RNA components in BSMV antigen-antibody precipitates from artificial and real mixtures were analysed. As before only BSMV-RNA was detected in precipitates from artificial mixtures prepared from tissue from all four leaf positions. This was also true for precipitates from real mixtures from leaves 1 and 2. Both TMV-RNA and BSMV-RNA were detected in the BSMV antigen-antibody precipitates from real mixtures from leaves 3 and 4. The TMV-RNA was particularly obvious in the leaf 4 sample (figure 30-3), more so than in experiments 1 and 2. Experiment 3 was chronologically the first experiment and at that time co-analysis of proteins had been conceived, but not developed.

In the final experiments (descriptively and chronologically experiments 4 and 5), it was decided not to use artificial mixtures. A considerable saving of purified TMV and also antibody was achieved by this decision. The combined protein and RNA analysis of each precipitate made the use of artificial mixtures less necessary. As was described in part 2D of this section, the source of any TMV-RNA detected in a BSMV antigen-antibody precipitate from a real mixture can be decided upon by the presence or absence of TMV protein in the corresponding protein analysis.

In experiment 4, only pooled doubly infected tissue from leaves 3 and 4 was analysed. TMV-RNA, BSMV-RNA and BSMV protein were detected in washed BSMV antigen-antibody precipitates, and only TMV-RNA and TMV protein were detected in washed TMV antigen-antibody precipitates (figure 31).

Figure 30. Experiment 3. RNA components from washed antigen-antibody precipitates from artificial and real mixtures of BSMV and TMV prepared from leaf 4.

Stained 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer.

1. RNA from BMV.
2. Washed BSMV antigen-antibody precipitates from the artificial mixture.
3. Washed BSMV antigen-antibody precipitates from the real mixture.
4. Washed TMV antigen-antibody precipitates from the artificial mixture.
5. Washed TMV antigen-antibody precipitates from the real mixture.


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Figure 31. Experiment 4. RNA and protein components from washed antigen-antibody precipitates from real mixtures of BSMV and TMV prepared from leaves 3+4.

Stained 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the real mixture.

2. Washed TMV antigen-antibody precipitates from the real mixture.

Stained 5% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in protein dissociation buffer.

3. Washed BSMV antigen-antibody precipitates from the real mixture.

4. Washed TMV antigen-antibody precipitates from the real mixture.


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3
4

In experiment 5, BSMV antigen-antibody precipitates were analysed from all four leaf positions from doubly infected plants. BSMV-RNA and BSMV protein were detected in the precipitates from all four leaf positions. TMV-RNA was detected in the precipitates from the third and fourth leaves, but not from the first and second leaves. No TMV protein was detected in precipitates from any leaf position, including those which contained TMV-RNA (figure 32). The gels illustrated in figure 32 are shorter and wider than previously illustrated and the RNA bands are fainter. These were the gels cast in quartz tubes which were scanned at 254 nm before being removed from the tubes to be stained. The bands are fainter because only 25µl, rather than the standard 50µl, were layered on a gel surface with a greater area than normal. Larger quantities resulted in off scale absorbance profiles. The protein analysis was on standard gels using 50µl samples.

A point of interest is the quality of the BSMV-RNA. The single major components detected in samples from leaves 3 and 4 resolved as two narrowly spaced bands in samples from leaves 1 and 2. The splitting of the BSMV-RNA major band was also recorded, but not photographed, in the leaf 1 sample prepared from artificial and real mixtures in experiment 2. A single major component was detected in all other samples from leaves 1 and 2 in the other experiments.

The absorbance profiles of the gels illustrated in figure 32 are shown in figure 33. The following peaks, corresponding to stained bands, were detected. The major BSMV peak was half way along the profile and to the right of this was the minor BSMV peak. The two components in the

Figure 32. Experiment 5. RNA and protein components from washed antigen-antibody precipitates from real mixture of BSMV and TMV from leaf 1, 2, 3, and 4, respectively.

A. Stained 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 1.

2. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 2.

3. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 3.

4. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 4.

5. RNA from TMV

6. RNA from BMV.

B. Stained 5% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in protein dissociation buffer.

1. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 1.

2. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 2.

3. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 3.

4. Washed BSMV antigen-antibody precipitates from the real mixture from leaf 4.

5. protein from BSMV

6. protein from TMV.

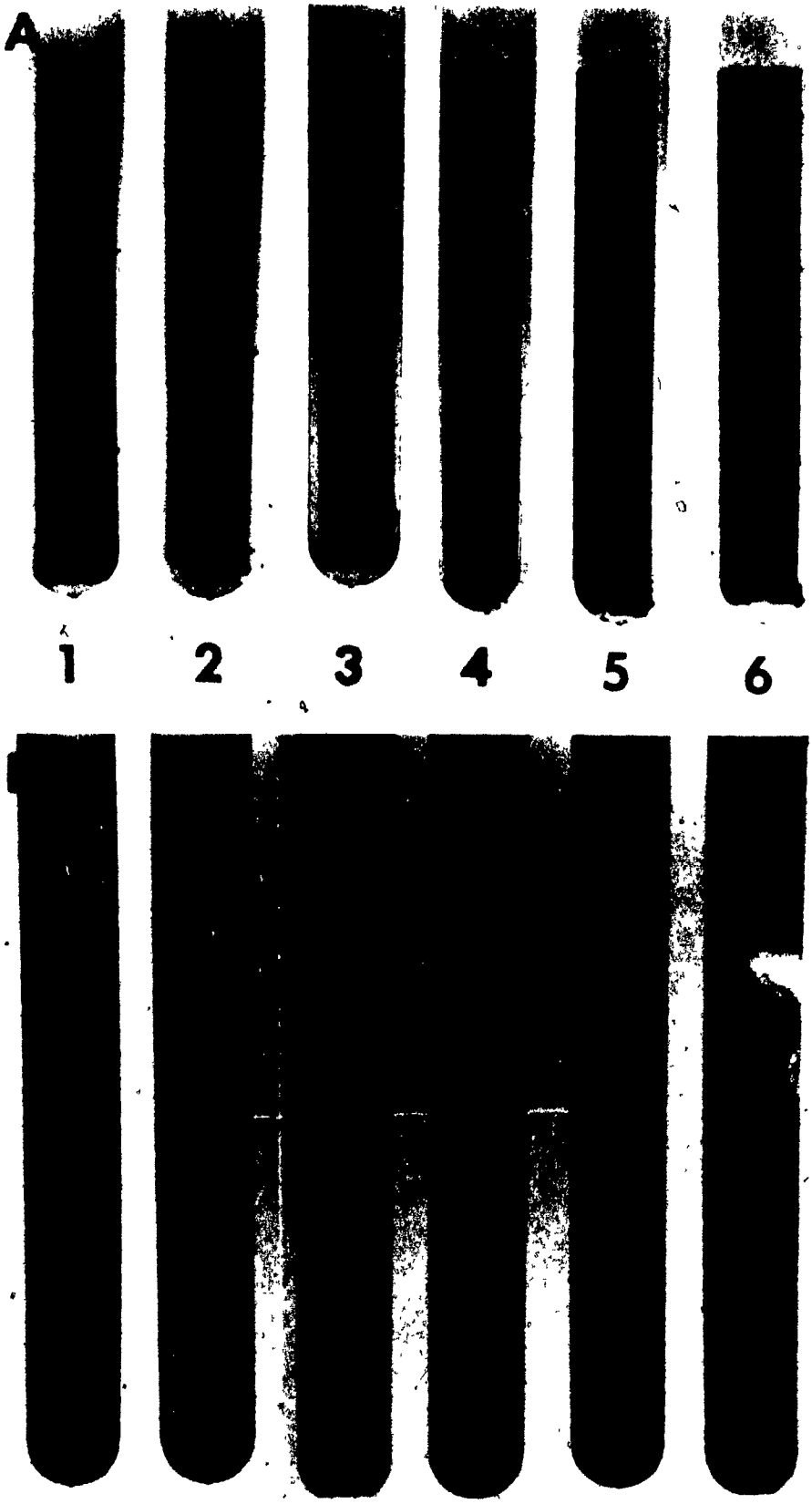
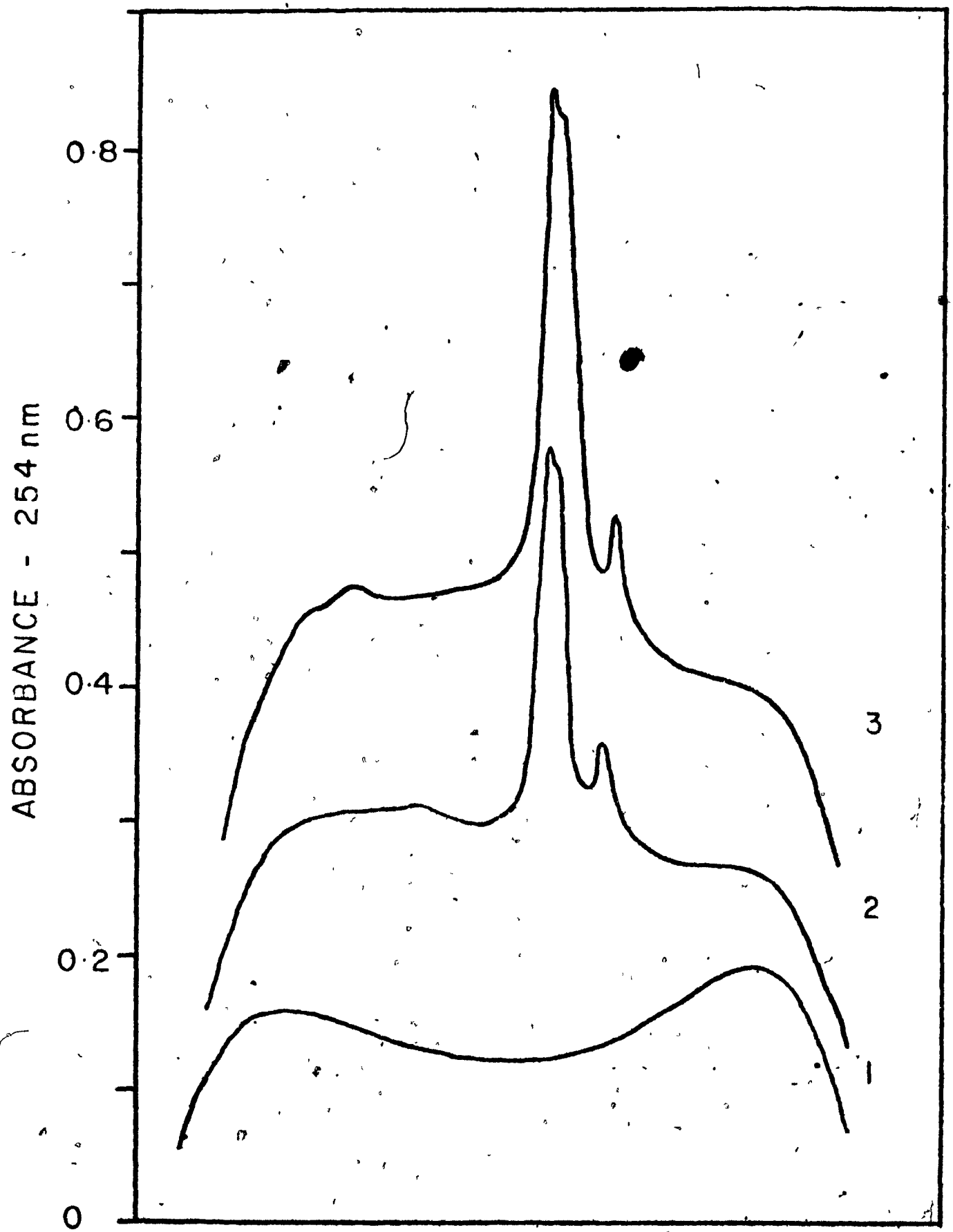
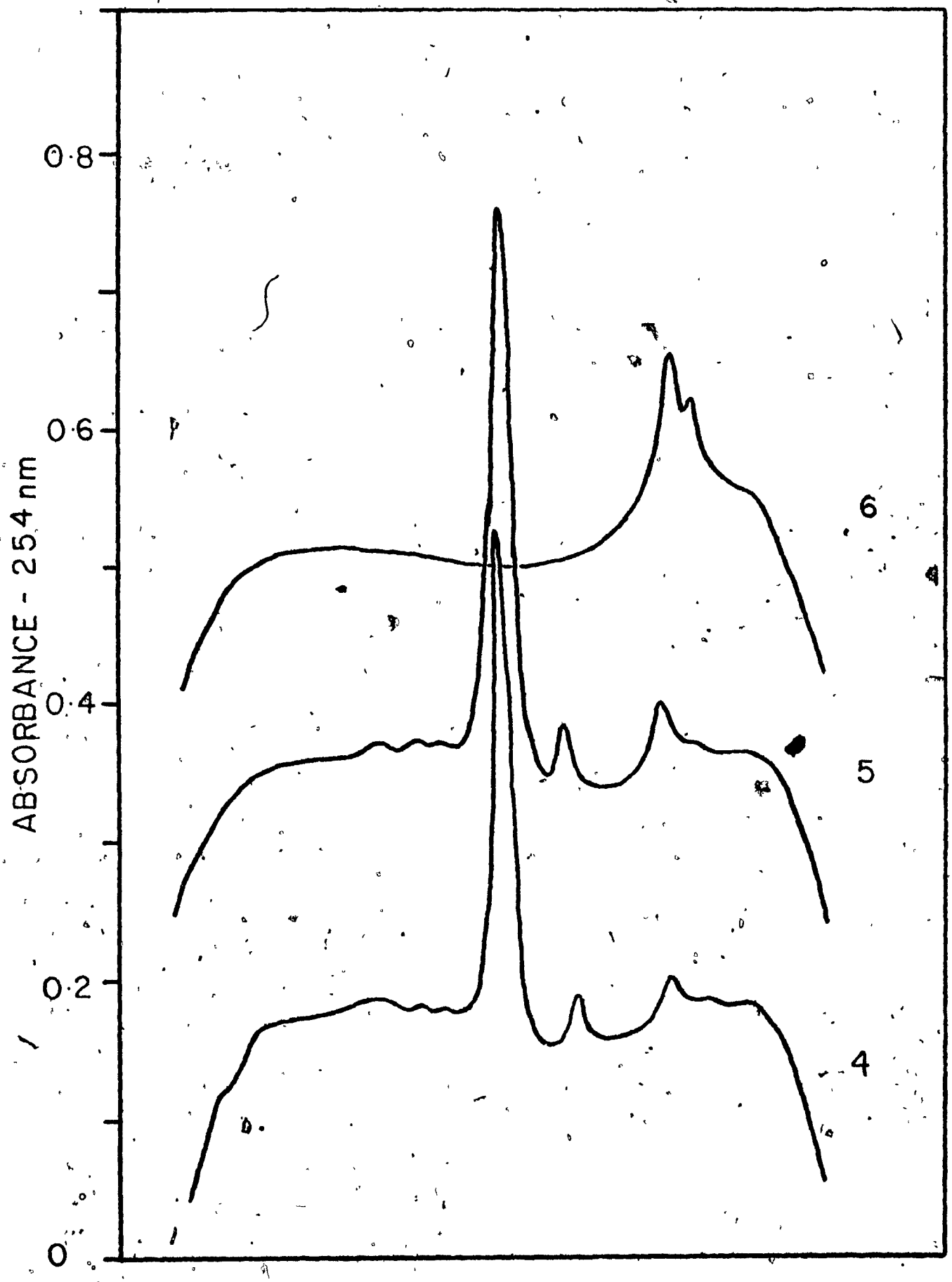

A
1
2
3
4
5
6

Figure 33. Experiment 5. RNA components from washed antigen-antibody precipitates from real mixtures of BSMV and TMV prepared from leaves 1, 2, 3, and 4 respectively.

Absorbance profiles at 254 nm of 2.4% polyacrylamide gels after electrophoresis of antigen-antibody precipitates resuspended in RNA dissociation buffer. Direction of electrophoresis from right to left.

1. Absorbance baseline after electrophoresis of dissociation buffer.
2. Washed BSMV antigen-antibody precipitate from the real mixture from leaf 1.
3. Washed BSMV antigen-antibody precipitate from the real mixture from leaf 2.
4. Washed BSMV antigen-antibody precipitate from the real mixture from leaf 3.
5. Washed BSMV antigen-antibody precipitate from the real mixture from leaf 4.
6. RNA from TMV.


ABSORBANCE - 254 nm
0·8
0·6
0·4
0·2
0
3
2
1


0·8
0·6
0·4
0·2
0
ABSORBANCE - 254 nm
6
5
4

major peak from the leaf 1 and 2 samples were resolved, but less clearly than in the stained gels. Some very minor peaks were resolved; these had electrophoresed beyond the major BSMV-RNA components. They were not visible in the equivalent stained gels but similar bands were just detectable in the stained gels shown in figure 30. TMV-RNA is still absent in leaf 1 and 2 samples, but is clearly resolved in leaf 3 and 4 samples, near the top of the gel. The TMV-RNA peak was superimposed upon the lower molecular weight component of the TMV-RNA standard, which resolved as two peaks.

The areas beneath each of the peaks are proportional to the amount of RNA contained in the bands they represent, and these areas were estimated with a planimeter. The percentage of TMV-RNA in the total RNA (BSMV-RNA + TMV-RNA) in antigen precipitated by BSMV antibody can then be estimated. The percentages are shown in table 9, along with the data on the quantitative recovery of virus in the same experiment.

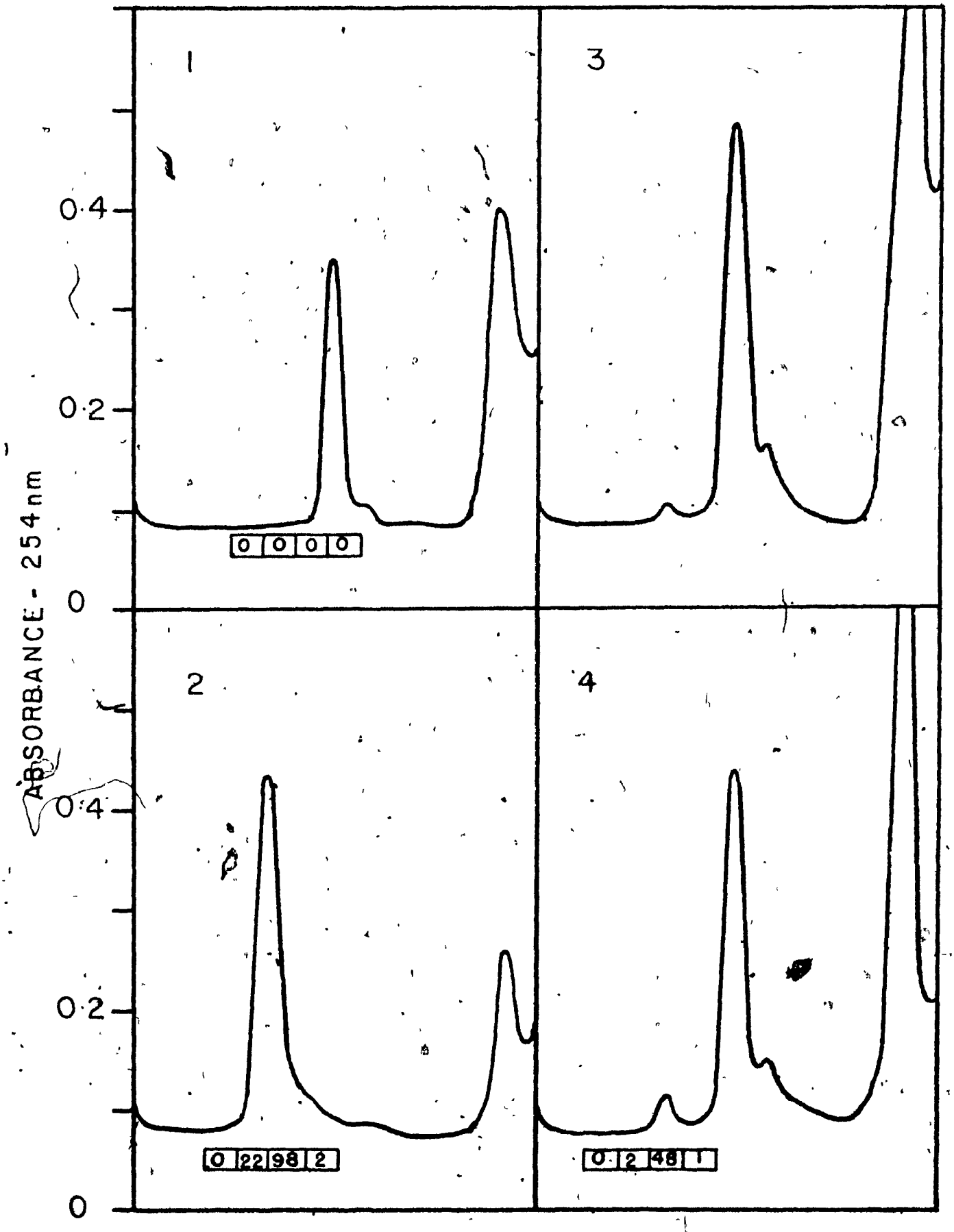
The RNA components from the leaf 3 and 4 samples and the RNA from BSMV and TMV were also analysed on linear log sucrose density gradients and the absorbance profiles of centrifuged gradients are shown in figure 34. These results were the same as those described above, but presented in a different way. BSMV-RNA resolved as a major peak which had sedimented half-way down the gradients. The major BSMV-RNA component had a shoulder of slower sedimenting RNA associated with it. The failure to resolve the 1.5×10^6 minor BSMV-RNA component detected by polyacrylamide gel electrophoresis, which should have sedimented deeper than

Figure 34. Experiment 5. RNA components from washed antigen-antibody precipitates from real mixtures of BSMV and TMV prepared from leaves 3 and 4 respectively.

Absorbance profiles at 254 nm of linear-log sucrose density gradients after centrifugation of antigen-antibody precipitates resuspended in RNA dissociation buffer. Direction of sedimentation from right to left.

1. RNA from BSMV.
2. RNA from TMV.
3. Washed BSMV antigen-antibody precipitate from the real mixture from leaf 3.
4. Washed BSMV antigen-antibody precipitate from the real mixture from leaf 4.

The bars beneath the profiles mark the zones collected for infectivity tests. The numerals between the bars are the average numbers of TMV lesions produced on one leaf of Chenopodium amaranticolor by the zone sample.


1
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0·4
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0
0·4
0·2
0
ABSORBANCE - 254 nm
0 0 0 0
0 22 98 2
0 2 48 1

the major component, has also been observed by Jackson and Brakke (1973). They suggest that the minor component has an unusual conformation which results in it co-sedimenting with the 1.2×10^6 major component, in sucrose density gradient centrifugation. TMV-RNA sedimented about 2/3 the depth of the gradient. The samples from both leaf 3 and leaf 4 contained BSMV-RNA and a small amount of TMV-RNA. The absorbance areas under these peaks were also measured and computed as before and the results are included in table 9.

The component from BSMV antigen-antibody precipitates from real mixtures, which has been described as TMV-RNA because it co-electrophoresed with TMV-RNA, could foreseeably be an electrophoretic artifact. The fact that this component also co-sedimented with TMV-RNA in sucrose density gradient centrifugation indicates that, if it is an artifact, it must have the same molecular weight and have many other properties in common with TMV-RNA. In the next part of this section, the component is confirmed as TMV-RNA by infectivity.

This, the major section of the thesis, can be summarised as follows. Only BSMV-RNA and BSMV protein were detected in 9 washed BSMV antigen-antibody precipitates from artificial mixtures. Four of these, before washing, had been contaminated with large amounts of TMV. The washing procedure was therefore adequate for the removal of TMV particles from BSMV antigen-antibody precipitates. In the three experiments where leaves 1 and 2 were analysed, the BSMV antigen-antibody precipitates collected from real mixtures contained only BSMV-RNA and BSMV protein.

Table 9. Experiment 5. Virus recovery and genomic masking of TMV-RNA in different leaves from doubly infected plants.

Leaf[a] position	Sampling[b] date	mg virus/g leaf[c] BSMV	TMV	% genomic[d] masking	
1	7	0.7	0.1	0[e]	-[f]
2	10	1.8	1.0	0	-
3	14	1.8	3.5	5.5	4.5
4	20	1.5	4.5	8.5	8.0

[a] 1 is first leaf to emerge, inoculated 7 days after seeding. 2, 3, 4 are successively younger, uninoculated leaves.

[b] Days after inoculation of leaf 1.

[c] Determined by quantitative sucrose density gradient centrifugation of antibody treated extracts.

[d] Expressed as percentage of RNA in antigen precipitated by BSMV antibody that was TMV-RNA.

[e] Determined from absorbance profiles of polyacrylamide gels.

[f] Determined from absorbance profiles of linear, log suerose density gradients.

In each of 5 experiments that analysed leaves 3 and 4, either separately or as pooled tissue, the washed BSMV antigen-antibody precipitates collected from the real mixtures contained both BSMV-RNA and TMV-RNA, but only BSMV protein (protein was not analysed in one experiment). When a quantitative comparison was made between leaves 3 and 4 (table 9), more TMV-RNA (8% of the total RNA) was detected in antigen precipitated by BSMV antibody from fourth leaf samples than from third leaf samples (5% of total RNA).

The inability of the washing procedure to remove TMV-RNA from BSMV antigen-antibody precipitates from the real mixtures, and the inability to detect TMV protein in these same precipitates will be discussed in the conclusions section of part II. However, genomic masking of TMV-RNA in BSMV protein in doubly infected plants is the simplest explanation of these results.

In contrast to the above results, in two analyses, genomic masking of BSMV-RNA in TMV protein was not detected in leaves 3 or 4 from doubly infected plants.

4. Fidelity of genomically masked TMV-RNA

In the last section, the detection of TMV-RNA as well as BSMV-RNA in dissociated BSMV antigen-antibody precipitates from real mixtures was described. The TMV-RNA was resolved in 2.4% polyacrylamide gels and also in linear log sucrose density gradients. Fractions from the sucrose density gradients illustrated in figure 34 were collected and

inoculated to Chenopodium amaranticolor. The numbers of lesions produced by each fraction are shown in figure 34. The samples which produced most lesions were those collected from that part of the gradient which contained TMV-RNA. This confirms that the unexpected component extracted from BSMV antigen-antibody precipitates was biologically active TMV-RNA. The results of experiments which began with the lesions produced on Chenopodium amaranticolor are shown in table 10.

TMV particles 300 nm long were detected in negatively stained extracts prepared from ten lesions induced by TMV-RNA extracted from BSMV antigen-antibody precipitates (genomically masked TMV-RNA). TMV particles were also detected in each of five negatively stained extracts from lesions induced by normal TMV-RNA. No particles were detected in each of five extracts from tissue pieces taken from uninoculated Chenopodium amaranticolor leaves.

Other lesions were also excised, extracted, and inoculated to Nicotiana glutinosa and Nicotiana tobaccum. The extracts from lesions induced by genomically masked TMV-RNA produced few or no lesions on Nicotiana glutinosa, and this was also true for extracts from lesions induced by normal TMV-RNA. This could be because Chenopodium amaranticolor contains an inhibitor of TMV infectivity (Suckbir et al, 1964). Extracts from healthy leaves produced no lesions. All the extracts from lesions induced by either genomically masked TMV-RNA or

Table 10. Properties of virus isolated from genomically masked TMV-RNA

Inoculum	Lesions sampled[a]	300 nm particles[b]	Infectivity on N. glutinosa[c]	N. tobaccum[d]	Isolate code	Virus yield mg/g[e]	Normal TMV profile[f]	Absorbance area[g]	TMV antigen in barley[h]
Genomically masked TMV-RNA	1	+	4	+	GM_1	2.5	+	221	+
	2	+	1	+	GM_2	3.0	+	264	+
	3	+	1	+	GM_3	2.5	+	220	+
	4	+	0	+	GM_4	3.0	+	259	+
	5	+	12	+	GM_5	3.5	+	355	+
	6	+	0	+	GM_6	2.0	+	174	
	7	+	15	+	GM_7	3.0	+	269	
	8	+	29	+	GM_8	4.0			
	9	+	0	+	GM_9	3.5			
	10	+	0	+	GM_{10}	3.5			
Normal TMV-RNA	1	+	0	+	N_1	2.5	+	216	
	2	+	23	+	N_2	4.0	+	361	
	3	+	3	+	N_3	3.0			
	4	+	2	+	N_4	3.5			
	5	+	4	+	N_5	3.0			
Uninoculated	1	-	0	-					
	2	-	0	-					
	3	-	0	-					
	4	-	0	-					
	5	-	0	-					

Table 10. continued

[a] produced on Chenopodium amaranticolor.

[b] detected by electron microscopy of negatively stained extracts from lesions produced on Chenopodium amaranticolor.

[c] lesion/leaf after inoculation with an extract from lesions produced on C. amaranticolor.

[d] ± systemic infection after inoculation with an extract from lesions produced on C. amaranticolor

[e] from systemically infected tobacco plants.

[f] after scanning centrifuged gradient tubes at 254 nm.

[g] corresponding to virus in centrifuged gradients.

[h] twenty days after inoculation with BSMV and TMV isolates GM_1-GM_5.

normal TMV-RNA elicited a systemic infection in Nicotiana tobaccum. No infections in tobacco were observed after inoculation with healthy tissue extract.

The symptoms on tobacco were the same, regardless of the source of inoculum, and were typical of those induced by TMV. In tobacco plants, therefore, there were 10 isolates of TMV derived from genomically masked TMV-RNA (GM_1 - GM_{10}) and five isolates derived from normal TMV-RNA (N_1 - N_5). The yield of virus from each of these 15 plants was approximately the same, between 2.0 and 4.0 mg/g which is a normal value for TMV.

Seven of the partially purified isolates of TMV derived from genomically masked TMV-RNA and two of the isolates derived from normal TMV-RNA were diluted to about 0.5 mg/ml and analysed by sucrose density gradient centrifugation (figure 35). The actual dilutions were such as to indicate the relative recovery of virus from equal amounts of tobacco tissue. A preparation of standard TMV, which was essentially unaggregated virus, sedimented to about 2/3 the depth of the gradient. The isolate derived from normal TMV-RNA (N_1) had an unaggregated component, but also a series of components in different states of aggregation which sedimented progressively further into the gradient. This is quite characteristic of TMV purified by the polyethylene glycol method. The two isolates derived from genomically masked TMV-RNA (GM_1, $_2$) had the same aggregated quality. The other isolates analysed (GM_3 - GM_7, N_2), but not illustrated, also had the same quality.

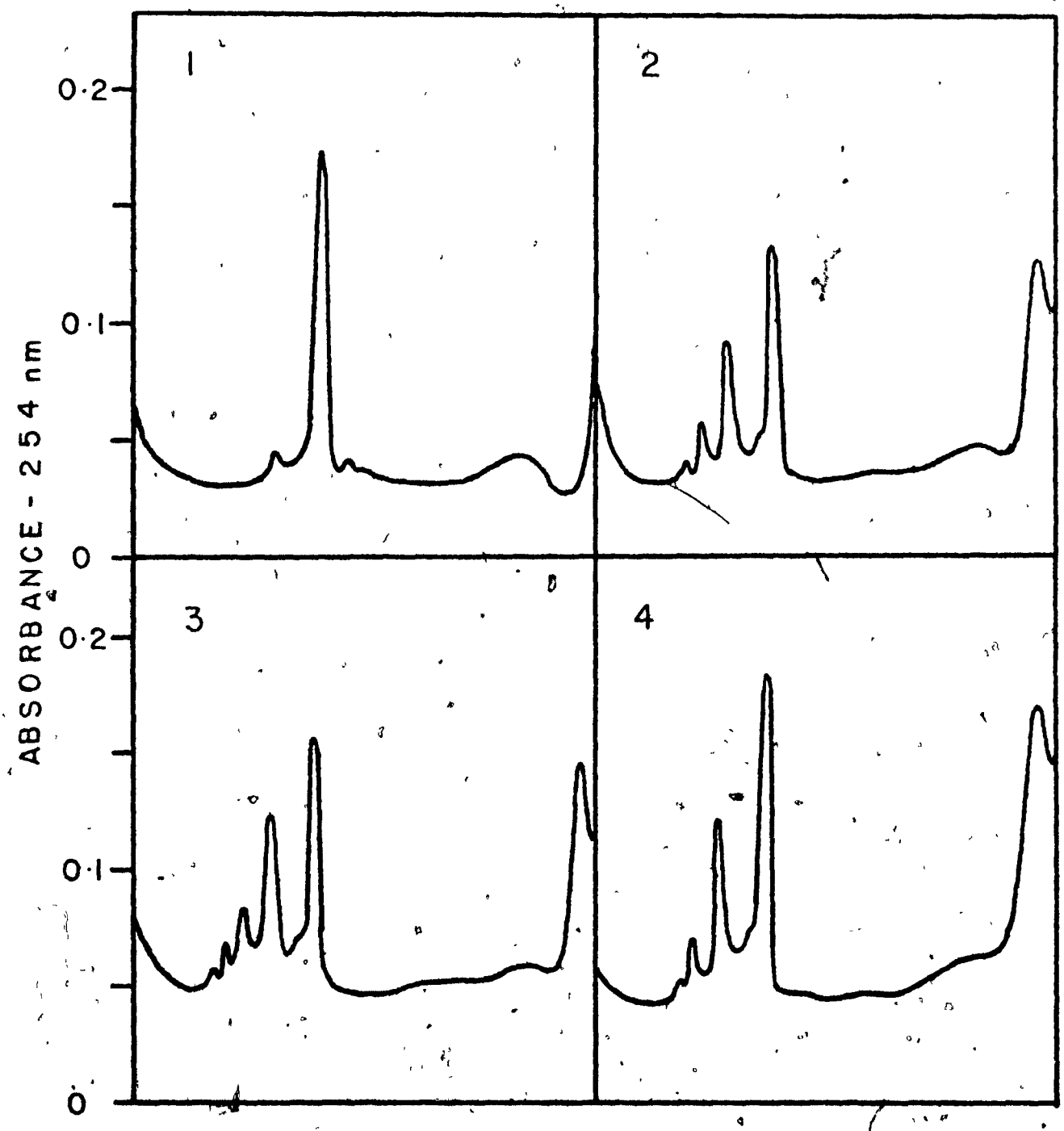
Figure 35. Quality of TMV derived from genomically masked TMV-RNA in sucrose density gradient centrifugation.

Absorbance profiles of 10-40% linear sucrose gradients in 0.01 M phosphate buffer pH 7.0. Samples were centrifuged for 45 minutes at 40,000 rpm in a Beckman SW41 rotor. Direction of sedimentation from right to left.

1 Pure TMV (unaggregated).

2 TMV derived from normal TMV-RNA.

3,4 TMV derived from genomically masked TMV-RNA.


1
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ABSORBANCE - 254 nm

The amount of virus recovered from equal amounts of tobacco tissue for each isolate (GM_1 - GM_7, N_1 - N_2) was indicated by the absorbance area corresponding to virus in each absorbance profile. Visual inspection of the profiles (figure 35) and the actual areas determined by planimetry (table 10) indicated that the recovery of virus, as well as its quality, was about the same for all isolates regardless of source.

Five isolates (GM_1 - GM_5) derived from genomically masked TMV-RNA were co-inoculated with BSMV to barley seedlings, which were then grown to the fourth leaf stage at 30°. Extracts from fourth leaves from each of the five sets of barley plants reacted positively with TMV antibody in the microprecipitin test. TMV particles, as well as BSMV particles, were readily detected in negatively stained preparations from these extracts.

The partially purified TMV isolates were also analysed in Ouchterlony double diffusion tests. The results are shown in figure 36. Control reactions are illustrated in plate A. Five antigens were used, virus solvent (water), healthy tobacco sap, the purified TMV illustrated in figure 35, a partially purified isolate derived from normal TMV-RNA (N_1) and a partially purified isolate derived from genomically masked TMV-RNA (GM_1). None of the antigens reacted positively with normal serum or BSMV antibody. Water and healthy tobacco sap reacted negatively with both TMV antibodies tested, but all three TMV preparations reacted positively. With TMV antibody-1, all TMV preparations reacted by producing a dense precipitin line close to the antigen well. These

Figure 36. Serological relatedness of TMV derived from either genomically masked TMV-RNA or normal TMV-RNA.

Ouchterlony double diffusion plates made from 1% agar in 0.01 M phosphate buffer pH 7.2.

A. Antibody wells

A TMV antibody-1
B BSMV antibody
C TMV antibody-2
D Normal serum

Antigen wells

1 TMV from genomically masked TMV-RNA (GM_1)
2 Tobacco sap
3 Water
4 Tobacco sap
5 Purified TMV
6 TMV from normal TMV-RNA (N_1)

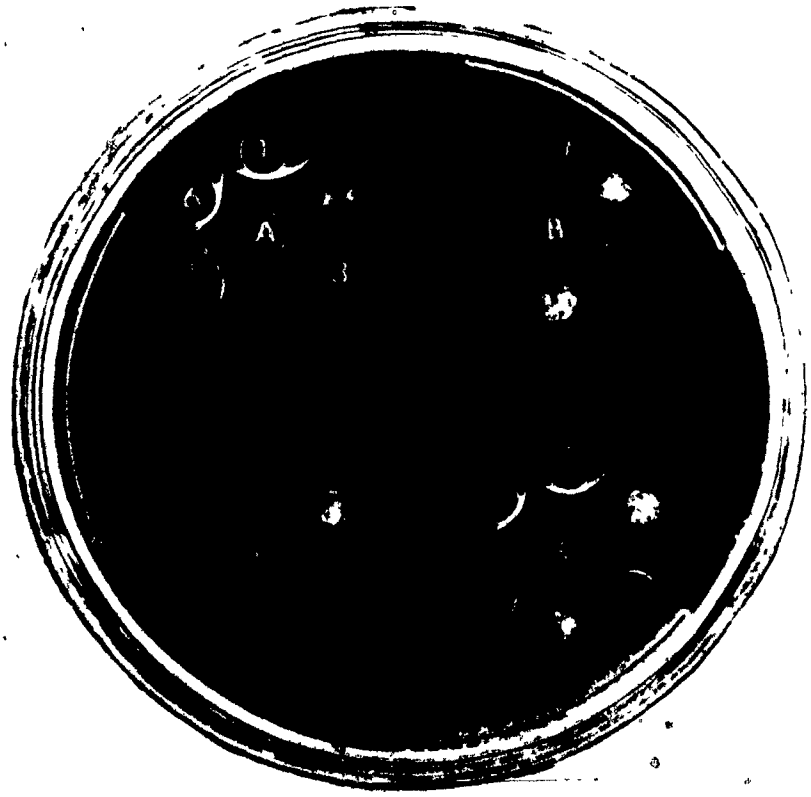
B. Antibody wells

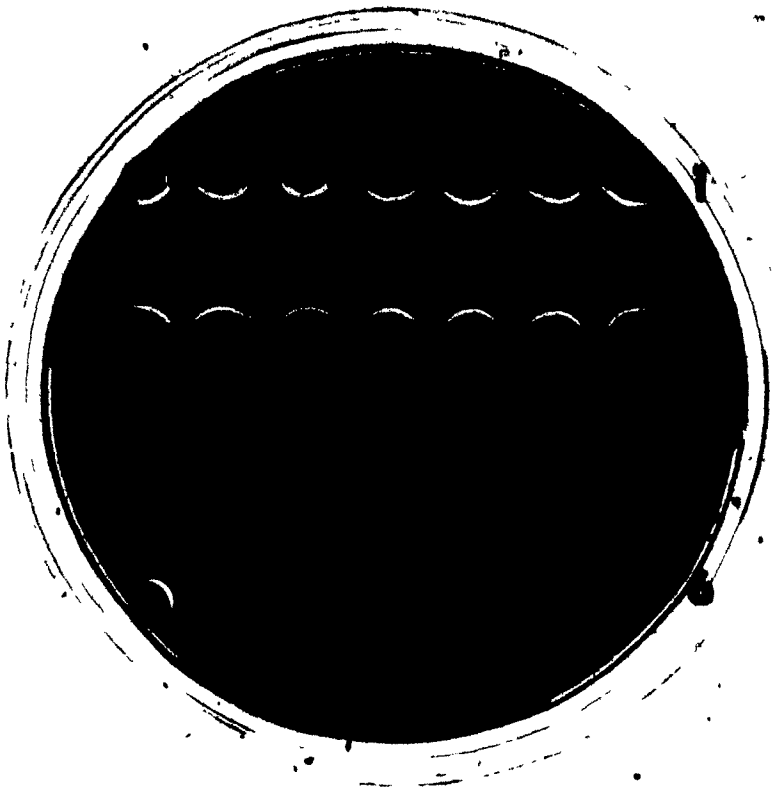
Row 2 TMV antibody
Row 5 Normal serum

Antigen wells

Row 1, 4 from left to right: GM_1, N_1, GM_2, N_2, GM_3, N_3, GM_4.

Row 3, 6 from left to right; GM_5, N_4, GM_6, N_5, GM_7, N_6, GM_8.


A
B



lines fused with one another completely. In addition, a second precipitin line formed mid-way between each TMV antigen well and the TMV antibody well. This second line was absent in the test using TMV antibody-2, but the major line was present. The major line close to the antigen well is characteristic of slowly diffusing antigens such as TMV particles. The origin of the minor band using TMV antibody-1 is uncertain. It was not a host component contaminant since tobacco sap did not react, and the purified TMV should have been free of host components. The most likely explanation is that TMV antibody-1 contained a substantial amount of antibody capable of reacting strongly with fast diffusing TMV-A protein (low molecular weight viral protein aggregates). Such antibody was presumably absent in TMV antibody-2.

TMV antibody-1 was used in figure 36-B to test the relatedness of the TMV isolates derived from either genomically masked TMV-RNA or normal TMV-RNA. These two kinds of TMV isolates were placed in alternate antigen wells as explained in figure 36. It is apparent that the precipitin lines produced by reaction with TMV antibody fused with each other completely. Had there been any major serological differences between the two kinds of isolates, this would have been expressed by the formation of spurs, or precipitin lines extending beyond the points of fusion. None were detected.

In summary, 10 TMV isolates, derived from genomically masked TMV-RNA isolated from a BSMV antigen-antibody precipitate, could not be distinguished from 5 TMV isolates derived from normal TMV-RNA. The original genomically masked TMV-RNA inoculum produced lesions on Chenopodium amaranticolor typical of TMV. These lesions contained typical TMV-rods. The infectivity extracted from these lesions was low; few lesions were produced on Nicotiana glutinosa. This could have been because Chenopodium amaranticolor contains an inhibitor of TMV infectivity (Suckbir et al, 1964). All lesion extracts were able to infect tobacco, however, and produced normal symptoms of a TMV infection. The yield of virus from each tobacco plant and the quality of the partially purified TMV after sucrose density gradient centrifugation were typical of normal TMV. The TMV isolates derived from genomically masked TMV-RNA, when inoculated together with BSMV, were able to infect barley grown at 30°. These isolates were also indistinguishable from normal TMV in Ouchterlony double diffusion tests against TMV antibody.

CONCLUSIONS

Two methods were used to separate BSMV from equivalent artificial and real mixtures of BSMV and TMV. The first method, removal of TMV by precipitation with TMV antibody, successfully eliminated all TMV infectivity from BSMV derived from artificial mixtures. The BSMV derived from equivalent real mixtures, however, retained TMV infectivity when similarly treated.

The second method was to collect BSMV from a mixture in the form of a BSMV antigen-antibody precipitate. The precipitate was then washed free of conta minating TMV particles. Washed BSMV antigen-antibody precipitates derived from artificial mixtures contained BSMV-RNA but not TMV-RNA, indicating that the separatory method was successful. Washed BSMV antigen-antibody precipitates from real mixtures, however, consistently contained BSMV-RNA and TMV-RNA.

Two different methods, therefore, failed to separate either TMV infectivity or TMV-RNA from BSMV derived from real mixtures of BSMV and TMV, even though separation was complete from equivalent artificial mixtures.

One possible explanation for these results is genomic masking of TMV-RNA in BSMV protein in doubly infected plants. Such particles would behave antigenically as if they were BSMV particles. They would not be precipitated by TMV antibody when using the first separatory method and would be left in suspension along with normal BSMV. They would then impart TMV infectivity to the BSMV from real mixtures. Similarly, because genomically masked TMV-RNA would behave antigenically as if it were BSMV, it would become part of the BSMV antigen-antibody precipitate collected from real mixtures, when the second separatory method was used. No amount of washing would remove such TMV-RNA and so, after the precipitate was dissociated, TMV-RNA and BSMV-RNA would be detected.

Any alternative explanation would have to imply a major qualitative difference between quantitatively equivalent artificial and real mixtures. This difference would have to result in the inability of TMV antibody to

precipitate all normal TMV from real mixtures, when using the first method, even though this was possible from artificial mixtures. The TMV infectivity associated with BSMV derived from real mixtures would then be the consequence of normal TMV particles being present. Similarly, when the second method was used, normal TMV particles would somehow be held more firmly in BSMV antigen-antibody precipitates from real mixtures than from artificial mixtures.

Confirmatory experiments distinguished between these alternatives. The TMV infectivity associated with BSMV derived from the real mixture was neutralised by BSMV antibody, but not by TMV antibody. This is the expected result for genomic masking of TMV-RNA in BSMV protein. Had the source of TMV infectivity been normal TMV particles, the neutralisation results would have been reversed. Similarly, even though both TMV-RNA and BSMV-RNA were detected in washed BSMV antigen-antibody precipitates from real mixtures, no TMV protein was detected in these precipitates, only BSMV protein was detected. Again, this is the expected result for genomic masking of TMV-RNA in BSMV protein. Control experiments demonstrated that the TMV protein would have been detected had the source of TMV-RNA been contaminating normal TMV particles.

The conclusion is therefore inescapeable that genomic masking of TMV-RNA in BSMV protein takes place in doubly infected barley plants. The component analysis of washed BSMV antigen-antibody precipitates answered the questions to what extent and in which leaves this phenomenon occurred.

Four leaf positions were analysed. Genomic masking of TMV-RNA was not detected in the inoculated leaf (leaf 1) or the first leaf to emerge after inoculation (leaf 2) from doubly infected plants. These leaves synthesise large quantities of BSMV, but low quantities of TMV. It was in leaves 3 and 4, which synthesise large amounts of BSMV and even larger amounts of TMV, that genomic masking of TMV-RNA was consistently detected. In these leaves, between 5-8% of the total RNA in BSMV-like particles was TMV-RNA, the percentage being highest in leaf 4, which also synthesises the most TMV. This may not represent a maximum percentage, as results of the next section will illustrate.

The TMV-RNA which became genomically masked was biologically normal. It was infectious and the product of infection was normal TMV particles in both singly infected tobacco and doubly infected barley.

Despite the detection of genomic masking of TMV-RNA by BSMV protein, the reverse phenomenon, encapsulation of BSMV-RNA in TMV protein, was not detected when TMV antigen-antibody precipitates from real mixtures were analysed.

SECTION III. SEED TRANSMISSION FROM BARLEY PLANTS DOUBLY INFECTED WITH BSMV AND TMV

INTRODUCTION

Although the mechanism involved in seed transmission of plant viruses are poorly understood (Bennett, 1969), the various physical barriers proposed to account for the lack of transmission of many viruses, including TMV, do not apply in the case of BSMV. This virus is readily transmitted in barley: up to 100% of seeds set on infected plants can transmit the virus. The mechanism of seed transmission of BSMV involves direct infection of the embryo, within which virus particles have been observed by electron microscopy of thin sections of seeds (Carroll, 1972). It is possible that virions consisting of TMV-RNA within BSMV protein might also form in embryos, along with normal BSMV virions, and these could effect seed transmission of TMV, even if normal TMV could not be transmitted.

MATERIALS AND METHODS

To test this possibility, double and single infections of BSMV and TMV were established at 30° in three barley varieties, Atlas, Compana and Champlain. When at the four leaf stage, heat clarified sap from the terminal half of fourth leaves of BSMV and doubly inoculated plants was assayed for the presence of BSMV and TMV by the microprecipitin

test. Only a small volume (four drops) of the 1.0 ml of clarified sap from each leaf was used for the microprecipitin test. The remaining clarified sap from those leaves which proved to be doubly infected was pooled and assayed for possible genomic masking. An equal volume of clarified sap from BSMV infected plants to which purified TMV was added was used to make an artificial mixture (control). Each mixture was halved and from one half BSMV antigen was precipitated with BSMV antibody and from the other half TMV antigen was precipitated with TMV antibody. These antigen-antibody precipitates were washed with PBS and finally resuspended in RNA dissociation buffer. The suspensions, after overnight incubation at 10°, were analysed by density gradient centrifugation through linear-log sucrose gradients to establish whether genomic masking was detectable in the plants being used in these seed transmission experiments.

After selection of definitely doubly infected plants, the number of such seedlings was trimmed to one per pot. An equal number of BSMV infected and doubly infected plants were allowed to mature at temperatures lower than 30° which, although necessary for the establishment of good double infections, is not compatible with good seed set. In the case of Atlas, an early maturing variety, 9 plants for each treatment were transplanted to 8 inch clay pots and placed in a greenhouse in mid-May, 1972. In the case of Champlain and Compana, varieties selected for the Quebec growing season, 17 and 9 plants respectively for each treatment

were transplanted to separate rows in the experimental plot of the Plant Pathology Department, Macdonald College on June 5, 1972.

Each singly and doubly infected plant of all varieties was rechecked by the microprecipitin test for the presence of TMV and BSMV, once in July and once in August. The test was on heat-clarified sap from two young leaves from each plant.

In September, 1972, seeds were collected from all plants and those from each treatment were pooled. During 1973 the seeds were germinated and allowed to grow to the fourth leaf stage at 30°. They were scored for symptoms of BSMV infection and then heat clarified sap from selected plants or leaves was assayed for BSMV and TMV by the microprecipitin test, and for TMV by inoculation to two leaves of Nicotiana tobacum var. Xanthi or Chenopodium amaranticolor for each sample.

RESULTS

1. Establishment of double infections at 30°

The three varieties Atlas, Compana and Champlain were doubly inoculated at the one leaf stage and the numbers of doubly infected plants at the fourth leaf stage were as follows; Atlas 9/18 tested, Compana 13/62 tested, Champlain 23/56 tested. Samples of clarified sap from doubly infected plants chosen for transplanting (9 Atlas, 9 Compana, 17 Champlain seedlings) all gave very heavy precipitates with TMV antibody in the microprecipitin test. None of the BSMV infected plants tested were contaminated with TMV. None of the clarified sap samples from either singly or doubly infected plants gave precipitates with normal serum.

Thus, at the time of transplanting, the doubly infected plants selected were heavily infected with both BSMV and TMV for all three varieties.

2. Genomic masking at transplanting time

The RNA species extracted from the washed BSMV and TMV antigen-antibody precipitates from real and artificial mixtures prepared from clarified sap of fourth leaves from Compana and Champlain are shown in figure 37.

The RNA from the BSMV antigen-antibody precipitates from artificial mixtures from both varieties (A_1, B_1) can be characterised as only BSMV-RNA, consisting of a major species, sedimenting to about one third the depth of the gradient, and a minor species which sedimented slightly slower. Similarly, only TMV-RNA, which sedimented further than BSMV-RNA, was extracted from TMV antigen-antibody precipitates from both artificial (A_3, B_3) and real (A_4, B_4) mixtures from both varieties. Genomically masked TMV-RNA (arrowed), along with BSMV-RNA, was extracted from the BSMV antigen-antibody precipitate from real mixtures (A_2, B_2) from both varieties. For Compana, the TMV-RNA represented only a small portion of the total RNA in virions coated with BSMV protein, a level of genomic masking similar to that found in Black Hulless. For Champlain, on the other hand, a very high level of genomic masking was detected, since more TMV-RNA than BSMV-RNA was extracted from virions coated in BSMV protein. For Atlas, no genomic masking was detected and so the density gradient profiles are not shown.

Figure 37. Levels of genomic masking in doubly infected plants used for seed transmission tests.

Absorbance profiles of linear-log sucrose gradients in SSC pH 7.0, centrifuged for 4 hours at 40,000 rpm in a SW40 rotor. Direction of sedimentation from right to left.

0.5 ml of PBS-washed antigen-antibody precipitates in RNA dissociation buffer were layered on the gradients before centrifugation. Antigen-antibody precipitates were collected with either BSMV antibody or TMV antibody from artificial or real mixtures in clarified sap prepared from fourth leaves of barley.

A - samples from Compana barley

B - samples from Champlain barley

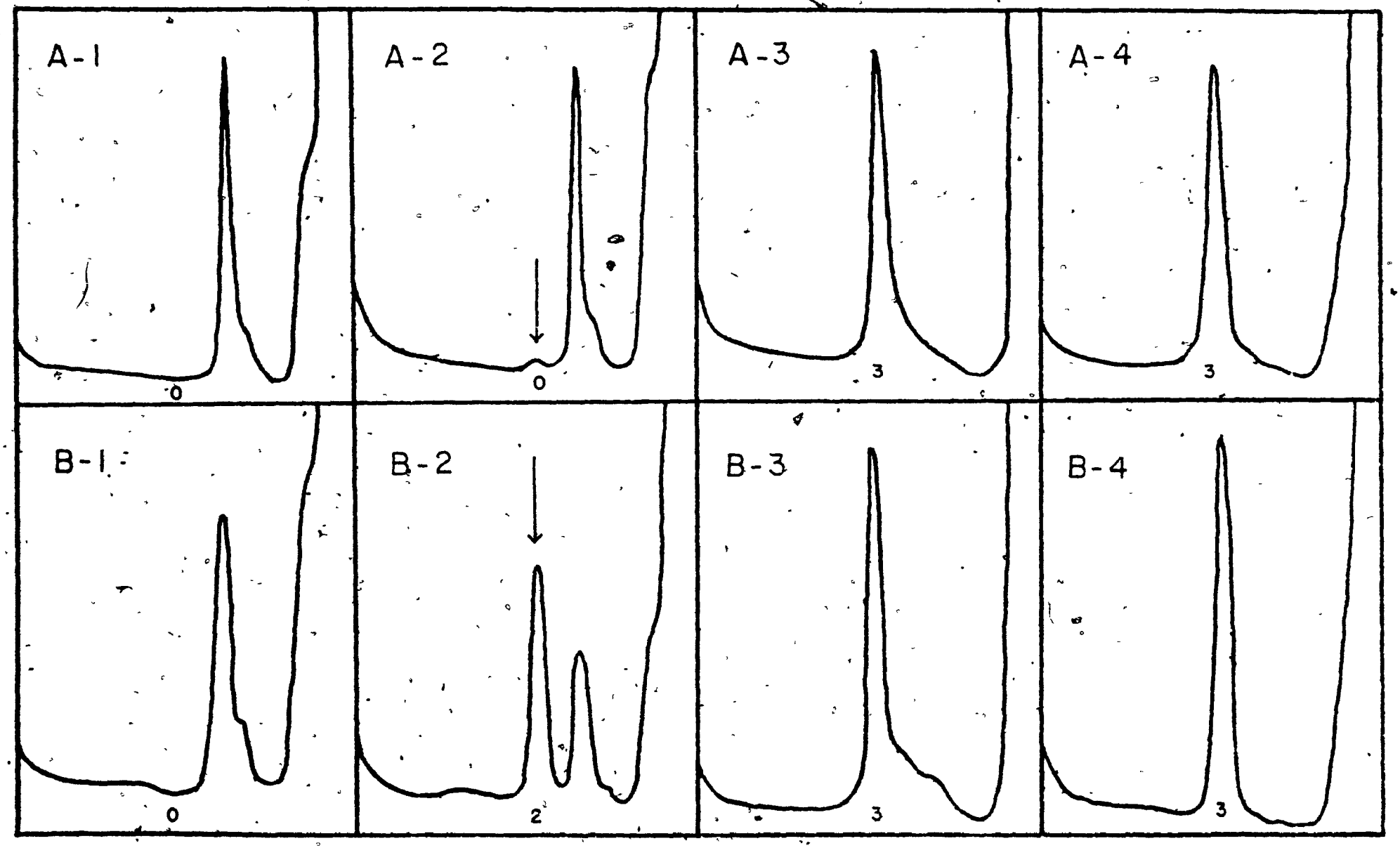
1.- RNA from BSMV antigen-antibody precipitates from artificial mixtures.

2.- RNA from BSMV antigen-antibody precipitates from real mixtures. The arrow indicates genomically masked TMV-RNA.

3.- RNA from TMV antigen-antibody precipitates from artificial mixtures.

4.- RNA from TMV antigen-antibody precipitates from real mixtures.

The numeral beneath each profile indicates the position in the gradient from which a sample was taken for inoculation to 3 tobacco plants. How many of these three that became infected with TMV is given by the number.


A-1
A-2
A-3
A-4
B-1
B-2
B-3
B-4
0
0
3
3
0
2
3
3
RELATIVE ABSORBANCE - 254 nm

That part of each of the eight gradients shown in figure 37 to which TMV-RNA would have sedimented was collected and inoculated to three White Burley tobacco plants, and the subsequent development of a TMV infection was recorded and the results are given in figure 37.

These results indicate that the absorbance peaks described above as TMV-RNA were able to establish TMV infections in tobacco, confirming their identity. This applied to TMV-RNA from TMV antigen-antibody precipitates, and also to genomically masked TMV-RNA from BSMV antigen-antibody precipitates. Concentration, however, seems to have been a factor since the fairly large amount of TMV-RNA extracted from all four TMV antigen-antibody precipitates infected all three plants inoculated. The lesser amount of genomically masked TMV-RNA from Champlain failed to infect one of the three plants inoculated. The small amount of genomically masked TMV-RNA from Compana was too small to be detected by inoculation to tobacco.

In summary, in addition to having established double infections in all three varieties as starting material for these experiments, in the case of Compana, and to an even higher degree in Champlain, there was an appreciable amount of genomic masking of TMV-RNA detectable in the doubly infected plants. In Atlas, however, no genomic masking was detected.

3. Maintenance of double infections

Clarified sap from all singly and doubly infected plants from all three varieties reacted positively with BSMV antibody in microprecipitin tests carried out one and two months after transplanting. However, none of these same samples reacted with TMV antibody at either sampling date. Therefore, TMV appears to have stopped replicating in doubly infected barley plants upon transplanting them from 30° to the greenhouse (Atlas) or to the field (Compana and Champlain).

4. Tests for seed transmission of TMV

The number of seeds harvested at the end of the growing season for Champlain, and particularly Compana, was not great, largely because most seeds had been eaten by birds. It was possible, however, to plant 200 seeds of Atlas and Champlain and 50 seeds of Compana, either from singly or doubly infected plants (table II). For all varieties, emergence was approximately 50% and of these plants 20-33% developed symptoms of BSMV infection. Taking Champlain as an example, 25 plants from seeds from doubly infected plants showed BSMV symptoms. An equal number of like plants which did not show BSMV symptoms were also chosen for analysis. All plants were tested with BSMV antibody and TMV antibody by the microprecipitin test using clarified sap from pooled third and fourth leaves as test antigen. A direct correlation was observed between the presence or absence of BSMV symptoms and the presence or absence of BSMV antigen

Table II. Data on seed transmission of BSMV and TMV from doubly infected barley.

Variety	Parental infection	Seeds planted	Seedlings emerged	Infections detected BSMV[a] Number	%	TMV[b] Number
Atlas	BSMV	200	81	22	28	0
	BSMV & TMV	200	95	29	31	0
Compana	BSMV	50	25	5	20	0
	BSMV & TMV	50	21	7	33	0
Champlain	BSMV	200	123	27	22	0
	BSMV & TMV	200	110	25	23	0

[a] Assayed by symptomatology and serology.

[b] Assayed by infectivity and serology.

in clarified sap. A reaction with TMV antibody was not detected in any sample. All samples failed to elicit lesion formation on either N. tobaccum var. Xanthi, or C. amaranticolor. The same analysis was carried out on first and second leaves from 10 of the 25 BSMV-infected or uninfected plants and again no TMV was detected. Identical results were obtained with the 27 BSMV-infected plants and the 27 plants selected for their healthy appearance grown from seed from BSMV infected plants.

The Compana and Atlas seedlings were analysed as described for Champlain, and, as in the tests on Champlain, TMV was not detected by serology or infectivity in BSMV infected or apparently healthy plants grown from seed from singly or doubly infected plants.

CONCLUSIONS

Good double infections of BSMV and TMV were established at 30° in three barley varieties, Atlas, Compana and Champlain. When plants were at the four leaf stage, genomic masking of TMV-RNA in BSMV protein was detected in Compana and Champlain. The level of genomic masking was very high in Champlain. No genomic masking was detected in Atlas.

TMV antigen could not be detected in formerly doubly infected plants allowed to grow to seed set in the greenhouse or the field. BSMV could be detected in these plants. Seed from doubly infected plants of all three varieties transmitted BSMV. TMV was not transmitted through seed from doubly infected plants, in any variety, either along with the transmission of BSMV or in apparently healthy plants from these seeds.

SUMMARY OF RESULTS

1. Direct evidence for double infection of barley cells with BSMV and TMV was obtained by electron microscopy of thin sections of doubly infected fourth leaves sampled twenty days after inoculation of first leaves. Doubly infected fourth leaves are known to contain more TMV than BSMV. The majority of cells from these leaves were doubly infected.
2. The only feature unusual to doubly infected cells was the common occurrence of virus-like particles with the characteristic length of TMV (300 nm) and the characteristic width of BSMV (20 nm).
3. Two kinds of TMV infectivity were detected in extracts from barley leaves doubly infected with BSMV and TMV. The first could be separated from BSMV by incubation of real mixtures with TMV antibody. The second resisted this treatment. No TMV infectivity resistant to TMV antibody was detected in artificial mixtures of BSMV and TMV. This is evidence for genomic masking of TMV-RNA in BSMV protein.
4. The TMV infectivity resistant to TMV antibody described above was neutralised by BSMV antibody at the same time that BSMV infectivity was neutralised by the same treatment. This confirms genomic masking of TMV-RNA in BSMV protein.

5. Viral antigen held within washed BSMV antigen-antibody precipitates, collected from extracts prepared from doubly infected leaves, contained BSMV-RNA and TMV-RNA. Washed BSMV antigen-antibody precipitates from equivalent artificial mixtures contained BSMV-RNA but not TMV-RNA. This is evidence for genomic masking of TMV-RNA in BSMV protein.
6. BSMV protein, but not TMV protein, was extracted from washed BSMV antigen-antibody precipitates from real mixtures which contained both BSMV-RNA and TMV-RNA. Only BSMV protein was extracted from washed BSMV antigen-antibody precipitates from artificial mixtures which contained only BSMV-RNA. This confirms genomic masking of TMV-RNA in BSMV protein.
7. Washed TMV antigen-antibody precipitates from both artificial and real mixtures of BSMV and TMV contained only TMV-RNA and TMV protein. There was no evidence for genomic masking of BSMV-RNA in TMV protein.
8. Genomic masking of TMV-RNA was not detected in doubly inoculated barley leaves (leaf 1) or in the first leaves to elongate after inoculation (leaf 2). These leaves synthesised less TMV than BSMV. Genomic masking of TMV-RNA was consistently detected in leaf 3 and 4. These leaves produced more TMV than BSMV. In one experimant, the approximate percentage of RNA within BSMV coat protein that was TMV-RNA was 4-8% in BSMV prepared from doubly infected Black Hulless barley and 50% in BSMV prepared from doubly infected Champlain barley.

9. Genomically masked TMV-RNA was infectious and produced typical TMV lesions on Chenopodium amaranticolor. The virus produced in several of these lesions was inoculated separately to single tobacco plants. The virus produced in each tobacco plant was indistinguishable from normal TMV.

10. BSMV, but not TMV, was transmitted through the seed collected from three varieties of barley plants doubly infected with BSMV and TMV. At the fourth leaf stage all the plants contained both BSMV and TMV and in two varieties, genomically masked TMV-RNA was detected. At a later stage of maturity, BSMV, but not TMV, was detected in the same plants.

CLAIMS OF ORIGINAL WORK

1. The detection of the RNA of tobacco mosaic virus (TMV) encapsulated in barley stripe mosaic virus (BSMV) protein in doubly infected barley plants is the first confirmation of genomic masking between this or any other pair of structurally dissimilar, unrelated plant viruses.
2. The first isolation of genomically masked viral nucleic acid (TMV-RNA) in amounts sufficient for physical characterisation and quantification.
3. The first use of dissociated, washed antigen-antibody precipitates as a separatory tool for the recovery of specific viral RNA or protein from mixtures of viruses.
4. The first ultrastructural analysis of barley cells doubly infected with TMV and either BSMV or brome mosaic virus (BMV).
5. The clearest demonstration to date that vesicles, found in the periplastidial space of chloroplasts from BSMV-infected plants, are associated with the outer of the two chloroplast limiting membranes.

DISCUSSION

In the introduction, it was mentioned that the study of double infections is a useful exercise because information about virus behavior can sometimes be found that would escape detection in single infections. The subject of this thesis, the encapsulation of TMV-RNA in BSMV coat protein in doubly infected barley plants grown at 30°, is one such example. At first sight this would appear to be an unusual event, particularly since the two viruses are unrelated and structurally dissimilar, and so a critical discussion of the methods used to detect such a particle is in order.

In most studies of genomic masking, a key observation is that the infectivity of one virus cannot be separated from the other virus following a physical separation of the two viruses from a real, or natural, mixture. Separation is complete, however, from an artificial mixture.

In this thesis, TMV infectivity could not be separated from BSMV derived from doubly infected barley plants, even though this was the case when artificial mixtures were used. Based on this result, it was predicted that TMV-RNA would also be detected in the BSMV population from doubly infected plants, from which TMV had been separated, and this was the case. Again, separation of TMV-RNA from BSMV was complete from equivalent artificial mixtures. In another double infection study in barley quite similar to this one, Peterson and Brakke (1973) could not separate the infectivity of BSMV from brome mosaic virus (BMV, a spherical virus) derived from natural mixtures, whereas separation was complete from most artificial mixtures. On the basis of this

evidence alone, these authors concluded that genomic masking of BSMV-RNA in BMV protein had been detected. They were unable to detect BSMV-RNA in the BMV population which had both BSMV and BMV infectivity associated with it because the analytical method used, linear-log sucrose density gradient centrifugation, could not adequately resolve the RNA componentsof BSMV and BMV when only small quantities of BSMV-RNA were present relative to the amount of BMV-RNA.

In this present study, the local lesion hosts used to assay the TMV infectivity associated with BSMV from real mixtures, Chenopodium amaranticolor and Nicotiana glutinosa, were not infected by BSMV. The small amount of TMV infectivity was therefore readily detected and the susceptibility of this infectivity to BSMV antiserum and TMV antiserum could be studied. In Peterson and Brakke's study the BSMV infectivity associated with BMV from real mixtures was assayed on barley, a host infected systemically by both BSMV and BMV. Control experiments had demonstrated interference in barley when the inoculum potential of BMV was higher than that of BSMV in a mixed inoculum. This resulted in limited production of BSMV in doubly infected plants. Because of this, the authors had to concentrate extracts from the assay plants in order to detect the production of BSMV. In that study, and in a parallel study (Morris, 1970) a serological analysis for the presence of BSMV in the assay plants was negative.

Whether the kind of evidence described so far is sufficient to prove rather than to indicate genomic masking is open to question. An assumption is generally made that if artificial and real mixtures are quantitatively equivalent, failure to separate the infectivity of virus-A from virus-B from real, but not artificial mixtures, is a direct indication of genomic masking. It is not beyond reason, however, to propose that qualitative differences between such mixtures might lead to incomplete separation of the two viruses from real mixtures even when separation was complete from artificial mixtures. The infectivity of virus-A associated with virus-B derived from real mixtures would then be the consequence of contaminant virus-A, rather than genomic masking of virus-A genome.

In Peterson and Brakke's study it is difficult to imagine what the qualitative peculiarity would be in real mixtures of BMV and BSMV that would lead to the presence of infectious BSMV particles in the BMV zone in only real mixtures after separation of BMV and BSMV by sucrose density gradient centrifugation, the separatory method used. Even so, although their results are best interpreted as evidence for genomic masking, this interpretation has yet to be confirmed.

An analysis for TMV protein was used in the present study to distinguish between genomic masking or contamination by TMV particles as the explanations for the association of TMV infectivity and TMV-RNA with BSMV derived from real mixtures. If contamination by TMV particles was the explanation, the TMV protein around such particles would make

them susceptible to TMV-antiserum. Incubation with TMV-antiserum did not neutralise the TMV infectivity. Likewise, TMV protein, as well as BSMV protein, would be detected in a physical analysis of the BSMV which had TMV-RNA associated with it. TMV protein was not detected. Control experiments had demonstrated that the methods used to detect TMV-RNA and TMV protein were such that TMV protein was always detected in samples in which TMV-RNA was detected, if the source of TMV-RNA was normal TMV particles.

If genomic masking of TMV-RNA in BSMV protein was the explanation, then such particles would be precipitated by BSMV-antiserum. Incubation with BSMV-antiserum did neutralise the TMV infectivity. Likewise, only BSMV protein would have been detected after a physical analysis of the BSMV which had TMV-RNA associated with it, and this was the case. These two tests confirm that genomic masking of TMV-RNA in BSMV protein is the explanation for the failure to separate TMV infectivity and TMV-RNA from BSMV derived from natural mixtures of BSMV and TMV extracted from doubly infected barley plants grown at 30°.

There is another, but improbable, explanation for these results, which is that TMV-RNA can be adsorbed on to the surface of BSMV particles in doubly infected plants. This kind of explanation has not been mentioned in any report on genomic masking or phenotypic mixing, but it was considered by Siegel (1971) in his investigation of TMV pseudovirions. The failure to offer this as an explanation for results

better interpreted as genomic masking is presumably because it is unlikely that externally adsorbed nucleic acid would be able to survive the methods used for extraction, purification, separation and analysis of the viruses being studied. Siegel (1971) incubated TMV in ribonuclease in an attempt to eliminate hypothetical adsorbed RNA. In the present study this treatment was essentially used, since the viruses were incubated at 40° for 1 hour in plant sap, which contains ribonuclease, during the preliminary clarification step. Because of this, and for the reasons mentioned above, adsorption of TMV-RNA onto the surface of BSMV particles is not considered a possible explanation for the results presented in this thesis.

As mentioned earlier, at first sight genomic masking between structurally dissimilar unrelated viruses would seem to be an unusual event, particularly since assembly of rod shaped viruses such as BSMV and TMV probably involves a specific initiation step. Since it has been detected between TMV-RNA and BSMV protein, it is worth considering the conditions which might favour this phenomenon. A clear requirement is for unassembled TMV-RNA and BSMV protein to be present at a given time in a pool, which may or may not also contain BSMV-RNA and TMV protein, from which they can be withdrawn as particle assembly progresses. Presumably these conditions are best found in doubly infected cells. If the two viruses were assembled separately in different cells, fairly large scale transfer

of TMV-RNA to BSMV infected cells would have to be proposed. While this may not be impossible, the high frequency of doubly infected cells detected by electron microscopy of thin sections of doubly infected leaves suggests it was in these cells that genomic masking occurred.

Specific recognition of homologous virus protein by viral RNA is probably the major factor which prevents genomic masking in doubly infected cells. If this specificity were poor, in vitro studies having demonstrated that heterologous assembly could occur, then intracellular compartmentalisation of homologous assembly would prevent genomic masking.

If genomic masking is detected, any specificity of homologous assembly has been overcome. One way this could happen would be if there were compartments or environments within doubly infected cells in which only the heterologous components were present, in this case TMV-RNA and BSMV protein, which are known to assemble in vitro (Atabekov et al, 1970). Such intracellular "compartments or environments" could be temporal as well as structural. The replication cycle of BSMV might be faster than that of TMV such that at a given time, when most BSMV-RNA had already been assembled into BSMV particles, excess BSMV protein might interact with TMV-RNA synthesised prior to the accumulation of TMV protein. No information is available on the rates of synthesis of BSMV and TMV in doubly infected barley plants. An investigation of rates of synthesis needs to be undertaken.

In the absence of compartmentalisation, the production of a large quantity of heterologous RNA might be a mechanism for overcoming specificity during assembly. This could be the mechanism in the present system since much more TMV than BSMV was produced in those leaves in which TMV-RNA became genomically masked. If TMV protein is not produced in any quantity until after BSMV protein has accumulated, then the kind of ratio of BSMV-RNA : TMV-RNA : BSMV protein might exist which results in heterologous, as well as homologous, assembly in vitro (Fritsch et al, 1973). In that study, when the ratio of TMV-RNA : TYMV-RNA : TMV protein was 1:10:20, as much as 40% of the RNA in the product assembled at pH 7.0 was heterologous TYMV-RNA. It is worth noting that in such a system, either in vitro or in vivo, the majority of the excess heterologous RNA remains unassembled, and could reassemble with its homologous protein at a later time, or at another site of assembly. The mechanism just discussed is a very attractive possibility for the results obtained in this thesis since most genomic masking of TMV-RNA was detected in those leaves in which the ratio of TMV to BSMV was the highest. Further experiments on the correlation between the amount of genomic masking and the ratio of TMV to BSMV are needed. Different leaf positions in a given variety could be compared. Also barley varieties could be used which support different levels of BSMV synthesis. Very high ratios of TMV to BSMV might then be possible in varieties which produce low amounts of BSMV, and infact this was

probably the case in Champlain barley in which a very high level of genomic masking was detected (p 152).

Fritsch et al (1973) also demonstrated that heterologous assembly was very efficient in conditions sub-optimal for homologous assembly, even in the presence of homologous RNA, when heterologous RNA was in excess. Environments in doubly infected cells unfavourable for specific homologous assembly probably do occur, since a change from pH 7.0 to pH 6.0 was all that was required in the in vitro study. This suggests another mechanism for overcoming the specificity of homologous assemblies. Apart from the brief report by Atabekov et al (1970), there is no information on the conditions or component ratios which favour heterologous assembly of TMV-RNA and BSMV protein, in the presence or absence of BSMV-RNA. Some in vitro studies along these lines are called for.

The arguments just developed call for the presence of BSMV-RNA, an excess of TMV-RNA, and BSMV protein, but not TMV protein, preferably in conditions unfavourable for homologous BSMV assembly. If TMV protein was also present conditions would have to be unfavourable for homologous TMV assembly, since such assembly would lead to a lowering of the critically high concentration of TMV-RNA.

If, however, no TMV protein at all was produced, then the above conditions would be met. It has been demonstrated that genomic masking of the RNA of defective strains of TMV occurs readily

in double infections with competent strains. The possibility exists, therefore, that genomic masking of TMV-RNA in the present study was the consequence of the production of such defective mutants in certain doubly infected barley cells. Doubly infected plants were grown at elevated temperatures (30°) which might favour mutation. The virus produced from genomically masked TMV-RNA was biologically and physically the same as normal TMV. It produced normal TMV particles in either singly infected Chenopodium amaranticolor or tobacco grown in the greenhouse or in doubly infected barley grown at 30°. This indicates that it was not an unusual population of TMV-RNA, unable to direct the synthesis of its own protein, which became genomically masked.

It is interesting that genomic masking was unilateral, since BSMV-RNA was not encapsulated in TMV protein. This result supports the theory that the mechanism for overcoming specific homologous assembly requires an excess of heterologous RNA. In the leaves which were analysed for genomic masking of BSMV-RNA (leaves 3 and 4), BSMV was not in excess. Genomic masking of BSMV-RNA was not analysed in leaves 1 and 2 which produced more BSMV than TMV, but this should be done.

On the basis of this and many other studies which have demonstrated genomic masking or phenotypic mixing, it is worth considering how significant or common such phenomena might be outside of the laboratory.

The main significance for plant pathology lies in dependent vector transmission; the transmission of genomically masked RNA by the vector of the virus which donated the protein capsid. It has been suggested that genomic masking could be a contributory factor in the distribution of different isolates of barley yellow dwarf virus in New York State (Rochow and Jedlinski, 1970). Other similar examples have yet to be demonstrated but wherever a virus, such as the MAV isolate of BYDV, is consistently found in double infections with other viruses, genomic masking might be suspected, particularly if the vector of the virus is not known to occur in the area, or if it has no known vector.

The literature on in vitro and in vivo phenotypic mixing suggests that this is an almost predictable consequence of double infection with two closely related virus strains. Natural double infections between closely related strains is not too likely, however, because of the phenomenon of interference. In fact, tomatoes are deliberately infected with a mild strain of TMV to protect them against a subsequent infection with more severe strains (Rast, 1972). That this does not happen with the two barley yellow dwarf isolates studied by Rochow, together with the observation that they are serologically unrelated and have different specific vectors in single infection, indicates that even though they are structurally similar and produce similar diseases, they are only distantly related and they may even be unrelated (Aapolo and Rochow, 1971).

In double infections between distantly related or unrelated, structurally similar or dissimilar viruses, genomic masking is the most likely structural interaction. Double infections with such viruses are quite common since interference between them is not usually a problem. The observation of Kassanis and Conti (1971) that the infectivity of defective TMV could not be protected by viruses from several different groups of plant viruses other then the TMV group suggests that genomic masking is not a predictable consequence of double infections with unrelated viruses. If this is true, then genomic masking is unlikely to be of general significance to vector transmission or any other phenomenon involving virus phenotypes. However, there are three reports of genomic masking between unrelated plant viruses (Rochow, 1970: Peterson and Brakke, 1973 and the present study) and as Rochow (1972) has pointed out, "the phenomenon (dependent vector transmission) could occur as a result of interaction among only one infected plant, only one aphid and only one phenotypically mixed (genomically masked) virus particle".

An attempt was made in the present study to investigate the possible significance of genomic masking to seed transmission of TMV. It was postulated that like BSMV, TMV-RNA encapsulated in BSMV protein might have survived in the embryos of barley plants. Seed transmission of TMV was not detected, however. This is not surprising since there was an apparent loss of TMV from new tissue after transferring

doubly infected plants from 30° to lower temperatures. This, together with the observation that the frequency of double infection only approaches 100% at 30°, requires more attention.

Although genomically masked TMV-RNA was isolated from BSMV antigen-antibody precipitates in amounts suitable for physical characterisation, the genomically masked particles have not been isolated in pure form. There is therefore, no direct evidence for the morphology of such particles, although Atabekov et al (1970) have presented in vitro evidence for the most predictable structure, a rod shaped particle with a width of 20 nm, determined by the BSMV protein, and a length of about 300 nm, determined by the size of the TMV-RNA. Such particles were searched for deliberately in the present study, in thin sections of doubly infected cells and were, in fact, readily found, usually side by side in files either free in the cytoplasm or attached to chloroplasts. In plants singly infected with BSMV, such groups of particles were not found, although occasional abnormally long particles did exist. Although the common occurrence of 20 nm x 300 nm rods in doubly infected cells is indirect evidence for genomic masking of TMV-RNA, electron microscopy of thin sections cannot distinguish such particles from pairs of BSMV particles aggregated end to end. Such aggregation could be the consequence of double infection.

Aside from genomic masking, the fine structural studies produced three other interesting results. The micrographs demonstrating the vesiculation of chloroplasts (figure 7) provide clearer evidence for the attachment of vesicles to the outer of the chloroplast limiting membranes than the published observation on vesiculation associated with BSMV infection (Carroll, 1970). Carroll did, however, conclude that this was the relationship. Secondly, the micrographs of cells doubly infected with BMV and TMV or BSMV and TMV demonstrate the remarkable capacity of plant cells to support the synthesis of massive amounts of virus. In the former case, this occurs without the development of severe symptoms in the doubly infected plants, and at the ultrastructural level, the cell organelles appeared normal. Thirdly, in common with other ultrastructural studies on plant cells doubly infected with unrelated viruses, crystallisation of TMV and BSMV could be either mixed or essentially separate. Some evidence was presented for a common influence on the crystallisation of both BSMV and TMV (figure 17-A).

A final point, again unrelated to genomic masking, is the different degrees of heterogeneity detected for BSMV-RNA. In some experiments, the RNA from virus produced in leaves showing acute symptoms (leaves 1 and 2) resolved into two major components of approximately 1.2×10^6d and a single minor component of 1.5 x

10^6d. From chronically infected leaves (leaves 3 and 4) of the same plants similar quantities of BSMV-RNA were resolved into only a single major component of 1.2 x 10^6d and a single minor component of 1.5 x 10^6d. Jackson and Brakke (1973) have reported strains of BSMV which have either one or two major components of about 1.2 x 10^6d. The present results indicate that either BSMV-RNA can exist as different numbers of components at different stages of infection, which would be a unique result for multicomponent viruses, or the BSMV isolate used in this study was a mixture of strains, of which the one with the least number of components predominated in later stages of infection.

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