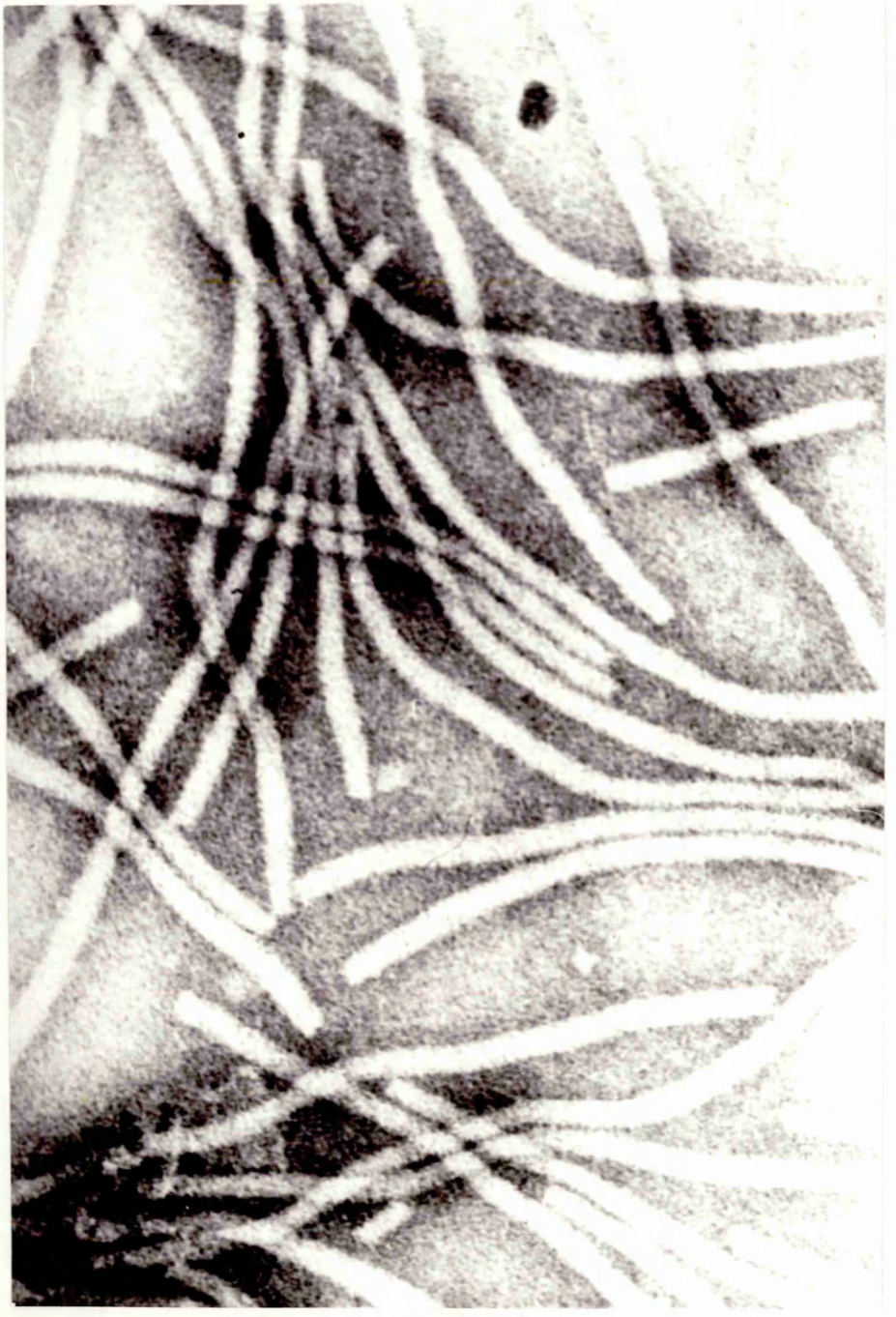
ANALYSIS OF METHODS FOR SCREENING PLANT VIRUSES.

D.W.J.AW, B.Sc.

Thesis presented for the Degree of Doctor of Philosophy of the University of Glasgow in the Department of Botany.



Electronmicrograph of Potato Virus X (PVX) Particles (Strain N, Group 3) in the Crude Sap of Infected Nicotiana tabacum plants (x 165,000 magnification).

Acknowledgements

I would like to thank Rose Millar for all her help in the preparation of gels and hybridisation of samples in the nick translation experiments, also for the preparation of the N.tabacum seedlings. My thanks also to Eain Robertson for the preparation of the electron micrographs. I would also like to thank Christine, Candy, Robert and my family for their patience and support throughout the thesis. Finally, I would like to thank Dr J. Milner for his advice throughout this project.

I wish to certify that, apart from the people listed above, all experiments were planned and carried out by myself.

This work was supported by a grant from the Ministry of Agriculture, Fisheries and Food.

June, 1987. D.W.J.AW

CONTENTS.

ABBREVIATIONS

The following abbrevations are used in the text.

h	=	hour
min	=	minute
s	=	second
l	=	litre
ml	=	millitre
µl	=	microlitre
cm	=	centimetre
G	=	relative centrifugal force
rpm	=	revolutions per minute
M	=	molar
mM	=	millimolar
g	=	gram
mg	=	milligram
µg	=	microgram
psi	=	pounds per square inch
Ci	=	Curie
mCi	=	milliCurie
cpm	=	counts per minute
ATP	=	adenosine triphosphate
DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
cDNA	=	complementary DNA
ds cDNA	=	double-stranded complementary DNA
BPB	=	bromophenol blue
XC	=	xylene cyanol
EDTA	=	disodium ethylene diamine tetraacetic acid

DTT	=	dithiothreitol
TEMED	=	N,N,N',N'-tetramethylethylenediamine
Xgal	=	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
SDS	=	sodium dodecyl sulphate
PA Œ	=	polyacrylamide gel electrophoresis
V	=	volts

SUMMARY.

Potato virus X, belonging to the group of viruses known as the potexviruses, is an economically important virus, affecting potato crops worldwide. The detection of this and other plant viruses has, so far, mainly been dependent upon the antigen-antibody assays such as the enzyme-linked immunosorbent assay (or ELISA). By using the ELISA: 1) a large number of samples can be handled at the same time; 2) the antigen-antibody reaction is specific for the virus; 3) results can be obtained within a couple of hours using prepared trays, 4) the assay uses an immunoglobulin marker in place of a radiolabelled isotope, resulting in negligable loss of activity after extended periods of time, and 5) the assay is sensitive with small amounts of enzyme-labelled immunoglobulin, thereby dispensing with expensive detection systems.

The use of the ELISA for the detection of PVX strains in crude plant sap showed a high degree of sensitivity. The assay could detect isolates of both strain-group 3 (PVX/PVX N) and strain-group 2 (PVB) to at least 10^{-3} x dilution (reaching 10^{-6} x dilution with one isolate of strain-group 3). The assay was also very sensitive in the detection of purified PVX/PVB viral particles, detecting 20 ng of viral particles or 200 pg RNA with purified RNA samples (equivalent to approximately 33 ng virus particles).

However, the main disadvantage of using serological assays, even with one as sensitive as the ELISA, is that only viral antigens are detected, giving no information about the infectious RNA within. An alternative system was, therefore, developed with the aim that it would be as sensitive as the ELISA, easy to handle, and one which allowed the detection of the infectious viral RNA. The assay was a dotblot system using nitrocellulose to which the RNA was bound by baking. Aliquots of infected sap, purified viral particles or RNA were

applied and bound to sheets of nitrocellulose. The filter, containing the samples, was then be hybridised with labelled complementary DNA probes, detecting the RNA.

Two systems were devised for the hybridisation stage of the assay using two different probes, one radiolabelled and the other a biotinylated probe. Both ^{32}P-labelled and biotinylated nucleotides were incorporated into cDNA probes, using PVX N RNA as template. These probes hybridised to complementary sequences, detecting the signals on the filters by autoradiography for the ^{32}P-labelled probe and with an enzyme reaction (streptavidin conjugated to biotinylated alkaline phosphatase) for the the biotinylated probe. The alkaline peroxidase-conjugated streptavidin, in the presence of substrates, converted the substrates into visible precipitates on the filters. With the radiolabelled probe, RNA in the viral particles of crude sap could only be detected to 10^{-1} x dilution with both PVB and PVX isolates (reaching 10^{-2} x dilution maximum), compared to 10^{-3} to 10^{-6} obtained with the same sample using ELISA. For the detection of purified viral particles, the radiolabelled probe was sensitive to 20 ng and to 200 pg with purified RNA. Results for the biotinylated probe were identical, although the radiolabelled probe could be increased in sensitivity when the exposure time to X-ray film was increased.

A third probe, produced by cDNA cloning a section of the PVX N RNA template and labelled by nick translation, was also used to detect complementary RNA on filters. The final clone (pPVX21 N) used for hybridisations contained an insert between 550-600 bp in length but only detected PVX RNA in the PVX (strain-Group 3, PBI) crude sap samples to 10^{-2} and purified RNA to 20 ng with a 7 day exposure, results which were much less sensitive than either of the cDNA probes by at least 2 logs. However, an increase of non-specific hybridisation

was also noted with the filter. The nick-translated probe did not detect PVB (strain-Group 2, PBI) or PVX N in the crude sap isolates or purified viral particles at all, unlike the cDNA probes.

In addition to the development of these assays for the detection of viral RNA, studies were undertaken to obtain structural information on the 3' terminal region of the RNA. These studies included the synthesis of complementary DNA from an RNA template in the presence and absence of various primers. Initial results conflicted with the published literature of Sonenberg (1978) which reported the absence of poly (A) sequences at it 3' terminus, but confirmed the report of a poly (A)-rich region in PVX RNA by Morozov et al. (1983). These results were checked by affinity chromatography on oligo d(T) cellulose columns which selectively bound poly (A)-rich regions in RNAs. These experiments were further backed by selectively degrading the RNA with enzymes which could then be sized on polyacrylamide gels.

The position of the poly (A) sequences was determined using RNase H, an enzyme which specifically attacked and degraded these poly (A) nucleotide sequences when bound to oligo d(T), leaving the RNA in two or more fragments which could then be analysed by polyacrylamide gel electrophoresis. Lastly, the size and position of these poly (A) sequences were checked by direct RNA sequencing, a method dependent upon a series of base-specific RNases. PVX RNA was labelled, purified on a 4 % polyacrylamide gel to remove excess label, digested with the enzymes and analysed on a thin gel by thin-layer, polyacrylamide gel electrophoresis and autoradiography. The size of the poly (A) region in PVX N RNA was found to be between 8 and 16 nucleotides long and positioned at the 3' terminus rather than being an internal sequence.

CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION:

Viruses can be considered as entities which only multiply within cells and have the ability to cause disease in all types of organisms.

Matthews (1981) defines a virus as: a set of one or more nucleic acid template molecules, normally encased in a protective coat or coats of protein or lipoprotein, which is able to organise its own replication only within suitable host cells. Within such cells, virus production is i) dependent on the host's protein-synthesising machinery, ii) organised from pools of the required materials rather than by binary fission, and iii) located at sites which are not separated from the host cell contents by a lipoprotein bilayer membrane.

In addition, Matthews (1981) states that a virus must be shown to be transmissable, to cause disease in at least one host, and to have some or all of the following features:

i) The nucleic acid may be DNA or RNA and single-stranded or double-stranded.

ii) The mature virus particle may contain polynucleotides other than the genomic nucleic acid, e.g. some cucumber mosaic virus isolates (CMV) contain a satellite RNA that is dependent on, but unrelated in sequence to any of the CMV RNAs.

iii) Where the genetic material consists of more than one nucleic acid molecule, each may be housed in a separate particle or all may be located in one particle, e.g. tobacco mosaic virus (TMV).

iv) Enzymes specified by the viral genome may be present in the virus particle. Most of these enzymes are concerned with nucleic acid synthesis.

v) Replication of many viruses takes place in distinctive virus-induced regions of the cell.

vi) Some viruses share with certain nonviral nucleic acid molecules the property of integration into host-cell genomes and of translocation from one integration site to another.

vii) A few viruses require the presence of another virus for their replication.

In addition to infecting bacteria, animals, and birds, a large number of plant diseases are caused by a wide range of viruses. Many plant diseases are of considerable economic importance in relation to food production and the decorative plant industries. Unlike the animal response to viral infection, plants have no antibody reaction with which they can resist invasion by disease-causing agents. Although the plant cell is protected by a relatively thick cellulose cell wall, a relatively high proportion of virus diseases are spread through large insect vector populations, such as aphids, greenflies, jassids, and leaf-hoppers. In other instances, disease can be transmitted by root contacts between plants, soil-borne viruses or by nematode vectors (Horne, 1978). Therefore, the need for the detection of plant viruses is vital. Difficulties arise in plant virus detection since symptoms caused by plant viruses mostly resemble those caused by mutations, nutrient deficiencies or toxicities, insect secretions, by other pathogens, and other factors. The determination, therefore, that certain plant symptoms are caused by viruses, involves the elimination of every other possible cause of the disease, and the transmission of the virus from diseased to healthy plants in a way that would exclude transmission of any of the other causal agents. Improvement of current methods of plant viruses is important, not only economically, but also since their small size restricts the use of usual detection methods used for other pathogens.

1.2. Potexviruses.

In the initial classification scheme developed by Brandes and Wetter (1959), rod-shaped viruses were divided into 12 groups which was dependent on particle length. From this original scheme (Brandes and Bercks (1965)), sections 4,5, and 6 were combined into a single category which was designated the potato virus X group. Plant viruses belonging to this group all possessed slightly flexible particles 11-13 nm in diameter and lengths ranging from 480-580 nm. In 1971, the Plant Virus Subcommitee of the International Committee for the Nomenclature of Viruses (now called the International Committee for Taxonomy of Viruses) changed the group name to 'potexvirus' (Harrison et al., 1971; Fenner; 1976).

Other main characteristics of the members are: (a) they are usually quite stable in vitro and are readily mechanically transmissable, although most members have no known natural vectors (Matthews, 1979); (b) they are often present in high concentrations in infected plants, and their virions can be detected by light and electron microscopy as characteristically banded inclusion bodies; and (c) they are good immunogens (Purcifull and Edwardson, 1981).

1.2.2. Members.

The International Committee for Taxonomy of Viruses lists 12 plant viruses as members belonging to the potexvirus group (Harrison et al., 1971; Fenner 1976; Matthews, 1979). These are: (a) cactus virus X (CaVX); (b) cassava common mosaic (CCMV); (c) clover yellow mosaic (ClYMV); (d) Cymbidium mosaic (CybMV); (e) hydrangea ringspot (HyRSV); (f) narcissus mosaic (NaMV); (g) nerine virus X (NeVX); (h) Papaya mosaic (PaMV); (i) pepino mosaic (PepMV); (j) potato virus X (PVX); (k) Viola mottle (VioMV); (l) white clover mosaic (WCl MV).

Other possible members include the artichoke curly dwarf (ACDV); boletus (BeV); and the potato aucuba mosaic (PoAMV) (Koenig and Leseman, 1978).

1.2.3. Physiochemical Properties.

Members of the potexvirus group typically possess slightly flexible particles with lengths in the range of 470-580 nm (Koenig and Lesemann, 1978) and widths of 11-13 nm (Brandes and Bercks, 1965). Varma et al., (1968) studied negatively stained mounts under the electron microscope of four potexviruses (potato virus X, potato aucuba mosaic virus, white clover mosaic virus, and hydrangea ringspot virus) and determined that their particles showed crossbanding, with a helical construction and a pitch of about 34-37 A. The group also reported the presence of a central canal in the particles of PVX (Varma et al., 1968), but these canals are not typical in the potexviruses (Lesemann and Koenig, 1977).

Potexvirus nucleoproteins typically sediment as a single component with a sedimentation coefficient between 118-121 S (Varma et al., 1970; Bercks, 1970). Paul (1959) reported an extinction coefficient value of 2.97 for PVX at 260 nm for 1 mg/ml.

The molecular weight of potexvirus RNAs range from 2.1-2.6 x 10^6 and constitute between 5-7% of the particle weight. The RNA is single stranded and adenine is usually the most common base for the potexviruses (Lesemann and Koenig, 1977). However, with cactus virus X, the most common base is cytosine (Attathom et al., 1978). In addition, isolated potexviral RNA has been shown to retain some infectivity (Hiebert, 1970; Koenig, 1971). Sonenberg et al., (1978) reported a methylated guanosine at the 5' terminus of PVX.

Several potexviruses have coat proteins which contain a single type

of subunit, ranging from between 18,000 to 26,000, depending on the virus. With potato virus X, reported values for the subunit molecular weight have been 26,000 (Tung and Knight, 1972; Koenig et al., 1978), 31,500 (Lesnaw and Reichmann, 1970), and 22,300 (Miki and Knight, 1968). These values vary because the coat protein of PVX behaves anomalously during electrophoresis in sodium dodecyl sulphate-containing acrylamide gels, resulting in incorrect high molecular weight values. However, this is not the case for the reported low values which appears to be due to proteolysis of the subunit (Koenig et al., 1978).

The amino acid composition of PVX has been reported by Shaw et al., 1962; Miki and Knight, 1968; Goodman, 1975; Pierpoint and Carpenter, 1978; Koenig et al., 1978.

1.2.4. Antigenic Properties.

Antisera have been prepared to many of the potexviruses, but the most extensive studies on antigenic nature have been on PVX. Although native PVX protein is composed of a single type of subunit, various antigenic variations have been observed and these have been attributed to strain differences (Chester, 1936; Matthews, 1948b; Shepard and Shalla, 1972); to proteolytic degradation of the coat protein (Shepard and Secor, 1972; Koenig, 1978); or to differences in antigenic reactions of the intact virus and degraded protein derived by chemical treatment of the virus (Shepard and Shalla, 1970, Shalla and Shepard, 1970a).

Degraded protein subunits result from the treatment of PVX with 30% pyridine (Reichmann, 1960). This degraded protein preparation, called D-protein, consists of either dimers of the structural units or a mixture of monomers and dimers (Shalla and Shepard, 1970a). The

antigenic specificity of the D-protein differs markedly from that of the intact virus (Shepard and Shalla, 1970). Antiserum to formaldehyde-stabilised D-protein reacted weakly with intact virus, and similarly, the D-protein reacted weakly with antiserum to glutaraldehyde-stabilised virus. Shalla and Shepard (1970a) suggested that the protein structural units may undergo conformational changes which result in their altered antigenic specificity. The antigenic valence of the monomer is 3 or 4. However, Van Regenmortel (1978) has suggested that the valence of PVX subunits indicates the presence of cryptotopes since the small portion of the subunit exposed in the intact virus would be insufficient to accommodate that many antibody molecules.

In contrast, native protein obtained from treatment of PVX with lithium chloride demonstrated serological identity when compared with D-protein derived from pyridine degradation, (Goodman, 1975). The LiCl-derived protein was considered native because of its ability to reassemble with PVX-RNA (Goodman et al., 1975). Only the reconstituted product, but not the protein or RNA, reacted with antiserum to intact virus.

The use of sonication to fragment PVX resulted in products that were serologically indistinguishable from the intact virus and sonicated antigens in immunodiffusion tests indicated a relationship between parsnip virus 3 and PVX (Tomlinson and Walkey, 1967).

1.2.5. Relationships Between Members of the Potexvirus Group.

Serological tests have determined the degree of relatedness between different members of the potexvirus group (Koenig and Lesemann, 1978). In general, many relationships are distant and have been demonstrated with high titred antisera in liquid precipitin tests (Bercks and

Brandes, 1963; Koenig and Bercks, 1968) or in latex agglutination tests (Bercks and Querfurth, 1971). Serological relationships between PVX and cactus virus X have been demonstrated by immunodiffusion tests (Attathom et al., 1978). However, heterologous reactions of antisera may vary considerably during the course of immunisation and from one animal to another (Bercks, 1966; Koenig and Bercks, 1968).

Koenig (1978), using the method of enzyme-linked immunosorbent assays (ELISA), demonstrated that only weak cross reactions occurred in certain strains of cactus virus X. By using the double antibody sandwich form of the ELISA assay, native PVX and proteolytically degraded PVX (protein subunits in the intact virus were degraded at the N-terminus with reducing-agent dependent proteases and trypsin, or just proteases at the C-terminus) could be distinguished.

Although viruses in different taxonomic groups do not usually cross-react serologically, PVX has been reported to be serologically related to both a potyvirus (Koenig and Lesemann, 1974) and a carlavirus (Maat et al., 1978). However, the importance of these relationships is not clear.

Soluble antigens are serologically related to their corresponding virus coat proteins and these have been reported in clover yellow mosaic virus-infected pea (Purcifull and Shepard, 1964) and in PVX-infected tobacco (Shalla and Shepard, 1970b, Shepard and Secor, 1972).

Strains of PVX have been divided into 4 groups (Ireland, 1980) according to their interaction with the two dominant genes, Nb and Nx, which mainly determine the hypersensitive response of cultivated potatoes to PVX (Cockerham, 1954; Cockerham and Davidson, 1963). Strains in group 1 induce a hypersensitive reaction in plants containing either gene, strains of groups 2 and 3 induce

hypersensitivity with plants containing Nb and Nx respectively, while those of group 4 cause only systemic infection even in plants containing Nb and Nx. Potato isolated from growing potato crops in the U.K. usually belong to group 3 (e.g. PVX N); less commonly, isolated strains belong to groups 1 and 2, and seldom, if ever, to group 4.

Strains of PVX have also been detected on the basis of differences in symptomology, especially in solanaceous hosts, or on differences in host range (Matthews, 1948a, 1949; Salaman, 1938; Varma et al., 1970; Fribourg, 1975). Cross-absorption tests have been used to determine serological variants of PVX (Chester, 1936; Matthews, 1948b) although the strains were serologically related. Two strains which were serologically distinguishable (but related) on the basis of cross-absorption tests with their nucleocapsids were also distinguishable (but related) on the basis of antigenic properties of their denatured coat proteins (Shalla and Shepard, 1972). Other types of PVX variants include those which show differences in abundance and size of virus-induced inclusions (Bawden and Sheffield, 1944), differences in virus titre (Bawden and Crook, 1947), differences in thermal inactivation point (Fribourg, 1975), differences in heat tolerance in vivo (Mellor and Stace-Smith, 1970), and differences in the ability of mild strains to cross protect against severe strains (Matthews, 1948b).

Shepard (1975) recently discovered an alternative means of strain detection. He isolated protoplasts from tobacco plants infected with a mild mottle strain of PVX, and regenerated plants from infected protoplasts. About 1/10 of the protoplast-derived plants showed symptom variants. Necrotic ringspot types were frequently noted, but variants with different forms of mottle, or chlorotic spots, or no symptoms at all were observed. Thirty isolates examined in

immunodiffusion tests with D-protein antisera revealed no antigenic variants.

It is also, apparently, possible to select against certain strains by changing the culture host. Matthews (1948a) and Bercks (1970) reported that the maintenance of PVX strains in tobacco sometimes resulted in a loss of their ability to infect potato. Symptom variants were obtained with mixed infections of two PVX strains (Thomson, 1961b). The nature of the mechanism by which the new variants arose was not ascertained.

1.2.6. Purification.

Many of the potexviruses have been purified and characterised due to their stability and high concentration in infected plants. Lin et al., (1977) and Lisa and Dellavalle (1977) have reported yields of up to 0.25-3.0 g/kg tissue.

A variety of methods has been used to purify the different potexviruses and clarified sap has been obtained by treatment with organic solvents (Jones, 1977), by treatment with silver nitrate (Koenig et al., 1978), by absorption and filtration (Corbett, 1961; Francki and McClean, 1968), and by freezing and differential centrifugation (Pratt, 1961). Further purification and/or concentration have been achieved by precipitation with polyethylene glycol (de Bokx, 1965; Jones 1977), by density gradient centrifugation in sucrose (Brunt, 1966; Jones, 1977), or in caesium chloride (Miki and Oshima, 1972).

Certain problems have been encountered with the purification procedures for potato virus X and other elongated potexviruses, such as clover yellow mosaic virus. These include: (a) end-to-end (Bawden and Kleczkowski, 1948; Welsh et al., 1973) and side-to-side (Kassanis

and Govier, 1972) particle aggregation; (b) particle fragmentation (Miki and Knight, 1967; Chiko and Guthrie, 1969); (c) degradation of the protein coat (Tremaine and Agrawal, 1972; Koenig et al., 1978); (d) inability to remove host impurities (Schlegel and Delisle, 1971). The use of sodium dodecyl sulphate polyacrylamide gels to characterise protein has been reported to result in proteolysis of the protein subunits to produce heterogeneous fragments (Carpenter, 1971; Tung and Knight, 1972; Tremaine and Agrawal, 1972, Shepard and Secor, 1972). Koenig et al., 1978, proposed that the PVX subunit protein was partially degraded in the intact virus by plant sap proteins which were reducing-agent-dependent and which acted at the N-terminus of the PVX subunit peptide chain. Therefore, it was suggested that a reduction in the exposure to reducing agents would reduce the proteolytic activity.

Aggregation is another problem in the purification of certain potexviruses and results in lower yields and physical heterogeneity. The extensive in situ aggregation of PVX (Reichmann, 1959; Bercks, 1970) and clover yellow mosaic virus (Welsh et al., 1973) may contribute to the loss in yield and aggregation during extraction and purification procedures (Purcifull et al., 1966).

1.2.7. Preservation of Cultures.

The maintenance of a large collection of viruses obviously requires considerable effort and space in living plants. In addition, there may be the risk of cross-contamination when several viruses are kept in the same greenhouse. Alternative systems for the maintenance of viruses are therefore required.

One method is to store the virus in sap. The potexviruses are quite stable in vitro and retain their infectivity in sap for up to a year.

Cymbidium mosaic virus remains infective for 7 days at room temperature (Francki, 1970); hydrangea ringspot virus for 2-3 weeks at room temperature (Koenig, 1973); white clover mosaic virus for 10-99 days (Bercks, 1971); cassava common mosaic virus for 128 days at room temperature (Costa and Kitajima, 1972); potato virus X from weeks to several months (Koenig, 1978). The addition of glycerol to the sap has been reported to help retain infectivity of PVX for more than one year (Bercks, 1970).

For longer term storage of of plant viruses, pieces of infected leaf tissue are dessicated over calcium chloride and the dried leaf material then stored over magnesium perchlorate at 1°C (McKinney and Silber, 1968). Using this technique, potato virus X retained infectivity after 15 years.

Another useful storage method for certain potexvirues involves freeze-drying sap with 7% w/v glucose and peptone (Hollings and Stone, 1970) and storage under vaccumm at room temperature. Freeze-dried samples of PVX survived for at least 6-10 years at room temperature (Hollings and Stone, 1970).

1.2.8. Symptomology and Host Range.

Infected plants may show one or more of the following symptoms depending on the virus-host combination: mosaic, mottle, vein clearing, vein banding, distortion, stunting, ringspots, necrosis, and variegated colour patterns in petals. However, with some strains, macroscopic symptoms are mild or greatly reduced.

Various factors can affect the production of symptoms such as the strain of virus, the host cultivar, temperature, light, stage of development of the infection, and the presence of other viruses (Beemster and Rozendaal, 1972; deBokx, 1972). Systemically infected plants with "masked" or latent infections with no obvious signs of

disease usually undergo slight stunting of growth, and careful experiments were necessary to show that such infection reduced tuber yield by about 5-10%. The degree of stunting is generally correlated with the severity of other symptoms, particularly where loss of chlorophyll from the leaves is concerned. The host range of many individual potexviruses is fairly narrow (Fenner 1976), although PVX infects over 240 species in 16 families. Other plant viruses have a host range of over 400 species of plants in over 50 families, e.g. tobacco rattle virus (a tobravirus) (Harrison, 1970).

1.2.9. Transmission.

Potexviruses can be readily transmitted by sap inoculation using neutral or weakly basic phosphate buffers (Milbrath et al., 1973; Pratt, 1961). However, under natural conditions, tools and machines used in agricultural and horticultural operations were found to spread these viruses (Koenig and Lesemann, 1978). Todd (1957) found that PVX could be spread by man walking through potato plots after handling plants. In addition, infective PVX could be detected on the fabric of trousers a month after they were worn during a walk through infected potato plants, and in dog and rabbit hair a day after they had run among infected plants (Todd, 1957).

1.2.10. Plant-to-plant contact.

PVX can be spread by direct contact of healthy plants with diseased plants (Loughnane and Murphy, 1938). Roberts (1948) found that PVX was spread more easily amongst tomato than in potato, and that virulent strains were transmitted more frequently than avirulent strains. Similarly, PVX can also be transmitted from infected tubers to healthy ones which were stored in the same bag (Bawden et al., 1948).

1.2.11. Arthropod vectors.

In general, most potexviruses have no known natural arthropod vectors, although a few cases of of transmission by aphids or other insects have been reported. Pirone and Kassanis (1975) found that PVX treated with poly L-ornithine could be transmitted by Myzus persicae, but not untreated PVX. Another insect implicated in the transmission of PVX is the grasshopper (Walters, 1952). Orlob (1968) showed that the two-spotted spider mite took up PVX but failed to transmit the virus.

1.2.12. Fungal transmission.

The phycomycete, Synchytrium endobioticum, causes crown wart disease of potato. Nienhaus and Stille, (1965) reported that zoospores released from sporangia of fungus which developed from PVX-infected plants transmitted PVX to healthy potatoes. However, the zoospores failed to transmit PVX to tomato under the same circumstances (Lange, 1978) and PVX particles were absent from the fungus in this study.

1.2.13. Replication.

Not much is known about the replication of potato virus X (Koenig and Lesemann, 1978). Atebekov (1975) found that isolated PVX RNA had the same host range as the intact virus. It was found that a hybrid of PVX RNA encapsidated by protein of cucumber virus-4 (a tobamovirus) could infect Nicotiana glutinosa, which is a host of PVX but not of the tobamovirus. The same hybrid, however, failed to infect cucumber, which is a host for cucumber virus-4 but not for PVX.

The replication of PVX has also been studied using protoplast systems (Shalla and Petersen, 1973; Otsuki et al., 1974; Honda et al.,

1975). Treatment with poly L-ornithine was required for infection. Using electron microscope techniques, particles in protoplasts were shown to be absorbed to the plasmalemma and in vesicles which were formed by invaginations of the plasmalemma 10 minutes after inoculation (Honda et al., 1975).

Another characteristic of PVX-infected tissues are laminate inclusion bodies which consist of multilayered, proteinaceous sheets in the cytoplasm (Kozar and Sheludko, 1969; Stols et al., 1970; Shalla and Shepard, 1972). The laminate inclusions do not consist of PVX protein (Shalla and Shepard, 1972) and do not appear to be connected to virus synthesis since they only appear after considerable viral antigen and virus synthesis in PVX-infected protoplast (Shalla and Petersen, 1973; Otsuki et al., 1974) or in leaves (Allison and Shalla, 1974).

PVX production is suppressed in tobacco protoplasts by cycloheximide, but not chloramphenicol (Otsuki et al., 1974). This suggests that possible sites of protein synthesis are cytoplasmic ribosomes rather than chloroplastic ribosomes. Otsuki et al. (1974) also found that the addition of actinomycin D within 3 hours of inoculation also significantly reduced the percentage of infected protoplasts but not when replication had been initiated.

When compared to untreated protoplast after 24 hours, the infectivity of actinomycin D-treated protoplasts was much lower but climbed markedly after 48 hours. This indicated that the actinomycin D affected some initial process leading up to PVX replication but not with PVX replication itself or that an early step in PVX replication depended upon the product of a host gene (Otsuki et al., 1974). However, the replication of PVX in detached Datura stramomium leaves was stimulated by actinomycin D treatment, depending on the time of

application and the concentration of the antibiotic (Reunova et al., 1973).

Sonenberg et al. (1978) reported the presence of a 7-methyl guanosine at the 5' terminus of PVX RNA. This is also a feature of many messenger RNAs (Wodnar-Filipowicz et al., 1978) and a number of plant viral RNAs, e.g. BMV (Dasgupta et al., 1976; BSMV (Agranovsky et al., 1979). Sonenberg has also reported the absence of a poly (A) sequence at the 3' terminus of PVX based on affinity chromatography reults. These results, however, are contrary to those reported by Morozov et al. (1981) and Morozov et al. (1983). They report the presence of a 3' terminal poly (A) sequence of 50-200 residues in length.

PVX RNA is an efficient messenger in vitro, although capsid polypeptide was not detected in either the wheat germ or rabbit reticulocyte lysate systems (Ricciardi et al., 1978; Wodnar-Filopowicz et al., 1980). The major translation products were two polypeptides of 180 kD and 145 kD. These are probably identical at the N-terminus. Two apparently corresponding polypeptides are synthesised by TMV RNA in vitro. The genes for these ar 5' co-terminal, the larger resulting from suppression of an amber stop codon and subsequent read-through (Goelet et al., 1982). Morozov et al. (1983) report the presence of an open reading frame of 4711 bp located 784 bp from the start of the poly (A) tail at the 3' end of the RNA. This probably codes for the coat protein, presumably via a subgenomic mRNA. Based on these results, a tentative map of the location of genes on PVX RNA is shown in Fig.1. There may be additional open reading frames (ORFs) located between those for p180 and coat protein.

PVX replication is stimulated in plants doubly-infected with PVX and either potato virus Y (PVY) or tobacco mosaic virus (TMV) (Thomson, 1961a; Goodman and Ross, 1974c). Rochow and Ross (1955)

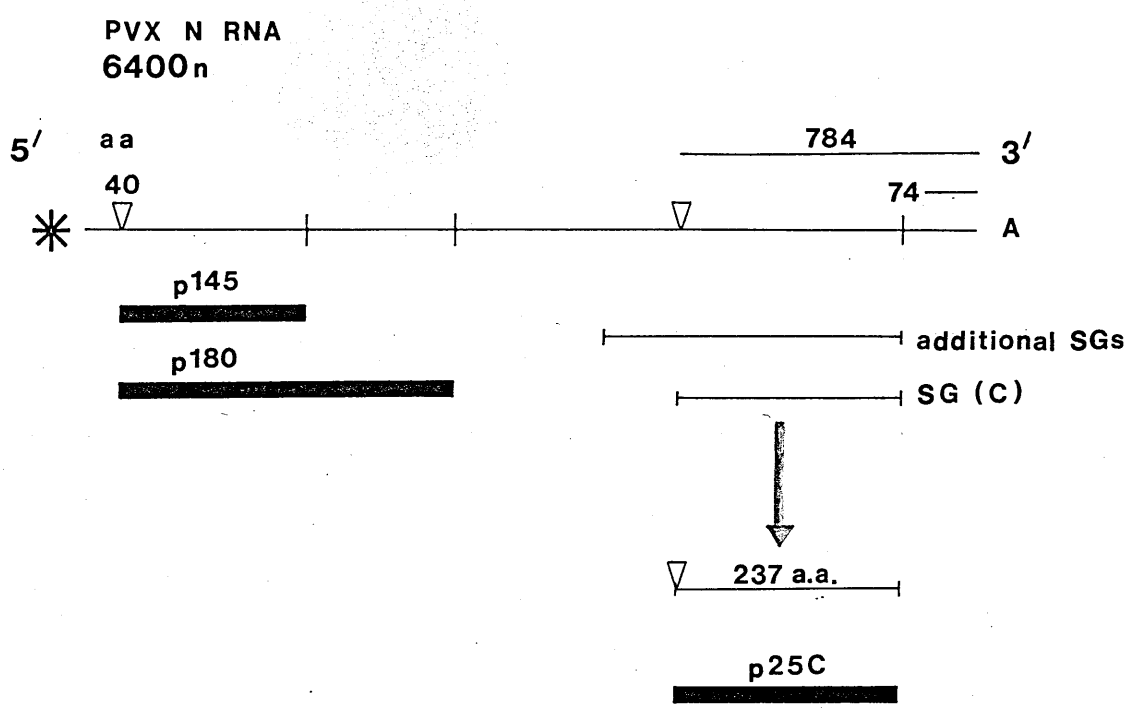


Fig.1: The genome of PVX, based on data by Morozov et al. (1981) and Morozov et al. (1983). The genome is 6,400 nucleotides in length and included in the diagram are the following:

∇ = translatioin initiation

$\ast$ = $m^7G^5ppp^{5'}$ cap

| = translation termination

A = poly A sequence

n = approximate number of nucleotides which comprise infectious RNA molecules

C = coat protein

⇩ = indicates the presumed derivation of subgenomic RNAs and of protein fragments

p = molecular weight x 10^{-3}

aa = amino acids

SG = subgenomic mRNA (hypothetical)

detected up to 10 times as much PVX in doubly infected plants as compared to plants infected with just PVX although this is affected by the timing of invasion of tissues by the two viruses (Goodman and Ross, 1974b).

Goodman and Ross (1974a), using ultrastructural studies and fluorescent antibody staining, showed that both viruses (PVX with either potato virus Y or tobacco mosaic virus) were replicating in the same cells and that the increased production of PVX was due to an increase in virus production per cell rather than an increase in the number of cells supporting PVX replication. Contrary to this, Skofenko et al. (1975) reported an increase in the frequency of PVX-infected cells over singly infected plants. Enhancement of PVX replication is apparently not due to heterologous encapsidation of PVX RNA by either PVY or TMV coat protein (Goodman and Ross, 1974c).

1.3. Comparative Diagnosis.

The diagnosis of plant viruses is important in certain situations: (i) plant samples may have to be analysed for quarantine reasons or grower information; (ii) disease surveys in crops or weeds; and (iii) the need for testing large numbers of samples such as propagating material for the absence of one or more viruses. The diagnostic method chosen will be dependent on several factors such as the availability of materials, the experience of the investigator; the economic importance of the problem, on practical limitations of time that can be expended on the project; and on available instrumentation such as an electron microscope.

The identification of potexviruses is similar to those of other plant viruses. Initial diagnostic work for a smaller number of samples usually involves a combination of several methods such as inoculation

of the sample to indicator plants, symptomology, light microscopy of inclusion bodies, electron microscopy of particles in extracts, and serology. The results from initial studies would indicate the virus or viruses as the causal agents of the disease. Depending on the initial results, further characterisation of the virus could be undertaken such as electron microscopy of tissue sections, physicochemical studies, in vitro stability of the virus, cross protection, and vector studies.

However, when mass testing of propagating material is required, usually only one method is selected depending on the preliminary comparison of various methods. Serological methods are quite useful for mass testing and they have been used extensively for indexing potato stocks for freedom from PVX and other viruses (de Bokx, 1972; Shepard and Carflin, 1975). The main methods used for diagnosing virus diseases of plants after purification can be divided into three main groups: 1) Transmission tests; 2) Serodiagnosis; and 3) Electron microscopy.

1.3.2. Purification.

Purified antigen is required for serological testing. Usually, the purification or isolation of viruses is most commonly obtained by ultracentrifugation of the plant sap. This involves 2 to 5 cycles of alternate high (40,000 to 100,000g or more) and low (3,000 to 10,000 g) speeds. The ultracentrifugation sequentially pellets plant material and debris during the low speed spin and RNA during the high speed spin. Therefore, ultracentrifugation concentrates the virus and separates it from host cell contaminants. Several modifications of the ultracentrifugation technique, such as density gradient centrifugation have also been employed. In these methods, the virus is obtained as a

colourless pellet which may then be used for infections, electron microscopy and serology. Other principles for purifying viruses are known. These include:

1) the use of organic solvents, e.g. chloroform and methanol;

2) the use of polyethylene glycol, e.g. in the purification of HIV;

3) by antibody precipitation;

4) filtration procedures, e.g. Celite filtration for SYNV;

5) density gradient centrifugation on sucrose gradients (rate zonal centrifugation);

6) equilibrium density gradients, e.g. caesium chloride gradients.

1.3.3. Transmission tests.

The technique of mechanical inoculation or sap transmission testing was established as a means of virus detection and diagnosis after Allard (1914) found that of the various plant species, tobacco mosaic virus (TMV) would only infect members of the Solanaceae, indicating the specificity of host/virus relationships. This was followed in 1959 by Hollings who, under standard conditions, determined the reactions of many plant viruses when inoculated to different plant species. From this, it was found that the detection and characterisation of viruses could be achieved with a relatively small range of test or indicator plant species including mainly two or three species of Nicotiana, two Chenopodium spp. and cucumber. The local and systemic reactions which occur following sap transmission of virus to these indicator plants are often sufficient to characterise viruses. The susceptibility of indicator plants to virus infection was found to increase when plants were kept in the dark for a period before inoculation (Bawden and Roberts, 1947).

1.3.4. Use of indicator plants.

The use of test plants for diagnostic purposes has several advantages: (i) they can be used in the identification of viruses (e.g. Hampton et al., 1978), (ii) they help to establish if a disease is viral in nature; (iii) mixtures of viruses can be detected and separated; (iv) they provide a source of virus for additional study; (v) infectivity tests can be comparatively sensitive (Hollings, 1974), and in certain instances may be even more sensitive than serological tests such as ELISA (McLaughlin and Barnett, 1978), and (vi) useful information concerning epidemiology and disease control may be obtained.

With potexviruses, indicator plants have been widely used for identification. Lists of experimental hosts for PVX and PoAMV have been published by Horvath (1978). Nicotiana debneyi has been reported by MacKinnon and Bagnall (1972) to be useful for the detection of potato viruses X, S and Y.

The methods adopted for sap transmission vary but are, some argue, the most sensitive tests available since a great proportion of viruses are mechanically transmissable. An advantage of the method is that sap transmission can be used for strain typing, for example the inoculation of TMV strains to isogenic tomato and pepper lines, and to separate virus mixtures when differential host plant susceptibility may be used to effect.

However, there are several disadvantages to the method. Engelbrecht and van Regenmortel (1968) reported erroneous results due to the presence of the latent virus sowbane mosaic in Chenopodium plants and virus reactions on test plants may be masked if the glasshouse environment is unsuitable. Also the test method has disadvantages; it requires the routine production of a range of many test plants which

may never be used in laboratories with occasional demand; efficiently maintained glasshouse accommodation is needed; and most importantly sap transmission is time consuming.

In addition, not all viruses are mechanically transmissable (e.g. barley yellow dwarf virus (BYDV) and potato leaf roll virus (PLRV)) and a natural virus vector may have to be used to demonstrate and identify the presence of the virus. Therefore, a diagnostic laboratory must maintain aviruliferous aphid colonies and be proficient in handling them in transmission tests.

In certain cases, viruses or virus-like agents can only be detected by graft transmission to susceptible hosts in which symptoms are produced.

Apart from these methods, there are two additional tests which can be grouped with the transmission tests. The first relies on the detection of virus induced-changes within the host cell such as callose formation in the phloem or phloem cell necrosis in potato stems and tubers infected with potato leaf roll virus (de Bokx, 1972). These tests require light microscopic examination of stained sections of tissue, they are subjective and may vary in reliability according to the cultivar under test. The second test is used for the detection of virus in farmers' own grown potato tubers. Eye pieces cut from a representative sample of potential seed tubers are grown in seed trays during the winter in glasshouses with 18 hours artificial light and a temperature of 15°C. After 6 weeks growth, plantlets have grown to about 300 mm high and the tuber-borne viruses can be detected by visual examination. However, like the above test, such testing is subjective, and development of leaf roll symptoms may be unreliable.

1.3.5. Serodiagnosis.

In recent years, significant advances have been made in the field of serodiagnosis. The specificity of the antigen/antibody relationship provides a versatile tool because of the simple nature of the proteins which make up the virus coat. These methods are dependent upon the specific responses between antibodies (or immunoglobulins) and antigens (or immunogens).

An antibody is a protein synthesised by an animal in response to the presence of a foreign substance. The antibody has specific affinity for the foreign material that elicited its synthesis. Antibody molecules are secreted by plasma cells, which are derived from B-lymphocytes (Stryer, 1981). Several classes of immunoglobulins have been recognised. The IgG and IgM immunoglobulins are the major types present in most sera and are the only ones that, so far, have been implicated in serological reactions with plant viruses (Matthews, 1981).

An antigen (immunogen) is a foreign macromolecule capable of eliciting antibody formation. Proteins, polysaccharides, and nucleic acids are usually effective antigens (Stryer, 1981). The specificity of an antibody is directed against a particular site on an antigen called the antigenic determinant. The antigenicity of a molecule is the ability of the antigen to combined with the specific antibody produced. The immunogenicity of an antigen is the ability to stimulate the animal to produce antibody that will react specifically with it, and in general, large molecules are usually more effective immunogens than small ones. Thus, plant viruses, contain protein multimers, are often very effective in stimulating specific antibody production.

Until recently, serodiagnosis was limited. The crudest method, the slide agglutination test relies on the trapping of chloroplasts by the antigen/antibody complex for positive diagnosis. This method is still

used as a field test for certification schemes but may be unreliable.

A second, more sensitive test is the microprecipitin test which requires preparation in the laboratory (Van Slogteren, 1955). Sap extracts from sample plant material are clarified by centrifugation before being mixed with dilutions of antiserum. The antigen and antibody preparation are mixed as small drops on a Petri dish. The dish is pre-coated with a plastic film so that the drops do not float off when liquid paraffin is poured on to the plate. This prevents evaporation so that the whole plate can be incubated for a period, after which precipitation reactions can be observed by dark-ground illuminated microscopy. This method has the combined advantages of economy of antiserum with economy of space. It is useful for both isometric and filamentous particle morphology.

A third, widely used serological test is the gel diffusion or Ouchterlony double diffusion test (Ouchterlony, 1953). In this method, antigen and antibody diffuse rapidly from wells cut in agar or agarose gel on microscope slides or on Petri dishes. Where the homologous components meet and mix in optimal proportions, precipitation occurs as a visible line in the agar. However, since the test relies on the diffusion of particles through the agar gel, it is only applicable to isometric viruses but not filamentous ones. Several modifications have been used to make this test available for filamentous viruses, such as fragmentation of the particles by ultrasound or by detergents. However, incomplete fragmentation may result in imperfect diffusion and may change the nature of the antigenic determinants and therefore the specificity of the test.

The main disadvantage of the gel diffusion test is that it takes several days to prepare and to complete. Precipitation lines may be indistinct or transient, and workers have to be proficient and

familiar with the test.

An improvement on this method was achieved by Abu Salih et al., (1968) who devised a method which depended upon the attachment of the virus specific antibody to a carrier material, in this case latex particles. When latex suspensions, sensitised with the specific gamma-globulin fraction of the antiserum, are mixed and stirred with the antigen preparation, complexes form and are visible as a granular white precipitate within a few minutes to an hour. In the diagnostic laboratory, this test has a number of advantages:

1. Sensitised latex can be prepared by a parent or central laboratory and stored (refrigerated at 4°C)
2. The technique is sensitive, up to ten times more so than the microprecipitin test, and many times more than the slide agglutination test.
3. The method is applicable to all viruses, whatever their morphology.
4. The test is economic on antiserum and does not require sophisticated equipment.

Querfurth and Paul (1979), modified the test and showed that the test worked more effectively with viruses of the potyvirus group, and with some sera if the latex used was first sensitised with protein A, a bacterial protein. It was suggested that this might make the final latex/antibody/virus complex more flexible and possibly orientate the gamma globulin molecule on the complex so that its receptor sites were better arranged to catch the virus.

In another modification (Lundsgaard, 1976), the test is carried out in capillary tubes which can be rotated to stir the contents and the reaction is then assessed miocroscopically. Therefore, smaller volumes of reagents are required and many more samples can be accommodated in a small space.

However, none of these serodiagnostic methods are suitable for testing large samples of the same disease, such as the need for potato tuber indexing. This has been achieved by the latest development of the enzyme-linked immunosorbent assay or ELISA.

1.3.6. Enzyme-Linked Immunosorbent Assay (ELISA Test).

This test was developed by Voller et al., (1976) for use originally with plum pox and arabis mosaic viruses. The use of ELISA in the detection of plant viruses is economical in the use of reactants, possessing relative sensitivity and simplicity as well as its adaptability to quantitative measurements and suitability for use with crude plant extracts. The ELISA, in addition, also offers a number of advantages over the use of radiolabelled materials which are used in the protocols for many screening methods. Recent developments have established that the method gives approximately the same sensitivity as radioimmunoassays, without utilising unstable, gamma-emitting isotopes.

In addition, radioactive isotopes have an inherent rate of decay which means that the radiolabelled reagents will lose their activity over time. Therefore, repeated relabelling, retesting, and restandardisation is required. Also, radiolabelled systems subject the users to a potential radiation hazard. Lastly, expensive equipment is needed to measure the radiation, thus restricting these assays to relatively large laboratories.

The development of the ELISA has, however, retained some of the advantages of the radioimmunoassays but has avoided some of the inherent probelms. ELISA is similar to radioimmunoassays except that an enzyme is used as the immunoglobulin marker in place of a radioactive isotope. Since a single molecule of enzyme can react with

a large number of substrate molecules, a very small amount of enzyme-labelled immunoglobulin can react with substrate to provide a visibly coloured reaction (Clark and Adams, 1977). Expensive detection equipment is, therefore, not required. Also, enzyme-antibody conjugates can be prepared which have negligible loss of activity after long periods of storage, therefore obviating the need for constant relabelling and retesting of reagents. ELISA systems can be designed to measure either antigen or antibody by means of a number of system designs. Obviously, in the case of an infectious agent such as PVX, the antibody is preferentially labelled in order to detect the antigen. In most cases, the system used is the direct or one-antibody sandwich (illustrated in Fig.2). In this system, an antibody, referred to as the capture antibody, is used to coat a solid phase. After a suitable incubation period, the unbound antibody is washed off and the test specimen is added. After another incubation period, the excess specimen is washed off and an aliquot of enzyme-labelled antibody is added. This will bind to any antigen bound to the capture antibody on the solid phase. After washing off the unbound enzyme-labelled antibody, substrate is added. Enzyme bound to the solid phase by the previous steps will convert the substrate to a visible form which can be seen by the naked eye or measured in a spectrophotometer. This system has the advantage of requiring only a single antibody. However, this single antibody approach means that a laboratory requires an enzyme-labelled reagent for each antigen to be tested, whereas a single labelled reagent would be more convenient. This can be accomplished by the use of an indirect ELISA where the antigen is bound directly to the plate. Unlabelled primary antibody is added and then quantitatively assessed by the use of an enzyme-labelled anti-immunoglobulin raised in a different animal to that which is used

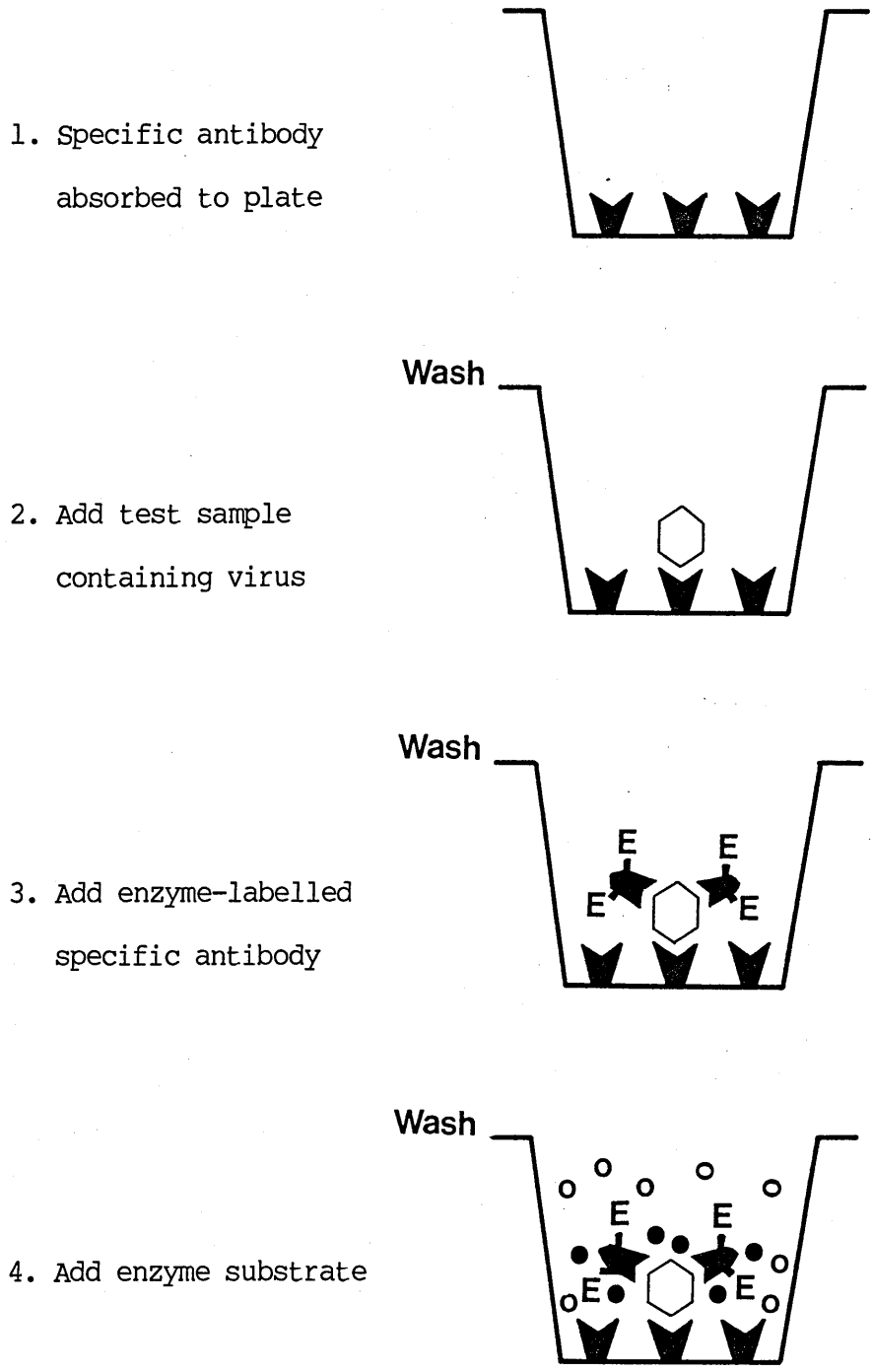


Fig.2. Principle of the ELISA technique for plant viruses, where colour intensity is proportional to virus concentration (Clark and and Adams, 1977).

to produce the primary antibody.

Obviously the enzymes used in the assay need to fulfil certain requirements, mainly that the enzyme be active when conjugated to an antibody molecule and that the conjugate be stable during storage. Two enzymes which have attained widespread usage are peroxidase and alkaline phosphatase (Yolken, 1979). Peroxidase has the advantage of being relatively inexpensive and having a large number of substrate systems which produce colour easily visualized by the naked eye. However, most of the proton receptors utilised precipitate after the reaction, thus decreasing the sensitivity of the assay. An additional disadvantage of the peroxidase is that it is inactivated by bacteriostatic agents, such as sodium azide, thus shortening its effective shelf life. Alkaline phosphatase, on the other hand, is stable in sodium azide and has a number of naturally-occurring substrates. However, alkaline phosphatase of specific activity high enough to be used must be obtained from mammalian tissue such as calf intestine. This source is expensive and the supply is often limited (Yolken, 1979).

Like the latex test, the ELISA is effective with either isometric or filamentous viruses. It is useful for rapid testing of samples from the field and is therefore valuable for virus indexing of plant material and in eradication campaigns and epidemiological studies, its sensitivity accelerates the work by bulking samples.

The main advantage of the ELISA in the screening and indexing of plants for viruses, in comparison with other serological tests, is that it can have low strain specificity and is, therefore, an advantage in detecting more than one strain. However, only viral antigens (such as the viral protein coat) are measured and not the infectious RNA.

Serology has been widely used for the identification of many plant

viruses, e.g. wheat streak mosaic virus, barley stripe mosaic virus and tobacco mosaic virus (Ball, 1974) but the importance of controls is of vital consideration. False positive results may occur due to reaction of host plant antigens with their corresponding antibodies which may contaminate a virus antiserum (van Regenmortel, 1963, 1966). In addition, an imbalance of reactants, insensitivity of a particular technique, or antigenic disparity between intact virus and its degradation products (Shepard and Shalla, 1970) can produce results which may be erroneously interpreted as negative. Distant serological relationships observed among potexviruses (Bercks, 1966) also must be considered in making specific identifications. Also, there are major limitations to all serological techniques if the virus to be examined is a poor antigen or is difficult to purify.

1.3.7. Electron Microscopy.

The morphology and normally high concentrations of potexviruses in infected tissues are useful for their detection with electron microscopy. Until recently, the use of the electron microscope was confined to the research laboratory where the equipment needed constant attention from technicians. However, with the development of more reliable machines and of quick methods for preparation and examination of plant material, the electron microscope is now used for virus diagnosis.

All the methods are essentially simple in preparation without the need for embedding and sectioning. These range from simple tissue maceration in heavy metal stains such as phosphotungstic acid, methylamine tungstate, ammonium molybdate and uranium acetate, to more involved extraction methods for the more difficult viruses. Viruses such as PVX, PVM, TMV, barley stripe mosaic virus, and tobacco rattle

virus, could be obtained from the spongy mesophyll of virus-infected leaves by peeling strips from the lower epidermis (Hitchborn and Hills, 1965). These strips were crushed in stain on a grid and were reported to give clean preparations.

Fenner (1976) reported a length range of 480-580 nm as characteristic of the group, although virus particles of between 400-600 were also considered as possible members. However, end-to-end aggregation resulting from purification (Welsh et al., 1973) or from the effects of environmental factors during the course of virus multiplication (Moore and Guthrie, 1965; Brandes, 1966) as well as fragmentation, can interfere with particle length determination. Potexviruses themselves are sometimes difficult to detect in extracts from affected leaves e.g. rhododendron necrotic ringspot disease (Coyier et al., 1977), but Sampson and Taylor (1968) found that electron microscopy was more sensitive than infectivity assays or microprecipitin tests for detection of PVX in potato tubers. Infectivity was slightly more sensitive than electron microscopy, however, for detection of PVX in leaf extracts. Sampson and Taylor (1968) also pointed out that PVX could be readily distinguished morphologically from potato virus S (a carlavirus) and potato virus Y (a potyvirus).

The use of electron microscopy, however, has its disadvantages. Properly calibrated, the electron microscope can detect moderate virus concentrations in crude preparations and the size and shape of the virus can be determined. However, in such preparations viruses with similar morphology cannot be distinguished and further testing may be needed.

In crude preparations, the detection of virus particles by electron microscopy may not be sufficiently sensitive where low concentrations

occur and some of the stains used may damage particles. Recent developments have overcome these problems by linking techniques of serology with electron microscopy.

The classic method by Ball and Brakke (1968), called the 'antiserum virus mixtures' involves mixing the virus extract with antiserum in optimal proportions before loading on to the carbon coated grid and staining. When virus and antiserum are homologous, 'clumping' of the antibody-virus complex occurs which fixes and protects the virus in the sap extract. In some of the clumps, where perhaps there may be antibody excess, virus particles have a coating of antibody stain mixture which can be readily visualised in the electron microscope. Therefore, using this method, there is both an increase in sensitivity of detection particularly of fragile virus particles and there may be specific virus labelling.

The use of the 'serum activated grid' or antiserum-coated grids (ACG's) was introduced by Derrick (1972). Grids are sensitised with a layer of antibody globulin by floating them, carbon coat downwards, on drops of diluted antiserum. The sample is then prepared and loaded on to the sensitised grid and stained or shadowed before examination (Derrick and Brlansky 1976; Milne and Luisoni, 1977). The virus is trapped by its homologous antiserum and fixed to the grid, increasing the sensitivity of detection by this method by some ten to a hundred times.

The third method (Milne and Luisoni, 1977) is a refinement of the first technique. Grids on to which virus has been loaded, either conventionally or by the 'serum activated grid' method may be floated again on antiserum, so that the virus particles become coated, or decorated, with globulin which stains darkly. This method, known as 'decoration' enables specific confirmation of virus identity, allowing

distinction of morphologically identical viruses on one electron grid.

1.3.8. Cytology.

Another method which has been used involves the use of a light microscope. In a study by Christie and Edwardson (1977), nearly all plant viruses were shown to induce some type of recognisable inclusion body or alteration in host cells. Diseased tissue can be stained and examined under a light microscope within 10-15 minutes, using epidermal strips, whole mounts or sections of the tissue. However, a magnification capacity of 1000 times is usually required although some inclusion bodies are observable under lower magnification. The use of light microscopy can also detect multiple infections. Information obtained using this method, such as size, shape, internal structure, types of tissue invaded, and the intracellular location of inclusions all provide diagnostic information. However, treatments for certain potexviruses may distort or destroy others. Christie and Edwardson (1977) found that the use of the nonionic detergent Triton X-100 helps in the detection of certain potyviruses by dissociating plastids but disperses the banded aggregates of potexvirus infections.

1.3.9. Advantages and Disadvantages of the Detection Systems.

From the methods described above, the choice of technique chosen must be determined by the quality of information required: the economy in terms of cost; staff time input, special training requirements; test duration and sensitivity; and the need for specialised equipment.

Transmission tests are accurate though wasteful, both in glasshouse space and test duration, i.e. the time required for results. They usually require a large number of specialised indicator plants which, unless there is a constant requirement for plants, means that time is needed to keep a constant stock at all times.

The use of the electron microscope is limited since specialised training is required for its use. It is not a reliable quantitative method for virus detection and cannot be used for large numbers of samples in field trials.

Similarly, serodiagnostic methods have their limitations: 1) they measure the virus protein antigen, not the amount of infective virus; 2) infectivity measurements can usually detect and measure about one-tenth to one-hundredth the concentration of virus required for the precipitation reaction carried out in tubes, although the microprecipitin test requires a lot less material than the infectivity tests; 3) with some rod-shaped viruses, end-to-end aggregation can markedly affect results of some of the assay methods (Welsh et al., 1973).

However, serodiagnosis has a number of advantages over the other methods such as the infectivity assays:

1. Results are obtained in a few minutes or hours compared with days for infectivity assays;
2. The methods give an answer that is directly proportional to virus concentration over a wide range of concentrations;
3. Serological tests are particularly useful with viruses that have no good local lesion host or which are not sap-transmissable;
4. Infectivity assays are limited by the number of plants which can be grown under comparable conditions; and
5. Antisera can be stored and comparable tests made over a period of years.

Although the enzyme-linked immunosorbent assay has several advantages over the other tests, it still has its limitations associated with the antigen/antibody reactions and the fact that it measures protein and not infectious RNA. Attempts to develop an alternative system bore two points in mind. Firstly, that the method

must be able to detect infectious viral RNA, and secondly, that the method should not require the use of radioisotopes.

The method chosen for the assay was a modification of the dot-blot assay whereby the viral samples, either crude sap, viral particles in solutions, or RNA, are applied to the binding medium, a nitrocellulose filter. After fixation of the samples,a complementary probe (a complementary DNA strand synthesised from the RNA template, or by nick translation of a clone) is hybridised to the samples and examined.

1.3.10 Hybridisation of Nucleic acids Immobilised on Solid Supports.

The theory behind the hybridisation assay is as follows. Duplex DNA is composed of two complementary polynucleotide chains which can be easily denatured by heat or by increasing the pH. This denaturation leads to a reversible separation of the two complementary strands. Under appropriate conditions, the strands will reunite and reform a duplex molecule. The reassociation is highly specific and the two separated polynucleotide chains will only reassociate if they are complementary. When foreign nucleotide chains are introduced to immobilised, denatured DNA, each strand will form duplexes with one of the strands provided that it shares a sequences with the denatured DNA. This reaction, which occurs for both RNA and DNA, is designated nucleic acid hybridisation and is the basis for the specific detection of bound viral RNA with appropriate probes.

There are several alternatives used in the detection of hybrids. Certain properties of nucleic acids can be exploited for this purpose; the absorbance of ultraviolet light differs between single and double stranded nucleic acids ('hyperchromicity') and this can be detected using a spectrophotometer. Other methods include the physical

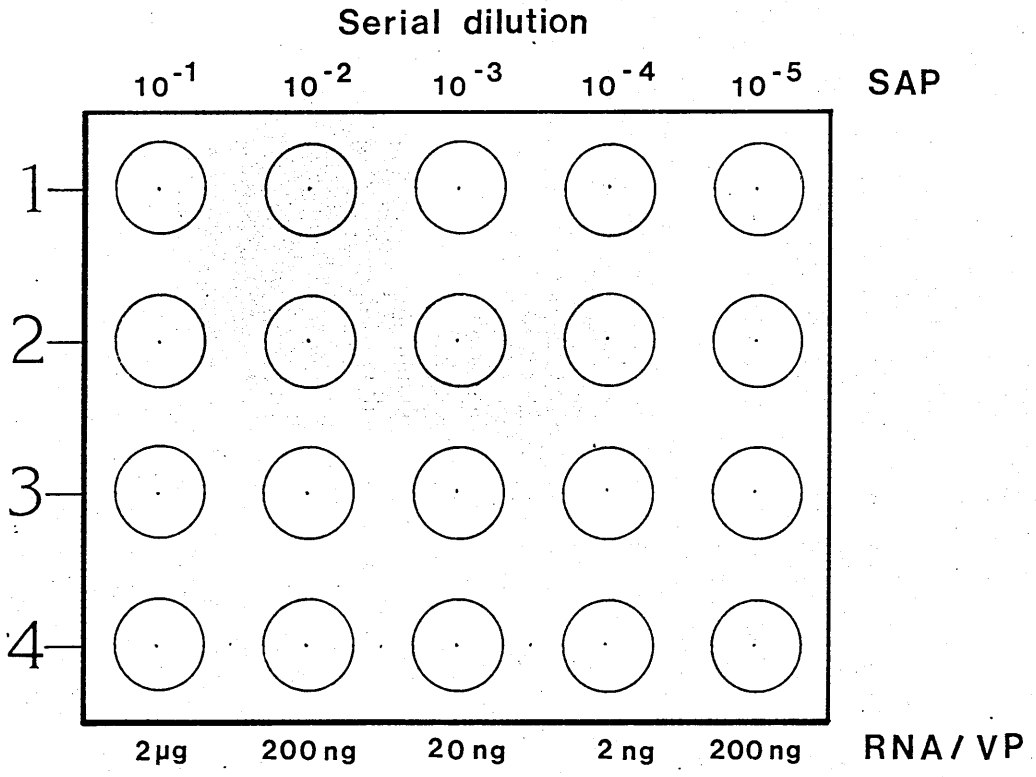


Fig.3: Diagrammetic representation of a sap spot filter for hybridisation with either ^{32}P or biotinylated probe. Each sample is spotted as 2 ul aliquots in a serial dilution of sap, viral particles or viral RNA. With RNA, dilutions were from 2 ug to 20 pg. With infected and healthy sap, aliquots of dilutions from 0.1 x to 0.00001 x dilution were spotted.

separation of the single and double stranded nucleic acids using hydroxylapatite chromatography. However, with these methods, pure DNA samples are required and the methods are, therefore, not applicable for the detection of nucleic acids in crude specimens.

The detection of hybrid formation is most commonly detected by immobilising nucleic acids on nitrocellulose (Nygaard and Hall, 1963; Nygaard and Hall, 1964; Gillespie and Spiegelman, 1965). In this method, the nucleic acid to be analysed is denatured and filtered through a nitrocellulose membrane. The single strands bind to the filter in such a way that they are able to interact with nucleic acids in a solution that is in contact with the filter. A radiolabelled probe is added, to detect the presence of a specific nucleic acid in the immobilised sample. If the strands are complementary between the probe and the immobilised nucleic acid, hybridisation will occur, which can be measured by scintillation counting or by subjecting the filter to autoradiography. This method, sometimes known as spot hybridisation, can be used for the detection of viral RNA. Serial dilutions of all isolates in sap, as purified viral particles and RNA are spotted on to the nitrocellulose and the filters then prepared as described (Materials and Methods 2.6).

The usage of radiolabelled isotopes is common in many assays and an alternative non-radioactive protocol for the visualisation of bound RNA is more difficult to achieve. The system which was finally chosen involves the production of a complementary DNA probe which had been labelled with biotin-11-dUTP and hybridised to the target RNA (Fig.5). The nitrocellulose was then incubated with streptavidin (a protein produced by Streptomyces avidinii with a high affinity for biotin) and a biotin-conjugated enzyme polymer, which bound to the biotinylated probe/target hybrid. When substrate dyes are added, the target DNA

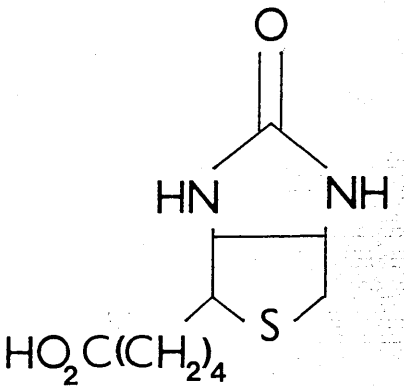
sequence is visualised as a pink/purple spot on the filter (Fig.3).

1.3.11. Biotin and Streptavidin.

The high affinity constant between the glycoprotein avidin and the vitamin biotin has prompted research into the nature of this complex. Biotin (also known as vitamin H) possesses several features which are advantageous in a probe (Langer et al., 1981). The interaction between biotin and avidin (a 68,000-dalton glycoprotein from egg white) has one of the highest binding constants known ($K_{dis} = 10^{15}$). When avidin is coupled to appropriate indicator molecules, fluorescent dyes, electron dense proteins, enzymes or antibodies, minute quantities of biotin can be detected. The specificity of the biotin-avidin complex has been exploited to develop methods for the visual localisation of specific proteins, lipids, and carbohydrates on or within cells, or more recently, for the detection of nucleic acids (Broker et al., 1978). Broker et al. chemically crosslinked biotin to RNA, via cytochrome C or polyamine bridges, and used these RNA-biotin complexes as probes for in situ hybridisation. The sites of hybridisation were visualised in the electron microscope through the binding of ferritin-avidin or avidin methacrylate spheres. Although this approach to the detection of polynucleotide sequences was successful in the specialised cases examined, a simpler and more general procedure for preparing biotin-substituted nucleic acids was desirable. Therefore, biotin directly attached to a nucleotide that functions as an efficient polymerase substrate would be more versatile, both in the experimental protocols and in the detection methods that could be used.

Langer et al., (1981) have synthesised a number of of nucleotide analogs that contain potential probe determinants (e.g. biotin,

1



2

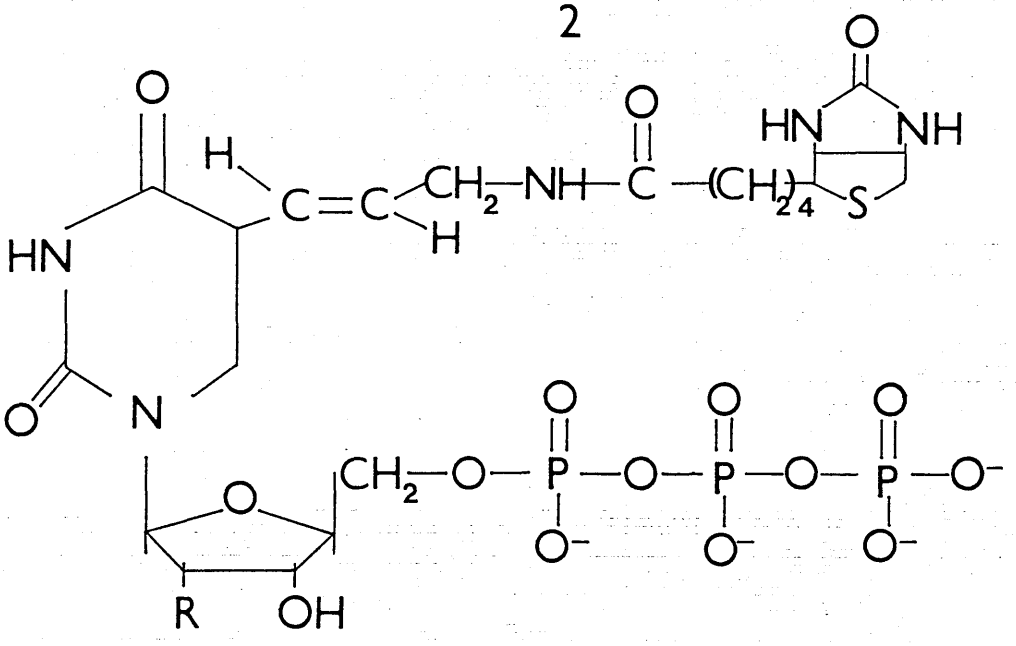


Fig 4. Diagram showing 1) Biotin (vitamin H) and 2) analogs of UTP and dUTP (R = OH or H respectively). The biotin molecule has been covalently bound to the pyrimidine ring through an allylamine arm. These biotin-labelled nucleotides are efficient substrates for a variety of DNA and RNA polymerases (Langer et al., 1981)

iminobiotin, and 2,4,-dinitrophenol groups) covalently attached to the pyrimidine or purine in the hope that one of them might prove useful as an affinity reagent. Biotin-labelled derivatives of UTP and dUTP are substrates for RNA or DNA polymerases. The properties of the resultant biotin-substituted polynucleotides appear to satisfy the basic criteria required of a good affinity probe.

Streptavidin, the other part of the complex, is an avidin analog (a protein of molecular weight 60,000) with four high affinity sites for biotin. It is isolated from the bacterium Streptomyces avidinii. Like avidin, streptavidin is a tetramer consisting of four subunits. While streptavidin and egg white avidin bind biotin with equally high affinity, streptavidin exhibits very little of the undesirable non-specific binding that is characteristic of avidin at physiological pH. This is due to two important differences between streptavidin and avidin.

1. Streptavidin has an isoelectric point close to neutrality. Avidin, however, has an isoelectric point of 10, which means that the protein is positively charged at pH 7.
2. Streptavidin contains no carbohydrate while avidin contains approximately 7 % carbohydrate. Avidin is a glycoprotein and will react with other biological molecules such as lectins via the carbohydrate moiety.

This affinity for biotin has led to the development of biotin-labelled probes which are hybridised to RNA or DNA immobilised to nitrocellulose filters. After removal of the residual probe, the sites of hybridisation are detected by incubation with a pre-formed complex with avidin-DH (or streptavidin) and biotinylated polymers of intestinal alkaline phosphatase. In the presence of substrates, purplish precipitates result where hybridisation has taken place

(Leary, Brigati and Ward, 1983). This protocol was tested for the detection of PVX RNA immobilised on nitrocelluose filters.

CHAPTER 2

MATERIALS AND METHODS

2.1. Growth Conditions and Inoculation of Nicotiana tabacum with Potato Virus X.

a) Germination.

Nicotiana tabacum (cultivar White Burley) seeds were surface sterilised in 1% Chloros solution for 16 h, dried, then scattered on to damp potting compost (John Innes, No. 5) previously layered with sterilised, fine sand. At a temperature of 22-23°C and 16 h constant light, seedlings usually appeared one week after sowing. These were pricked out and grown individually in 18 cm diameter pots.

All seedlings were grown under constant conditions in the greenhouse, at approximately 22-23°C, with alternating 16 h light and 8 h dark under Philips 400 watt mercury lamps at a height of 90-95 cm above the plants. During summer, each plant was watered every 1-2 days, and every 3-4 days during autumn and winter. All were fed with 50 ml Stort and Arnon's fertilizer solution weekly (from seedling stage to final post-inoculation harvest).

When the plants had grown to 7 cm in diameter (usually with 5-6 leaves) they were infected with the viral inoculum.

b) Virus Strains.

PVX strain N (Ireland, 1980; Matthews; 1946) was initially obtained from Dr. S.Pierpoint, Rothamstead. PVX strains (PVX-3) and PVB (strain group 2) were obtained from R.Boulton, Plant Breeding Institute. Inocula were originally supplied as freeze-dried, infected leaf material. Plants were subsequently inoculated with freshly infected leaves or with freeze-dried material. This was achieved by inoculating uninfected Nicotiana tabacum plants with crushed freeze-dried samples

of PVX-infected leaves and then using the freshly-infected leaves for further inoculation. Except where indicated, all experiments were carried out using PVX strain N.

In order to inoculate uninfected plants, both mortar and pestle, the diatomaceous earth (Celite) and distilled water used in the inoculation procedure were sterilised by autoclaving as a precaution against contamination by other viruses.

Two or three well-infected leaves were ground up in 3 ml distilled water. A double-layer 8 cm square of muslin was used as a pad to soak up the resultant plant sap. Prior to this , 25 mg diatomaceous earth (Celite) had been sprinkled on each of two growing leaves of each plant to be inoculated. The muslin, with the infected plant sap, was carefully wiped over the leaves with the Celite (which acts as an abrasive, helping the virus gain entry into the epidermal cells).

The post-inoculated plants were then grown under the above environmental conditions for at least six weeks before harvesting.

c) Purification of PVX From Infected Leaf Material.

The extraction protocol of viral particles from infected plant material was from Goodman, 1977.

Infected leaves were collected from plants six weeks after inoculation. In most cases, the final weight of the collected leaves ranged from 50-100 g. The leaves were frozen at -70°C, broken up into small pieces, and placed into a MSE ATO Mix Homogeniser with viral buffer (50 mM sodium orthophosphate, pH 7.6; 10mM EDTA) in the ratio of 1 g leaf to 2 ml buffer.

The leaf material was homogenised for 30 seconds and the homogenate then strained through four layers of muslin. Viral particle degradation during the extraction procedure is reported to be reduced at lower temperatures (Goodman 1977) so all buffers and the homogeniser were pre-chilled to 4°C, while the homogenate was kept on ice throughout the extraction. After straining, the remaining debris in the homogenate was precipitated by a low speed centrifugation step at 15,000 rpm at 4°C for 20 min in the MSE 8x50 rotor of an MSE centrifuge.

The supernatant, containing the viral particles, was layered over 7 ml 5mM EDTA, pH 7.6, containing 30% w/v sucrose in a 30 ml "Oakridge" tube, and centrifuged at 54,000 rpm for 45 min at 4°C (Sorvall OTD 65B Ultracentrifuge, T865 rotor). The resultant brown, opaque pellet was rinsed in deionised water to remove residual sucrose, then resuspended in 5 ml 5 mM EDTA. Viral aggregates in the suspension were broken up in a pre-chilled glass homogeniser, and the cycle of differential centrifugation then repeated.

The final purified virus pellet was resuspended in 0.25 ml 5 mM

sodium EDTA and stored at either -20°C or -70°C.

The viral particle concentration was determined by measuring absorbance at 260 nm. The extinction coefficient = 2.97, the literature absorbance for 1 mg/ml PVX virus (ISCO Tables: 7th Edition, Gunter Hofman, Ed.). A table of virus yields is given in Table 2. The yields are calculated as follows:

Absorbance of sample at A_{260} = X

X x dilution factor (d.f.) - extinction coefficient (2.97)

= Y mg viral particles/

Z g leaves.

The PVX RNA was then purified from the extracted viral particles. By assuming that PVX RNA constituted about 6 % of the particles, the expected yields of RNA could then be calculated (Bercks, 1970).

2.2. Purification of PVX RNA from Virus Particles.

The method for purifying PVX RNA was taken from Ricciardi, Goodman and Gottlier (1978).

PVX/PVB virus particles, resuspended in 5 mM EDTA, was diluted 1:4 in phenol extraction buffer (5 mM Tris-Cl, pH 7.6, 0.5 mM EDTA, 0.05 M NaCl, 0.25% (w/v) SDS). An equal volume of warmed (55°C) buffer-saturated phenol (the bottom phase of 10 ml phenol shaken with 10 ml phenol extraction buffer) was added to the virus solution, shaken at 55°C for 10 min, and centrifuged at 7,000 rpm for 10 min at room temperature in the 8 x 50 rotor of the MSE 18 centrifuge.

The top phase was removed with a Pasteur pipette into a sterile polypropylene tube and mixed with 0.1 vol 4 M potassium acetate, pH 5.5 and 1.1 vol cold isopropanol. The solution was left to chill at -20°C for 1-2 h, then centrifuged at 15,500 rpm for 30 min at 4°C to precipitate the RNA.

The PVX or PVB RNA pellet was resuspended in 0.4 ml deionised distilled water, reprecipitated with 4 M potassium acetate and isopropanol, centrifuged at 11,700 g for 10 min at room temperature in a MSE Microcentaur centrifuge.

The precipitated RNA was washed with 0.5 ml 100% cold ethanol and centrifuged at 11,700 g for 10 min at room temperature (MSE Microcentaur). The supernatant was removed and the pellet dried under a gentle stream of nitrogen. The dried RNA pellet was resuspended in 50-100 µl deionised distilled water and its absorbance checked at 260 and 280 nm. The purified RNA was stored at -20°C.

The calculations for the concentration of purified RNA were as follows:

A_{260}/A_{280} nm ratio x 2 = true A_{260}/A_{280} ratio.

RNA has an O.D. of 1 at 40 µg/ml.

Therefore, the concentration of RNA in the sample

= true A_{260}/A_{280} ratio x 40 x dilution factor

= X µg RNA/ml.

2.3. General Procedures:

The following procedures were widely used in several experimental protocols and are, therefore, included together below in a separate section.

1) Extraction of DNA with Phenol/Chloroform.

Proteins, which were present in solutions containing DNA during purification procedures, were removed by extraction with a 1:1 mixture of phenol (containing 0.1% quinolinol) and chloroform, usually in a siliconised Eppendorf vial, vortex mixed, then centrifuged at 11,700 g for 10 min at room temperature in an MSE microcentaur centrifuge.

Two phases form after centrifugation, the organic and aqueous phases. The upper aqueous phase was then pipetted into a clean Eppendorf vial and the phenol/chloroform phase was re-extracted with an equal volume of 0.1 x STE buffer (18 mM NaCl, 0.1 mM EDTA, 2 mM Tris-Cl, pH 7.5), and centrifuged at 11,700 g for a further 10 min. The two aqueous phases were combined and the DNA was precipitated with ethanol and potassium acetate (see below, section 2.3.2). The procedure was also used to inactivate and remove enzymes such as proteinase K after digestion of cell lysates.

The use of both phenol and chloroform to remove proteins was a standard procedure since deproteinisation is more efficient with two organic solvents than with only one. In addition, chloroform also removes any traces of the phenol from the nucleic acid preparation.

2) Concentration of Nucleic Acids.

A widely used method for concentrating DNA is by precipitation with either ethanol or isopropanol. DNA precipitates at low temperatures (-20°C) in the presence of monovalent cations such as potassium acetate. To the DNA solution, 0.1 volume 4 M potassium acetate was

added together with 1.1 volumes of chilled isopropanol or 2 volumes of chilled ethanol. The solution was vortexed, then chilled at -20°C for 30-60 min, centrifuged, washed with 70-100% ethanol, dried under a gentle stream of nitrogen, and resuspended in an appropriate buffer such as 0.1 x STE buffer (18 mM NaCl, 0.1 mM EDTA, 2 mM Tris-Cl, pH 7.5).

3) Cell Amplification of Escherichia coli stocks containing pUC 9.

Escherichia coli cells, strain JM 83 containing pUC 9 (Vierra and Messing, 1982) were cultured in L-broth (10 g tryptone, 5 g NaCl, 5 g yeast extract, per litre, adjusted to pH 8.0, and containing 50 µg/ml ampicillin). Cells (stored in 50% glycerol at -20°C) were amplified in 2 ml L-broth as above, then transferred to 50 ml L-broth containing ampicillin and shaken at 37°C, 220 rpm, for 2-3 h. Fifteen ml was then transferred to each of 2 x 1 litre flasks of L-broth and cell growth allowed to continue as above.

When cells had reached an $O.D_{600}$ of about 0.8 (late log phase), 150 mg chloramphenicol was added to each flask and the cultures were shaken at 37°C, 220 rpm, for 24 h. Chloramphenicol, while inhibiting chromosomal DNA, allows the replication of plasmid DNA to continue. Cells were harvested at 15,500 rpm for 5 min at 4°C.

4) Gel Electrophoresis of Nucleic Acids.

In this process, molecules separate in aqueous buffers supported within a polymerised gel matrix. For nucleic acid molecules, agarose (a polygalactose polymer) gels were most often used (Edgall, Hutchinson and Sinsheimer, 1969).

i) DNA Alkaline Gel:

Single-stranded DNA was analysed on alkaline agarose gels (15 x 20 cm) according to Maniatis (1982). Each gel was prepared by adding 1.4 g agarose to 10 ml 10 X Alkaline gel buffer (0.3 M NaCl, 10 mM EDTA) and 90 ml distilled water, then boiled for 15 minutes and poured into the cassette. When set, the gel was pre-electrophoresed in 1 x Alkaline electrode buffer (30 mM NaOH, 1 mM EDTA) at 25 mA for 1.5 h.

Sample Preparation:

Radiolabelled DNA samples and standards were diluted 1:0.4 with bromophenol blue sample buffer prior to loading.

All samples were electrophoresed at 25 mA (running buffer: 0.3 M NaOH, 10 mM EDTA) for 2.0-2.5 h until the marker dye had migrated 6.5-7.0 cm from the wells. After electrophoresis, the non-radiolabelled standards were cut from the gel and stained in ethidium bromide under usual conditions and examined under ultraviolet light for the DNA bands. The remainder of the gel was dried under vacuum at 80°C and set up for autoradiography at -70°C using X-Omat RP film in the presence of Dupont Cronex Lightning-Plus screens. The labelled DNA samples could then be directly compared with those of the standards.

ii) DNA Tris-borate/EDTA Gel:

A 1 % agarose TBE gel was prepared as above in a 15 x 20 cm gel cassette using 1 x TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA). No pre-electrophoresis was required for this gel.

Sample Preparation:

Each sample of DNA (1 µg) to be electrophoresed was made up to 20 µl with deionised distilled water and added to 5 µl bromophenol blue marker dye (450 mM Tris-borate, pH 8.3, 10 mM EDTA, 50 % glycerol, 0.1 % bromophenol blue). The samples then loaded into the wells in the gel and electrophoresed at 35 mA for 2-3 hours (until the marker dye had migrated 11-12 cm), stained in ethidium bromide solution (2 µg/ml) for 20-30 min and de-stained in 2 changes of distilled water for 45-60 min, and examined under UV light.

iii) RNA Gel Electrophoresis

The two systems most frequently used to measure the molecular weight of RNA and to separate RNAs of different sizes for "Northern" transfer or in vitro translation are:

i) Electrophoresis through agarose gels after denaturation of the RNA with glyoxal and dimethyl sulphoxide (Maniatis, 1982) .

ii) Electrophoresis through agarose gels containing methylmercuric hydroxide or formaldehyde (Maniatis, 1982).

In each case, the RNA is fully denatured and its rate of migration through the gel is in linear proportion to the log of its molecular weight.

RNA Formaldehyde Gel:

a) Preparation of Gel:

Electrophoresis was carried out by a modification of the technique of Rave et al. (1979) and Gustafson et al., (1982). 1.2 g agarose was dissolved by boiling for 15 min in 10 ml 10 x formaldehyde gel buffer

(0.2 M HEPES, 10 mM EDTA, pH 7.8) and 73 ml deionised distilled water. The melted agarose was cooled to 60°C and added to 16.7 ml 37% formaldehyde, mixed and poured to set in the gel cassette (15 x 20 cm).

b) Sample Preparation and Electrophoresis:

The sample RNA solution (containing 1-5 µg RNA) was diluted to 4 µl with deionised distilled water, and added to 23 µl Buffer X (150 µl deionised formamide, 30 µl 10 x formaldehyde gel buffer, 60 µl 37% formaldehyde). The sample was heated to 60°C for 10 min, then cooled on ice.

A 3 µl aliquot dye mix of bromophenol blue/glycerol (0.02% bromophenol blue with 50% glycerol and 1 x formaldehyde gel buffer), was mixed with the sample and loaded on to the gel. The gel was electrophoresed at 50-60 V, then stained. The electrode buffer was 1 x formaldehyde gel buffer, prepared by diluting 10 x formaldehyde buffer 1:9 with water.

c) Staining DNA/RNA in Agarose Gels.

DNA in agarose gels can be visualised in one of two ways: either with a 0.2% toluidine blue solution for 30 min (0.2% toluidine blue, 0.4 M sodium acetate, 0.4 M glacial acetic acid) and destained in several washes of water, or in an ethidium bromide solution (5 µg ethidium bromide per ml buffer (40 mM Tris-acetate, pH 8.2, 1 mM EDTA)) and examined under ultraviolet light for RNA/DNA.

Toluidine blue binds on to the phosphate groups of the nucleic acids while the glacial acetic acid and sodium acetate fixes the RNA/DNA in the gel, therefore preventing diffusion of the nucleic

acids. The gel is placed into 100 ml 0.2 % toluidine blue solution (0.2 % toluidine blue, 0.4 M sodium acetate, 0.4 M glacial acetic acid) for 30 min amd destained in 3 x 100 ml distilled water.

The method of staining gels with ethidium bromide was described by Sharp et al. (1973). Ethidium bromide is a fluorescent dye, containing a planar group that intercalates between the stacked bases of DNA. The bound DNA then causes an increased fluorescence when compared to the dye in the free solution. UV-irradiation absorbed by the DNA at 260 nm and transmitted to the dye, or irradiation absorbed at 300 nm and 360 nm by the bound dye itself, is emitted at 590 nm in the red-orange region of the visible spectrum. The gel is placed into the ethidium bromide solution made from either 300 ml 1 x TBE buffer (0.9 M Tris-borate, 0.9 M boric acid, 0.018 M EDTA, pH 8.3) or water with 10 mg ethidium bromide.

iii) Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) in urea gels has been used to separate polynucleotides differing in length by one base. This technique has been used in conjunction with a variety of DNA/RNA sequencing techniques (Sanger and Coulson, 1978). The nucleic acids are resolved on polyacrylamide gels which are formed as a result of polymerisation of acrylamide and N,N'-bis-acrylamide in the presence of a free radical system, which can be initiated by adding a free radical initiator such as ammonium persulphate to the catalyst, N,N,N',N'-tetramethylethylenediamine (TEMED). There are several modifications which use varying amounts of acrylamide, ammonium persulphate, and TEMED, with different gelling times (Kingsbury and Masters, 1970) but the acrylamide gels which were used followed the

method of Donis-Keller (1977) containing: 20% acrylamide, 0.67% bis-acrylamide and 7M urea, then adding 0.1 volume 10 x TBE buffer. The acrylamide was deionised with amberlite (stirring the solution with 1 g amberlite per 50 ml acrylamide solution for 3-4 h). All acrylamide stock solutions were stored at 4°C.

a) Preparation of Glass Plates for Acrylamide Gel Electrophoresis.

Acrylamide gels were poured and electrophoresised in pairs of glass plates (43 cm x 30 cm). Both plates were thoroughly cleaned with Decon 90, rinsed, wiped dry, rinsed with ethanol and left to dry. The smaller plate was siliconised with a pad soaked in dimethyldichlorosilane (2% in 1,1,1 trichloroethane, BDH Ltd.).

The larger, outer plate was laid flat on the bench with two spacers (0.5 mm thick) in place along the sides. The smaller, inner plate was laid in position, resting on the spacer bars. The entire length of the two sides and the bottom of the plates were bound with 3 M polyimide tape (3M Co.), making a watertight seal (as a slight modification, warmed plasticine was sometimes used for sealing the bottom gap instead of tape). The plates were kept in position with 4-6 large bulldog clips.

Just prior to use, 0.66 ml 10% ammonium persulphate solution was added to each 100 ml acrylamide solution. From this, 5 ml was removed and mixed with with 10 µl TEMED and injected between the sealed glass plates as a plug. Once the plug had set, 200 µl TEMED was added to the remaining 95 ml acrylamide solution, mixed and slowly injected into the glass plates. The appropriate comb (0.5 mm) was inserted at the top of the gel and the acrylamide gel then allowed to polymerise at room temperature for 1-1.5 hours. When polymerisation was complete,

the comb was removed and the wells then rinsed out with 1 x electrophoresis buffer (90 mM Tris borate, pH 8.3, 10 mM EDTA). The electrical tape was removed from the bottom of the gel and the gel was then attached to the electrophoresis tank, using the bulldog clips.

The reservoirs were filled with 1 x electrophoresis buffer. The wells were then flushed out again with 1 x electrophoresis buffer and loaded with RNA samples (usually in a marker dye) using a 10 µl glass micropipette (in some cases, the acrylamide gel had to be pre-electrophoresed, in which case, the gel was electrophoresed without the RNA samples at the appropriate voltage for 1-2 h, or until the marker dye (usually bromophenol blue with xylene cyanol) had migrated halfway down the gel). Electrodes were connected to the power pack and the gel then run at 1000-1800 volts until the bromophenol blue dye had migrated to halfway down the gel.

At the end of the run, the plates were detached from the tank and the tape removed from the side. The gel and the two glass plates were placed flat on to the bench, smaller glass plate uppermost. A corner of the upper, siliconised plate was lifted from the gel, leaving the gel attached to the lower plate. The remaining spacers were removed and the gel then completely covered with Clingfilm.

The gel was set up for autoradiography using X-Omat RP film with Cronex Lightning-Plus screens, at -70°C.

5) Restriction Enzymes: Setting Up Digestions.

The reactions usually contained 0.5-2.0 µg DNA in a total volume of 20 µl.

i) Solutions which contained the DNA were diluted with deionised distilled water to 18 µl and added to 2.0 µl of the appropriate 10 x

digestion buffer. Each enzyme came with its own recommended digestion buffer and these were prepared accordingly. For example, EcoRI (Escherichia coli RY 13) from Pharmacia P-L Biochemicals was supplied in 50% glycerol containing 5 mM potassium phosphate (pH 7.5), 300 mM NaCl, 0.1 mM EDTA, 2 mM 2-mercatoethanol, 0.15% Triton X-100, and 100 µg bovine serum albumin per ml. The 10 x EcoRI digestion buffer contained 100 mM Tris-Cl, pH 7.5, 1 M NaCl, 100 mM $MgCl_2$, 10 mM 2-mercaptoethanol.

ii) The solution was briefly vortexed and between 1-5 units of the restriction enzyme then added. The digestion mix was incubated for 1.5-2.0 h at the appropriate temperature (one unit of the enzyme is usually defined as the amount required to digest 1 µg of DNA to completion in 1 h in the recommended buffer and at the recommended temperature in a 20 µl reaction).

iii) Each reaction was terminated by the addition of 2 µl EDTA.

iv) The restricted DNA was then usually analysed by gel electrophoresis on a 1% agarose TBE gel, stained with ethidium bromide and examined under ultraviolet light.

a) Siliconising Agents for Eppendorf Vials, Polypropylene Tubes, and Glass Plates for Electrophoresis.

As a precaution against the loss of DNA by absorption on to the surfaces of the containers such as Eppendorf vials and polypropylene tubes, the vials and tubes were coated with a thin coat of silicone, using of the two siliconising agents, either dimethyldichlorosilane or "Repelcote" (2% dichlorosilane in carbon tetrachloride).

The method used for the smaller items followed that of Maniatis (1982). The Eppendorf vials were placed inside a large glass

desiccator. One ml of dimethyldichlorosilane was placed inside a smaller beaker inside the desiccator. The jar was attached through a trap to a vacuum, and the vacuum turned on. After 5 minutes, the vacuum was turned off and air allowed to quickly enter the desiccator. This allowed uniform dispersion of the gaseous dimethyldichlorosilane. The vacuum was turned on once again until a vacuum was achieved inside the desiccator. The system was then closed and left for 2 hours under vacuum. After siliconising the vials or tubes, each was rinsed with water, then autoclaved before use.

With gel plates, a pad of tissue soaked in dimethyldichlorosilane was used to wipe the inner surface of the smaller glass plate which was left to dry, then rinsed in water and dried.

6) Preparation of Agarose/Ampicillin Plates.

To one litre of L-broth (5 g NaCl, 5 g yeast extract, 10 g tryptone, pH 8), 1% agar was added, mixed, and the solution autoclaved for 15 min at 15 psi. The agar L-broth was cooled to 60°C and ampicillin added to a concentration of 100 mg/litre, mixed and approximately 10 ml poured into the base of an 82 mm Petri dish to set.

When agarose/ampicillin plates were needed for screening bacterial colonies containing inserts, the chromogenic substrate 5-bromo-4-chloro-3-indolyl- -D-galactoside (Xgal), was added at a final concentration of 50 µg/ml prior to pouring, mixed, and poured to set as above.

7) Proteinase K Treatment of DNA Samples.

In the smaller DNA preparations (less than 1 ml), 100 µg proteinase

K was added to the solution and incubated for 30 min at 37°C. The solution was then extracted with 1:1 phenol/chloroform mix as described above.

For larger volumes, proteinase K was added to a concentration of 100 µg/ml and incubated at 20°C for 20 min, then centrifuged for 5 min at 5,000 rpm at 20°C (MSE 18). The denatured proteins and other waste materials were pelleted and the supernatant was then extracted with phenol/chloroform as described above.

8) Preparation of a Sephadex G100 Column.

To a dry, clean 9 ml column, about 3 ml siliconising agent (2% dimethyldichlorosilane in 1,1,1 trichloroethane) was washed through to coat the glass with a thin layer of silicone. Pre-swollen Sephadex G100 (swollen in 0.1 x STE (18 mM NaCl, 0.2 mM EDTA, 2 mM Tris-Cl, pH 7.5) buffer, then autoclaved for 15 min at 15 psi) was cooled to room temperature before pouring into the column to settle (this prevented bubble formation in the column). A small amount of herring sperm DNA (250 µg as a 5 mg/ml solution) was loaded on to the column and washed through with 0.1 x STE buffer . After each use, the column was cleaned in one of two ways: i) about 10 ml concentrated chromic acid was run through the column and the base disc was allowed to sit in this for 30 min to prevent any blockages. The column was cleaned thoroughly in distilled water and left in 0.25 M EDTA for 30 min before re-equilibriating in 0.1 x STE buffer, ii) the column was washed in 1 M NaOH, rinsed with water, and re-equilibriated in 0.1 x STE buffer.

2.4. Detection System.

The DNA detection system can be considered as a non-radioactive alternative to autoradiography for the detection of nucleic acid sequences by utilising the high affinity of biotin-labelled nucleotides for streptavidin.

In general, the steps for labelling DNA with biotinylated nucleotide and its subsequent detection, are as follows:

1) incorporation of the biotinylated nucleotide into DNA, e.g. standard nick translation or cDNA synthesis protocols;

2) hybridisation of the labelled DNA probe to nucleic acid target sequences immobilised on an inert support; and

3) detection of the probe/target hybrid with steptavidin and a biotin-conjugated alkaline phosphatase (Langer, Waldrop and Ward, 1981; Leary, Brigati and Ward, 1983; Wahl, Stern and Stark, 1979). The procedure is shown in Fig.5 overleaf.

2.4.1. Preparation of Biotinylated Probes.

a) The Biotinylated Probe Reaction Mix:

Biotinylated and ^{32}P-labelled cDNA hybridisation probes, used throughout these studies, were synthesised using PVX N RNA as template.

The synthesis of cDNA for use as probe was randomly primed using calf thymus oligonucleotide primer (prepared as described by Maniatis, 1982) with biotin-labelled dUTP (Biotin-11-dUTP). The mixture contained 5 mM Tris-HCl, 137 mM KCl, 10 mM $MgCl_2$, 170 µg calf thymus primer, 21 mM 2-mercaptoethanol, 20 µM biotin-11-dUTP (Bethesda Research Laboratories), 20 µM dCTP, 0.5 mM dATP, 0.5 mM dGTP, 5 units reverse transcriptase (Anglian Biotech. "Super Reverse Transcriptase), 2 µg PVX N RNA, 10 µCi [^{32}P]-dCTP (Amersham Corporation, 3000 Ci/mmol), and deionised distilled water to a final volume of 40 µl. The reaction

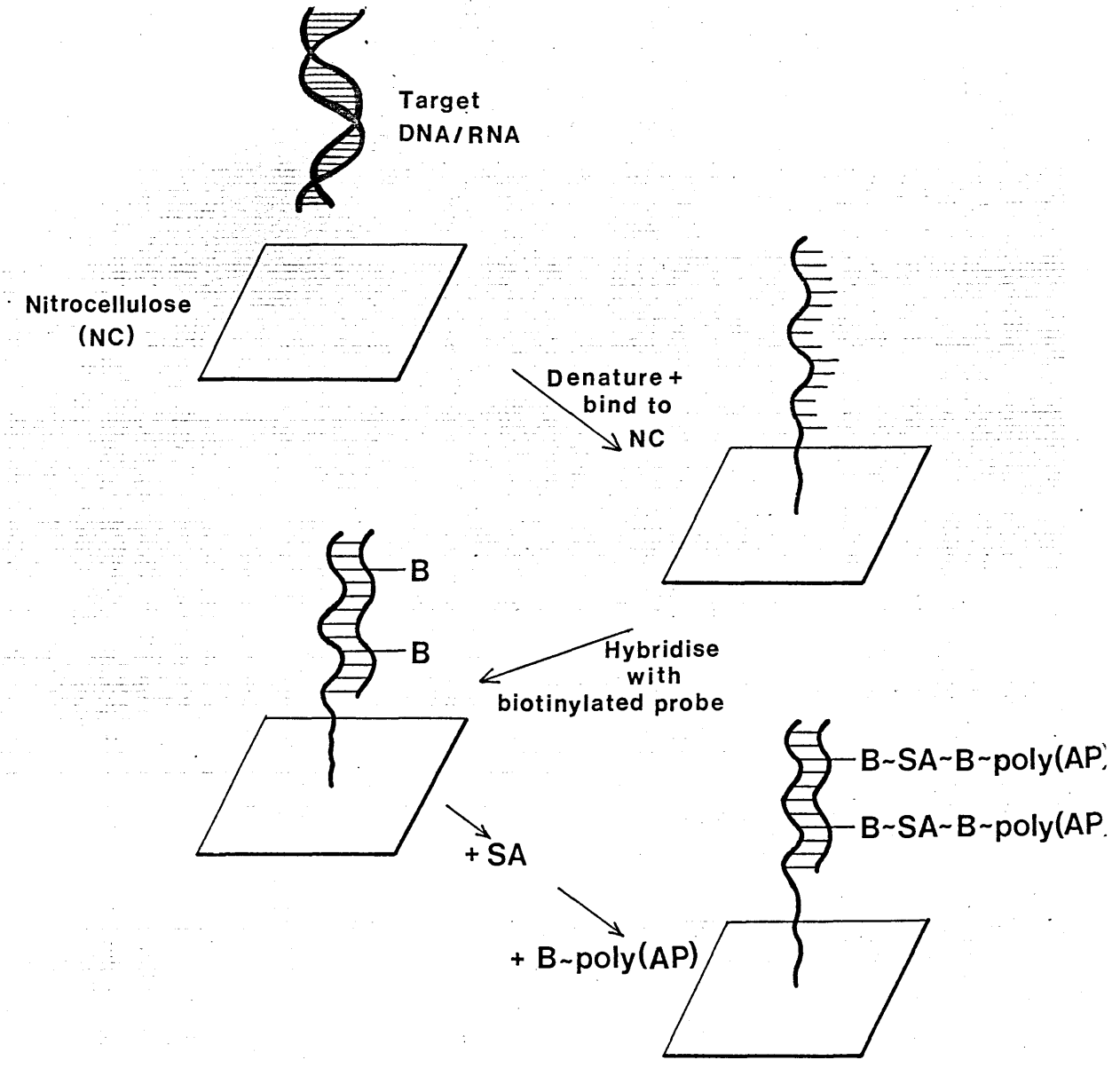


Fig.5: Diagrammatic Representation of the Detection of Target DNA with a Biotinylated Probe.

The biotinylated probe (B) is hybridised to the target DNA which has been immobilised on a nitrocellulose filter (NC). The nitrocellulose is then incubated with streptavidin (SA) and a biotin-conjugated enzyme polymer (B-poly(AP)), which binds to the biotinylated probe/target hybrid. When chromogenic substrates are added, the target DNA with its hybridised complex is visualised as a purplish spot (Langer,Waldrop and Ward, 1981; Leary, Brigati and Ward, 1983).

was incubated at 37°C for 1.5 h and terminated with 4 µl 0.25 M EDTA. From the reaction mix, a 2 µl aliquot was saved for passage through Sephadex G100, and the rest then alcohol precipitated as previously described and resuspended in 20 µl 10 mM Tris-Cl, pH 7.4, 1 mM EDTA. The reaction mix and protocol for both the biotinylated and radiolabelled probes were modifications of the protocol by Maniatis (1982). In this protocol, the dTTP in the reaction mix is replaced with 20 µM biotin-11-dUTP. In the absence of any "unlabelled" dTTP, biotinylated dUTP residues completely substitute for dTTP in any cDNA synthesised, resulting in maximal specific activity. Omission of unlabelled dTTP has the added advantage of obviating any possibility of differential incorporation of biotinylated and unlabelled nucleotides into cDNA by the reverse transcriptase. The addition of the ^{32}P-dCTP allows DNA synthesis to be easily monitored since ^{32}P-labelled cDNA is also fully biotinylated. Incorporation of ^{32}P-dCTP (and hence, biotin-11-dUTP) into cDNA varied from 15-77 %, similar to that obtained below for the reaction in the absence of biotin-11-dUTP.

2.4.2. Preparation of Radiolabelled and Nick Translated Probes:

1) Radiolabelled Probe Reaction Mix:

The reaction mix for the ^{32}P-labelled probe was similar to that described above except that the reaction mix contained 50 µCi [^{32}P]-dCTP (3000 Ci/mmol), and 0.5 mM dATP, dGTP, dTTP and 20 µM unlabelled dCTP. As before, the RNA used in all cDNA synthesis reactions came from the PVX N isolate. The reaction was incubated at 37°C for 1.5 h and terminated with 4 µl 0.25 M EDTA. Two microlitres were saved for passage through Sephadex G100. The efficiency of

incorporation of the radiolabelled probe for the reaction varied. In reactions for PVX N, the percentage incorporation for the ^{32}P-labelled probe ranged between 10-53 % (product/template ratios). The specific activity of the cDNA was 1.0×10^8 dpm/µg.

Under usual conditions for [^{32}P] probe, 6 µl 0.5 M NaOH was added to the remaining reaction mix and the cDNA then denatured at 65°C for 1 h. After neutralisation with an equal volume of 0.5 M HCl, the reaction mix was then phenol/chloroform treated, re-extracted with 20 µl 0.1 x STE buffer, and alcohol precipitated overnight. The radiolabelled probe was resuspended in 50 µl 0.1 x STE buffer. The size of the probes was determined by electrophoresis and found to range between 0.6-9 kbp. However, with biotinylated cDNA, this procedure was avoided as biotin molecules are sheared off in alkali. Therefore, the biotinylated cDNA was precipitated with 0.1 volume 4 M potassium acetate and 1.1 volume cold isopropanol.

In all hybridisation experiments, the radiolabelled or biotinylated probes were synthesised from PVX N RNA.

2) The Synthesis of Two PVX Group 3, and a Group 2 cDNA's.

Ten reaction mixes were prepared in total: one each containing PVX RNA Group 3 (original PVX N RNA), PVX RNA Group 3 (PBI), PVX RNA Group 2 (PBI), PVX RNA Group 3 (original PVX N RNA, 6 months storage), plus a duplicate set without oligo d(T) as controls. As an additional control, two vials containing TMV RNA were also prepared as above. The reactions were carried to completion as previously described (Section 2.11).

4) Labelling DNA by Nick Translation.

DNA was labelled by the procedure of Mackey et al (1977). DNAse I was stored at -20°C in 0,1 M HCl at a concentration of 1 mg/ml. The DNAse was thawed and diluted to 0.1 mg/ml in DNAse activation buffer (10 mM Tris base, pH 7.5; 5 mM $MgCl_2$; 100 µg/ml BSA) and allowed to stand for 2 hours on ice. Immediately before use, the DNA polymerase I solution was further diluted in DNAse activation buffer to a final concentration of 50 ng/ml.

Ten µCi of [^{32}P] dCTP (3000 Ci/mmol) was dried down in an Eppendorf vial. To this was added 0.1 mM dCTP, 0.4 mM dATP, dGTP, dTTP, Nick Translation buffer (50 mM potassium phosphate, 50 mM $MgCl_2$, pH 7.5, in a final volume of 3.7 µl. 0.4 µl of activated DNAse (50 ng/ml) was added.

The reaction mix was warmed to 19°C and 0.5 µl DNA (approximately 50 ng) added. The reaction mix was incubated for 60 seconds at room temperature, 0.5 µl DNA polymerase I (5 units) was added to the reaction, cooled on ice and incubated at 15°C for 8-16 h. The reaction was terminated with 50 µl 0.1 x STE buffer and 5 µl 0.25 M EDTA, pH 8.0. DNAse activity was destroyed by heating the solution at 70°C for 10 min. The DNA was precipitated with potassium acetate and isopropanol as previously described. The nick translated probe was denatured with 0.1 volume 1 M NaOH and heated at 65°C for 10 min prior to hybridisation. The denatured probe was cooled on ice and neutralised with 0.1 volume 1 M HCl, before adding to the hybridisation.

The specific activity of the nick translated probe was approximately 1.2×10^8 dpm/µg.

2.5. Enzyme Immunoassay (ELISA) for the in vitro Determination of Potato Virus X.

Assay Procedure:

Antisera, buffers, conjugates, etc. were obtained from Boehringer Mannheim, Biochemica. The antisera was a polyclonal antibody to PVX (strain-Group 3) strains. The assay procedure was a modification of the methods of Voller (1976) and Clark and Adams (1977).

The assay principle is as follows:
in the first incubation step involving the "sandwich assay", the wells of the plates are coated with the anti-potato virus X-antibody (a polyclonal antibody to PVX strains). Following this, the potato virus X of the sample is bound by the fixed antibody. Anti-potato virus X-antibody, labelled with alkaline phosphatase, then binds additionally to the virus. The amount of AP-conjugate bound is directly related to the potato virus content of the sample. Free AP-conjugate is removed by washing the wells. The bound alkaline phosphatase is determined after addition of the substrate as follows:

$$\text{4-Nitrophenyl phosphate} + H_2O \xrightarrow{AP} \text{phosphate} + \text{4-nitrophenol}.$$

The intensity of colour formed in the assay solution after a fixed reaction time is measured against the substrate solution either visually or spectrophotometrically at 405 nm. Each well was prepared as follows :

a) 0.2 ml antibody coating solution (1.8 µg protein/ml) was pipetted into each well of the microplates (Cook microtitre plates). The plates were then covered tightly and incubated for 2 h at 37°C.

b) The wells were emptied by suction or tapping on filter paper and washed at least three times with the wash buffer, ensuring that the

wash buffer was completely removed each time.

c) 0.2 ml of each crude sap or purified viral particle sample (serially diluted with 0.1 x STE buffer) was pipetted into the wells, covered and incubated overnight at 4°C.

d) The solution was removed from the wells by suction or tapping on filter paper, then washing the wells 3-4 times with wash buffer [phosphate-buffered saline (0.02 M phosphate, 0.15 M NaCl, pH 7.4) with 0.05 % Tween 20]. The wash buffer was removed carefully.

e) 0.2 ml antibody-AP-conjugate solution (2.0 µg/ml) was pipetted into each well, covered and incubated for 4 h at 37°C.

f) The antibody-AP-conjugate solution was removed from the wells by suction or by tapping over filter paper, washed 3-4 times with washed buffer and emptied as before.

g) 0.2 ml substrate solution was pipetted into the wells and incubated for 60 min at room temperature.

h) The substrate reaction was stopped with 0.05 ml 3 M sodium hydroxide solution, and then measured against substrate solution at 405 nm, or evaluated visually.

ELISA: 1 hour:

The following reactions were set up for assaying:

1) serially diluted crude sap samples from plants infected with PVX N (Gp 3), PVX Gp 3 (PBI), PVB Gp 2 (PBI). These crude sap samples were serially diluted to 10^{-6}. Healthy, uninfected sap was also included as control.

2) Purified PVX viral particles from PVX N (Gp 3), PVX (Gp 3, PBI), PVX (Gp 2, PBI) were assayed as above. serially diluted from 20

ug to 200 pg.

3) Positive reaction control, serially diluted as above. A positive (strain-Group 3) was supplied by Boehringer Mannheim, Biochemica.

4) Negative reaction control, serially diluted as above.

The above were incubated with the substrate solution for 60 min and colour development then stopped with 50 µl 3 M NaOH.

Positive reactions in the wells were identified visually, then at Hg 405. The serially diluted negative control was taken as the cut-off point at each dilution.

In some experiments, the samples were incubated with substrate for 2 or 5 hours. The samples for the assays were stored at -70°C, then used for comparison in repeat assays.

2.6. Preparation of Filters for Sap Spot Hybridisation.

Collection and Dilution of Crude Sap samples:

Sap samples from both infected and non-infected leaves were collected and stored at -70°C in 1.5 ml Eppendorf vials. Prior to spotting on to nitrocellulose, all sap samples were serially diluted (in 0.1 x STE buffer) to test the efficiency of the various probes.

1) Preparation of Nitrocellulose:

This method was supplied by R.Boulton (personal communication, PBI). The nitrocellulose membrane (Schleicher and Schuell nitrocellulose membrane, 0.45 µm 330 x 3000 mm, BA 85) was cut to a standard size of 7 x 9 cm, and marked into a grid with either a soft pencil or blue ball-point pen. This allowed 49-63 spots per filter (Fig.3). The filter number, date, and sample number were marked at the base of each grid.

Six sheets of Whatman 3 MM Chromatography paper were cut (9 x 11 cm wide). Three sheets were immersed in 20 x SSC buffer (3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0) for approximately 10 min, and the remaining three were stacked on a dry surface.

At the same time, the nitrocellulose sheet was immersed in deionised distilled water to wet (5 min), then into 20 x SSC (5 min).

The three 20 x SSC-soaked sheets were stacked on to the dry sheets and carefully layered on top with the nitrocellulose. Air bubbles were removed by rolling over the sheets with a glass rod.

Immediately, 2 µl spots of each crude sap sample were placed on to the filter, starting from undiluted sap through 10 x serial dilutions to 1 x 10^7 dilution.

In addition, dilutions of purified PVX viral particles and RNA were also spotted on to the nitrocellulose grid in a range of

concentrations from 1 µg/µl to 10 pg/µl.

As a control, the same range of concentrations of BMV RNA were spotted as an additional line to determine the degree of non-specific hybridisation between the PVX cDNA probe and the BMV RNA.

The nitrocellulose filter remained workable, i.e. drawing the sap sample through, for about 1 h. Duplicate filters (up to six filters) were prepared simultaneously, to be used for different probes and under varying conditions.

The completed filter was carefully removed from the pad and air-dried (15-30 min), then placed between two stapled sheets of Whatman 3 MM paper and baked in vacuo for 2-16 h at 80°C. To store, the filter was sealed in a heavy gauge polythene bag (10 x 14 cm), and stored at -20°C.

The filters were then hybridised with either a radiolabelled probe or a biotinylated probe.

1) Hybridisation conditions with the ^{32}P-labelled probe were the same as those for the prehybridisation and hybridisation of the in situ hybridisation of bacterial colonies (Section 2.16.3). Filters were washed in: a) 2 x SSC buffer + 0.1% SDS, pH 7.0, at room temperature for 15 minutes (two changes), and b) in 2 x SSC buffer + 0.1% SDS, pH 7.0, at 65°C for 1 hour (two changes), then autoradiographed at -70°C using X-Omat RP film in the presence of Dupont Cronex Lightning-Plus screens.

2a) The prehybridisation solution for the hybridisation of filters to a biotinylated probe was modified from Wahl et al. (1979). The solution contained 50 % v/v deionised formamide, 5 x SSC (0.75 M NaCl, 75 mM trisodium citrate), 5 x Denhardt's reagent (0.1% bovine serum

albumin, 0.1% PVP, 0.1% Ficoll), 25 mM sodium dihydrogen phosphate, ph 6.5, sheared, denatured herring sperm DNA (250-500 µg used per ml prehybridisation solution). To prepare the sheared, denatured herring sperm DNA, 250 mg DNA from herring testes was dissolved in 50 ml deionised distilled water. The DNA was sheared in a Polytron (setting 1.5) in bursts of 30 seconds. The shearing was repeated 10-12 times until the solution became less viscous. The sheared DNA was denatured by autoclaving.

Each filter was prehybridised in 10 ml prehybridisation solution for 4.0 h at 42°C.

b) Preparation and Denaturation of Biotinylated Probe.

Since biotin-labelled probes exhibit lower levels of non-specific binding to nitrocellulose (a higher signal-to-noise ratio) than radioisotope-labelled probes, higher probe concentrations can be used in the hybridisations. A probe concentration of 100 ng/ml was used in the majority of hybridisations, although lower concentrations were also tested. The biotin probes were heat denatured as described by Wahl etal. (1979). The biotinylated probe was resuspended in 20 µl 10 mM Tris-Cl, pH 7.4, 1 mM EDTA. The required amount of probe was heated in a water bath at 95°C for 5 min and quick-cooled in ice to denature the RNA-DNA hybrid.

c) Hybridisation:

The hybridisation solution for use with biotinylated probe was prepared as a modification of the procedure of Wahl et al. (1979). The solution contained per 100 ml: 10% (w/v) dextran sulphate, 45 ml deionised formamide, 1 x Denhardt's reagents (0.02% bovine serum albumin, 0.02% PVP, 0.02% Ficoll), 5 x SSC (0.75 M NaCl, 75 mM

trisodium citrate), 25 mM sodium dihydrogen phosphate, pH 6.5, sheared denatured herring sperm DNA (300 µg per ml hybridisation solution used).

The prehybridised filter was placed into 9 ml hybridisation solution in a strong, clear polypropylene bag. A further 1 ml was heated to 65°C and mixed with the denatured biotinylated probe before adding to the 9 ml and heat-sealing the bag. The sealed filter was hybridised at 42°C for 16h in a shaking water bath.

The post-hybridisation filter was washed in 1) 250 ml 2 x SSC buffer (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) + 0.1% (w/v) SDS for 3 min at room temperature (two changes), 2) 250 ml 0.2 x SSC buffer + 0.1 % (w/v) SDS for 3 min at room temperature (two changes), 3) 250 ml 0.16 x SSC buffer + 0.1% (w/v) SDS for 15 min at 50°C (two changes). The filter was then briefly rinsed in 2 x SSC buffer + 0.1% (w/v) SDS at room temperature.

d) Detection of Biotinylated DNA on Nitrocellulose Filters.

i) Filter Blocking:

The filter was washed and rehydrated for 1 min in Buffer 1 (0.1 M Tris-Cl, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% (v/v) Triton X-100) then incubated for 20 min at 42°C in pre-warmed buffer 2 at 42°C (3% (w/v) bovine serum albumin in buffer 1). The filter was gently blotted dry between two sheets of filter paper and then dried at 80°C between two sheets of 3 MM paper for 10-20 min *in vacuo*. Dried filters were stored desiccated for future use.

ii) Application of Detection System:

The stored, dried filter was thoroughly re-hydrated in Buffer 2 for 10 min and then drained. In a siliconised Eppendorf vial, 2 µl stock

solution streptavidin (1 mg/ml in 50 mM Tris-Cl, pH 7.5, 0.2 mg/ml sodium azide, BRL) was diluted to 2 µg/ml Buffer 1. The re-hydrated filter was incubated in the diluted streptavidin for 10 min with gentle agitation, occasionally pipetting over the filter, then decanted, and washed with a 40-fold greater volume of Buffer 1 than in the incubation step with the diluted streptavidin. The filter was gently agitated for 3 min in Buffer 1, and the solution then decanted. This step was repeated with two more changes of buffer.

In a second siliconised Eppendorf vial, poly (AP) (biotinylated calf intestinal alkaline phosphatase in 3 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 30 mM triethanolamine,pH 7.6) was diluted to 1 ug/ml Buffer 1. The filter was incubated in the diluted poly (AP) for 10 min, agitated gently, and occasionally pipetting the solution over the filter, before decanting the solution. The filter was washed again in a 40-fold greater volume of Buffer 1 than the incubation step with the diluted poly (AP), i.e. for every ml diluted poly (AP) used, 40 ml Buffer 1 was used to wash the filter. The filter was gently agitated for 3 min in buffer 1, and the solution then decanted. This wash step was repeated once with Buffer 1 and then with Buffer 3 (0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) with two changes of buffer.

iii) Visualisation of Biotinylated DNA:

In a siliconised polypropylene tube, approximately 7.5 ml of dye solution (33 µl nitro-blue tetrazolium (NBT: 75 mg/ml in 70 % dimethylformamide) solution to 7.5 ml Buffer 3 plus 25 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP: 50 mg/ml in dimethylformamide) solution) was freshly prepared (both NBT and BCIP were stored in 70% dimethylformamide). The filter was incubated in the dye solution within a sealed polypropylene bag. Colour development was

allowed to proceed in the dark for 4 hours. After colour development, the filter was washed in 20 mm Tris-Cl (pH 7.5) plus 5 mM EDTA to terminate the colour development reaction. The filter was then dried by baking at 80°C for 1-2 min *in vacuo* and stored in black plastic envelopes.

2.7. Affinity Chromatography to Isolate and the Chracterisation of PVX RNA using Oligo d(T) Cellulose.

Oligo d(T) cellulose chromatography was carried out by a modification of the procedure of Gilham (1964), Aviv and Lader (1972), Milner nd Jackson (1979).

A suspension of 0.15 g oligo d(T)$_4$ cellulose (PL Biochemicals, Type 7) in 5ml 10 mM HEPES, pH 7.5, was poured into a 12 cm X 0.5 cm X 0.5 cm column and washed through with 5 ml 10 mM HEPES (at approximately 20 ml/h using Eyela Microtube pump MP3). The oligo d(T) cellulose was equilibriated with two washes (10 ml each) Buffer A (0.5 M NaCl, 10 mM Tris HCl, pH 7.5, 1 mM EDTA, 0.5% (w/v) SDS) and stored at -20°C.

Passage of RNA Through Oligo d(T) Cellulose.

Removal of any RNases in the oligo d(T) cellulose was effected by passing 10 ml 0.5 M NaOH through the column and re-equilibrating with 10 ml Buffer A for 10 min (pump speed 3, equivalent to approximately 20 ml/h).

The Buffer A was pumped off and 50 µg PVX RNA in 4 ml Buffer A was loaded on to the column. The RNA solution was re-circulated through the oligo d(T) cellulose continuously for 1 h at 20 ml/h. The unbound RNA (given the term 'poly A-') in Buffer A, was pumped off and stored. The column was washed with a further 10 ml buffer A for 5 min.

The bound RNA (given the term 'poly A+') was eluted by adding 1 ml freshly prepared 10 mM HEPES/1 mM EDTA, pH 7.5 pumped through the cellulose, followed by a further 1 ml and then 3 ml. All three fractions were pooled and stored.

The concentration of the poly A+ and the poly A- fractions were assayed spectrophotometrically

To confirm that the RNA in the 'bound' fraction would bind again to oligo d(T) cellulose, the fraction was heated at 65°C for 5 min and

quick-cooled.This step broke up any aggregates which were present in the solution. One volume 2 x Buffer A (1 M NaCl, 20 mM Tris HCl, pH 7.5, 2 mM EDTA, 1 % (w/v) SDS) was added to the RNA which was again fractionated on the oligo d(T) cellulose column as above. The bound (second poly A+) and unbound (second poly A-) RNA were collected and the RNA concentration determined spectrophotometrically.

2.8. Isolation of Poly (A) Regions of PVX RNA and the Determination of their Sizes.

i) Poly (A) was isolated from PVX RNA (strain N) by the procedure of Agranovsky, Dolja, and Atabekov (1983). Purified RNA (100 µg), suspended in 150 µl deionised distilled water, was heated to 100°C for 3 min. The solution was cooled to 4°C and mixed with 7 µl of a solution containing 1 µg/µl RNase A and 1 unit/ul RNase Tl (Sigma). The reaction mixture was adjusted to a final concentration of 0.3 M NaCl, 2 mM EDTA, and 20 mM Tris-acetate, pH 7.6, then incubated for 1.5 h at 37°C, heated again at 100°C for 3 min, and supplemented with 15 µl of the fresh enzyme solution.

Incubation was resumed and continued for 30 min at 37°C. Sodium dodecyl sulphate (SDS) was added to 0.5% and the solution adjusted to pH 9 with 1 M Tris. This slight modification (El Manna and Bruening, 1973) was inserted after the detergent disruption by SDS but before phenol extraction and was reported to increase the RNA yield to over 90%.

The solution was treated with 10 µl proteinase K (5 mg/ml) for 20 min at 20°C, and shaken with an equal volume of a phenol-chloroform mixture (a combination of chloroform and distilled phenol (1:1 v/v), saturated with Tris-acetate). The RNase-resistant fragments were phenol-chloroform treated, then separated by centrifugation at 11,700 g for 10 min (MSE Microcentaur). The resultant aqueous phase was saved while the phenol phase was re-extracted with 300 µl deionised distilled water.

The RNase-resistant fragments were precipitated from the aqueous phase with ammonium acetate and ethanol as previously described (see Section 2.3.2) at 20°C for 16 h, and pelleted at 11,700 g for 10 min (MSE Microcentaur). The pellet was washed once with 100% ethanol and resuspended in 100 µl deionised distilled water.

ii) 5' Terminal Phosphorylation of RNA with [^{32}]P Phosphate.

This was carried out by a modification of the procedure of Donis-Keller, Maxam and Gilbert (1977). To 1 µl ethanol-precipitated, RNase-resistant fragments from PVX RNA above (0.5-1.0 µg RNA), 10 µCi [^{32}P]-ATP (Amersham, 3000 Ci/mmol), 1.5 µl 10 x kinase-labelling buffer (0.5 M imidazole chloride, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA), 8 µl deionised distilled water, and 0.25 µl (1 unit) polynucleotide kinase (P+S Biochemicals) were added. The reaction mix was incubated for 30 min at 37°C, after which, 100 µl 0.1 x STE buffer, 8 µl 5 mg/ml tRNA, 50 µl chloroform and 50 µl phenol (containing 0.1 % w/v quinolinol), were added and centrifuged at 11,700 g for 10 min. The [^{32}P]-labelled, RNase-resistant fragments were phenol-chloroform extracted from the aqueous phase, ethanol precipitated and pelleted as previously described. The labelled fragments were resuspended in 200 µl 0.1 x STE buffer.

iii) Polyacrylamide Gel Electrophoresis: RNA Sequencing Gel 1 .

A 20% polyacrylamide sequencing gel was prepared according to the method of Donis-Keller (1977) containing 20% acrylamide, 0.67% methylene bis-acrylamide, and 7 M urea in 1 x TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA, pH 8.3). The prepared gel was pre-electrophoresed at 800 V for 2 h in a Uniscience sequencing gel apparatus and the sample then loaded and electrophoresed at 1000 V for 2-3 h. After electrophoresis, the gel was set up for autoradiography at -70°C using X-Omat RP film with Dupont Cronex Lightning-Plus screens for 24 h.

2.9. Site Specific Enzymatic Cleavage of PVX RNA.

i) RNase H Digestion of PVX N RNA

To 30 µg PVX N RNA, dissolved in deionised distilled water at a concentration of 1 mg/ml, 12 µl oligo $d(T)_{12-18}$(0.14 mg/ml)(PL Biochemicals) was added. An equal volume of digestion buffer (20 mM Tris, pH 7.9, 0.3 M NaCl, 0.4 mM DTT) was added to the incubation mixture, then gradually cooled to 4°C and incubated for 5-10 min. The mixture was supplemented with 0.5 µl RNase H solution (2 unit/µl)(BRL) and incubated for a further 3.5 h at 4°C. The reaction was stopped by the addition of SDS and EDTA to 0.1 and 0.02% respectively.

ii) Labelling Reaction using DNA Molecules with Protruding 5' Phosphoryl Termini:

Since RNase H attacks only RNA-DNA hybrids, breaking the RNA endonucleolytically to produce a 3' hydroxyl and a 5' phosphate at the point of cleavage, the 5' phosphate termini can be labelled in an exchange reaction with [^{32}P]-ATP (Maniatis, 1982; Maxam and Gilbert, 1977).

The reaction mix was prepared as follows: 73 pmoles RNA (= 0.5 ug) with 5' terminal phosphates, 2 µl 10 x Exchange buffer (0.5 M imidazole chloride, pH 6.6, 0.1 M MgCl , 50 mM DTT, 1 mM spermidine, 1mM EDTA), 0.3 mM ADP, 6.6 pmol [^{32}P]-ATP (3000 Ci/mmole), 1.7 µM ATP, 2 units T4 polynucleotide kinase, and water to 20 µl.

The reaction mix was incubated at 37°C for 30 min, then stopped with 1.6 µl 0.25 M EDTA and extracted once with phenol/chloroform. The phenol phase was re-extracted with 11.8 µl 0.1 x STE buffer, and the aqueous phases then pooled. The RNA was precipitated and pelleted using the standard procedure, and resuspended in 20 µl deionised distilled water.

v) Sample Preparation:

The RNase H-treated RNA was resuspended in 20 µl deionised distilled water. A 1 µl sample was counted to determine the incorporation of ^{32}P.

To 3 µl 1 x TBE buffer, 1 µl of the resuspended RNA sample (containing about 5,000 cpm - sufficient to indicate labelled bands during autoradiography) was added. From this, 1 µl was added to 3.5 µl xylene cyanol/bromophenol blue loading buffer (20 mM sodium dihydrogen citrate, pH 5.0, 1 mM EDTA, 7 M urea, 0.02% xylene cyanol, 0.02% bromophenol blue) heated for 5 min at 50°C and quick-cooled.

vii) Polyacrylamide Gel Electrophoresis: Sequencing Gel 2:

A polyacrylamide sequencing gel gel was prepared according to the method of Donis-Keller, 1977) containing 5% acrylamide, 0.67% methylene bis-acrylamide, and 7 M urea in 1 x TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA, pH 8.0). The gel apparatus reservoirs were filled with 1 x TBE buffer, and the samples electrophoresed at 60-70 mA until the bromophenol blue dye had migrated halfway down the gel. The gel was then set up for autoradiography at -70°C using Kodak X-Omat S film in the presence of Dupont Cronex Lightning-Plus screens for 16-24 h.

2.10. Determination of the 3' Sequence of PVX RNA.

i) Labelling the 3' terminus of PVX RNA:

A reaction mix consisting of 3 µl 10 x ligase buffer (0.5 M HEPES, pH 7.5, 180 mM $MgCl_2$, 33 mM DTT), 21 µg PVX RNA, 6.6 µM ATP, 3 µl dimethylsulphoxide, 50 pmol [5-^{32}P]pCp (3000 Ci/mol)(Amersham) and water to 27 µl, was prepared. The reaction was initiated by the addition of 5 units T4 RNA ligase (RNase-free)(Pharmacia) and incubated at 4°C for 16 h. The reaction was terminated with 30 µl 4 M ammonium acetate and the labelled RNA then pelleted by adding 180 µl cold ethanol with 5 µg tRNA carrier, chilled on dry ice, then centrifuged for 10 min at 11,700 g.

The RNA was resuspended in 60 µl 0.5 M sodium acetate, pH 5.5, and re-precipitated in 150 µl cold ethanol and 5 µg tRNA after chilling at -20°C for 10 min.

The supernatant was pipetted off and the pellet then washed in 200 µl cold ethanol, chilled and centrifuged for 2 min (MSE Microcentaur). The supernatant was discarded and the pellet briefly dried under vacuum.

ii) Purification of 3'-labelled RNA by Polyacrylamide Gel Electrophoresis:

The [^{32}P]-labelled PVX RNA was resuspended in 10 µl 7 M urea, 1 mM EDTA, 0.05% each xylene cyanol and bromophenol blue, heated for 30 seconds at 90°C, and quick-chilled on ice. The sample was loaded on a 1.5 mm thick (15 x 20 cm), 4% acrylamide, 0.13 % bis-acrylamide gel cast in 50 mM Tris-borate, pH 8.3,1 mM EDTA. Electrophoresis was at 125 V until the xylene cyanol had migrated 20 cm (about 4-5 h, and which was equivalent to the comigration of about 460 nucleotide pairs (Maniatis, 1982)). The upper siliconised glass plate was removed and

the gel covered with plastic wrap, and exposed to X-ray film for 20 minutes. By using the developed autoradiogram as a guide, the position of the [^{32}P]-RNA could be determined. The PVX RNA, which migrated as a sharp band about 0.5 cm from the origin was excised with a sharp blade and placed into a siliconised blue (1 ml) Eppendorf tip which had been heat-sealed and plugged with siliconised glass wool. The gel was crushed with a siliconised glass rod and mixed with 0.5 ml elution buffer (0.5 M ammonium acetate,1 mM EDTA), then covered with parafilm and incubated at 37°C for 16 h.

Recovery of the solution was effected by cutting the pipette tip and allowing the solution to drain into a siliconised vial. The tip was rinsed with an additional 0.1 ml elution buffer. The RNA was precipitated with 2-3 volumes cold ethanol and 16 ug tRNA carrier as above. Once dried, the [^{32}P]-RNA was resuspended in sterile deionised distilled water and stored at -20°C.

iii) Sequence Analysis of Terminally-Labelled PVX RNA.

The 3' sequence of PVX RNA was determined by modifications of limited enzyme digestions according to Donis-Keller et al. (1977); Gupta and Randerath (1977); Lockard et al. (1978); Simoncsits (1977); and Levy and Karpetsky (1980). Enzymes were obtained from BRL.

i) Preparation of the Enzymes:

The following ribonucleases were diluted with their appropriate buffers as follows:

a) RNase T1 and Phy M to 0.5, 1.0 and 2.0 unit/µl with 25 mM citrate, pH 5, 0.7 M urea, 1 mM EDTA, 0.05% each xylene cyanol (XC) and bromophenol blue (BPB).

b) RNase U_2 to 0.5, 1.0 and 2.0 unit/µl with 25 mM citrate, pH 3.5, 7 M urea, 1 mM Na EDTA, 0.05% each XC and BPB.

c) RNase CL_3 to 0.5, 1.0 and 2.0 unit/µl with 10 mM sodium phosphate, pH 6.5, 0.01 mM EDTA.

d) RNase B.cereus to 0.5, 1.0 and 2.0 unit/µl with 25 mM sodium citrate, pH 5.0.

After dilution, the enzymes were kept on ice.

ii) Preparation of Digestion Mixes:

To ensure a satisfactory gel pattern, digestion of the RNA at varying enzyme concentrations was necessary. The following table was used to prepare digestion mixes sufficient for 5 reactions per enzyme (4 µl + 1 µl enzyme from each dilution).

Enzyme	Soln A	Soln B	Soln C	Soln D	[^{32}P]-RNA	tRNA
RNase Phy M/T1	2 µl	-	-	14 µl	3 µl	1 µl
RNase U_2	-	2 µl	-	14 µl	3 µl	1 µl
RNase CL_3	-	-	2 µl	-	17 µl	1 µl
RNase B.cereus	2 µl	-	-	-	17 µl	1 µl

Solution A: 0.25 M sodium citrate, pH 5.0

Solution B: 0.25 M sodium citrate, pH 3.5

Solution C: 0.1 M sodium phosphate, pH 6.5, 0.1 mM EDTA

Solution D: 10 M urea, 1.5 mM EDTA, 0.05% each XC and BPB

tRNA : 5 mg/ml

A minimum of 10^5 dpm [^{32}P]-RNA per reaction was used for Phy M, T1 and U_2 RNAses, and 2 x 10^5 dpm [^{32}P]-RNA per reaction for CL_3 and B.cereus RNAses (without intensification screens). The latter enzymes required twice as much of the [^{32}P]-RNA because the reactions were diluted 1:2 with solution D after digestion but prior to loading the

samples on the gel.

iii) The Sequencing Reactions:

The complete sequencing reaction mixes was distributed into siliconised Eppendorf tubes (4 x 4 µl) and kept on ice. To each of the 3 tubes, 1 µl of the diluted enzymes was added, using the fourth tube as a control (no enzyme).

The T1, Phy M, U_2 and B.cereus reactions were all incubated at 55°C, and the CL_3 at 37°C. All incubations were for 15 min. To terminate the reactions, T1, Phy M and U_2 were frozen in liquid nitrogen while 5 µl of solution D which were then added to the CL_3 and B.cereus reactions which were then frozen in liquid nitrogen. The digested RNAs were stored at -20°C prior to gel electrophoresis.

iv) Alkaline Hydrolysis of ^{32}P-RNA:

Limited hydrolysis at every base in a ^{32}P-RNA generates a ladder upon gel electrophoresis which helps in the interpretation of the enzymatic digestion. To a siliconised, sterile Eppendorf tube containing the [^{32}P]-PVX RNA, 1 µl 0.5 M sodium bicarbonate/carbonate, pH 9.2, 1 µl (5 µg) tRNA and water to 10 µl final volume, were added. At least twice the dpm in the alkaline hydrolysis lane as in the sequencing reactions were used because of the high degree of hydrolysis in this reaction and subsequent distribution of the [^{32}P]-label among many products. For each sequencing reaction, where 10,000 dpm had been used, 80,000 dpm was used for the alkaline hydrolysis, resulting in material sufficient for 4 ladders of 20,000 dpm each.

The contents of the Eppendorf tube were drawn into a 50 µl capillary tube which had been drawn at one end. Both ends were then heat-sealed.

The capillary was incubated at 90°C for 5-7 min, chilled on ice and transferred back to an Eppendorf tube, adding an equal volume (10 µl) solution D.

v) Separation of Partial Digestion Products on Sequencing Gels:

Sequencing ladders were generated by electrophoresis of the partial digestion products on 0.5 mm thick polyacrylamide gels. For reading up to 40 nucleotides from a labelled terminus, 20% acrylamide sequencing gels were used; a 10% acrylamide sequencing gel was used for reading deeper into the sequence (10->100 nucleotides).

i) Preparation of Acrylamide Sequencing Gels:

For a 10% gel, the following were prepared: 20 ml 0.5 M Tris-borate, pH 8.3/10 mM EDTA; 50g urea; 9.5g acrylamide; 0.5g bis-acrylamide and water to 100 ml.

For a 20% gel, 19 g acrylamide and 1 g bis-acrylamide were used.

All components were thoroughly dissolved and passed through a nitrocellulose filter to remove debris. The acrylamide was deionised with amberlite as previously described before the addition of Tris-borate and EDTA and the gel then polymerised with 100 mg ammonium persulphate (added as a solid) and 25 µl TEMED.

The gel was loaded on to the gel mould with a syringe and needle, allowing air bubbles to escape by supporting the mould vertically. The well former was inserted and the gel then left to polymerised for 30 to 60 min.

vi) Pre-electrophoresis and Electrophoresis of Gel:

Once polymerised, the well-former was removed from the gel and the wells immediately cleaned with electrophoresis buffer. The gel was

placed into the electrophoresis apparatus, and the chambers filled with electrophoresis buffer.

A single well was blown out with a syringe to remove urea which had leached out. Into this well, 5 µl solution D, diluted 1:1 with water, was loaded. The gel was then pre-electrophoresed at 20-25 watts (constant power: 1000 V inital) until the bromophenol blue had migrated halfway down the gel.

All [^{32}P]-labelled samples were heated at 90°C for 30 sec and quick chilled on ice just prior to loading. The urea from the remaining wells was blown out with a syringe as previously described and 5 µl of each sample then loaded.

The samples were then subjected to electrophoresis at 50 to 60 watts (constant power: 1800 V initial). On a 10% gel, the xylene cyanol co-migrates with a 55 nucleotide fragment and bromophenol blue with a 12 nucleotide fragment; on a 20% gel, xylene cyanol runs as a 30 nucleotide fragment and bromophenol blue as a 9 nucleotide fragment (Maniatis, 1982) .

At the conclusion of the electrophoretic separation, the siliconised plate was removed, still attached to the larger plate and the gel covered with plastic wrap. A sheet of X-ray film and intensification screen were laid on top, and the cassette exposed at -70°C for the appropriate length of time.

2.11. Preparation of Recombinant Clones.

Recombinant cDNA clones were prepared using PVX N RNA as template.

i) Synthesis of cDNA.

The standard reaction mix (Maniatis,1982) contained in a 20 µl volume: 10 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.3 at 42°C), 150 mM KCl, 28 mM 2-mercaptoethanol, oligo $d(T)_{12-18}$ (0.8 A_{600} units/ml), 10 µCi [^{32}P]-dCTP (3000 Ci/mmol, Amersham Corporation), 0.5 mM dCTP, dGTP, dTTP, dATP, and 2 µg PVX N RNA.

The reaction mixture was pre-incubated for 10 min at 42°C. Reverse transcriptase (11 units/µl) was then added at a concentration of 1 µl/2µg RNA and then incubation then resumed for 2 h at 42°C. The reaction was terminated with 2 ul 0.25 M EDTA.

The amount of first strand cDNA synthesised was estimated by gel filtration of a 2 µl sample on Sephadex G100. Twenty-five fractions (approximately 0.5 ml per fraction) were collected and 22 µl aliquots of each fraction was counted on the ^{32}P channel (Packard Liquid Scintillation Spectrometer, Model 3380).

The RNA from the RNA-DNA hybrid was hydrolysed by incubation with 0.5 M NaOH (3.0 µl/20 µl reaction mix) at 65°C for 1 h, then neutralised with 3.0 µl 0.5 M HCl. The cDNA was precipitated with 0.5 volume 7.5 M ammonium acetate and 3 volume 100% ethanol, pelleted by centrifugation at 11,700 g for 10 min (MSE Microcentaur), washed once in 100% ethanol and dried under vacuum for 10 min.

ii) Synthesis of Second-Strand Complementary DNA.

The dried cDNA pellet was resuspended in 50 µl 70 mM KCl, 10 mM $MgCl_2$, 100 mM HEPES, pH 6.9, 2.4 mM DTT, 0.5 mM dNTPs, 1% bovine serum albumin, 25 µCi [^{3}H]-dCTP (Amersham Corporation, 20 Ci/mmol) and 20

units DNA polymerase I (Anglian Biotechnology Ltd) per µg of cDNA.

The reaction mix was incubated at 15°C for 15-20 h, then terminated with 5 µl 0.25 M EDTA. The efficiency of the second-strand cDNA reaction was estimated by gel filtration, using a 2 µl sample on a Sephadex G100 column. A further 2 µl was stored for the determination of the size of the DNA. The remaining dsDNA was precipitated with 100% ethanol and 7.5 M ammonium acetate, and pelleted as previously described.

ii) Second-Strand Completion Reaction.

Maniatis (1982) recommends the use of both reverse transcriptase and DNA polymerase I in succession since it is thought that DNA polymerase I and reverse transcriptase may pause or stop at different sequences. Thus, partially synthesised second-strands produced by one enzyme may be extended to completion by the other.

The pelleted double-stranded cDNA was resuspended in 50 mM Tris-HCl (pH 8.3, 42°C), 140 mM KCl, 10 mM $MgCl_2$, 40 mM -2-mercatoethanol, 0.5 mM dNTPs, and 11 units reverse transcriptase. In some cases, the reaction contained 2 µCi [^{3}H]-dCTP.

The reaction was incubated at 42°C for 3 h and terminated with 4 µl 0.25 M sodium EDTA. As before, 2 µl was stored for size determination. The dsDNA was precipitated at -20°C with 1.1 volume isopropanol and 0.1 volume 4 M potassium acetate for 16 h, then pelleted at 11,700 g for 10 min (MSE Microcentaur), and dried under vacuum for 10 min.

iii) Cleavage of the Hairpin Loop with S1 Nuclease.

After the synthesis of ds cDNA, the two strands are covalently joined by the hairpin loop that was used to prime the second-strand synthesis. This loop was cleaved by the action of the

single-strand-specific nuclease S1.

To the dried down ds cDNA, 10 µl 10 x S1 buffer (300 mM sodium acetate, pH 4.6, 2 M NaCl, 10 mM Zn_2SO_4) and 88 µl deionised distilled water were added. One microlitre was removed and added to 25 µl 0.1 x STE buffer (18 mM NaCl, 0.1 mM EDTA, 2 mM Tris-Cl, pH 7.5) to be passed through Sephadex G100.

To the ds cDNA solution, 2 units S1 nuclease (BRL) were added per microgram DNA, and incubated at 37°C for 1 h. The reaction was terminated by adding 0.25 M EDTA, pH 8.0 to a final concentration of 10 mM. A 1 µl aliquot was removed and added to 25 µl 0.1 x STE buffer for passage through Sephadex G100.

The S1 reaction mix was extracted with a 1:1 phenol/chloroform mixture as previously described. The phenol phase was re-extracted with 50 µl 0.1 x STE buffer and the aqueous phases then pooled. The ds cDNA was then precipitated with isopropanol and potassium acetate as previously described, and resuspended in 50 µl of the appropriate buffer.

The efficiency of cloning was improved after the S1 nuclease treatment of the ds cDNA by treatment with the Klenow fragment of E.coli DNA polymerase I (Seeburg et al., 1977).

The ds cDNA was resuspended in 50 µl second-strand buffer (1 mM $MgCl_2$, 70 mM KCl, 2.5 mM DTT, 100 mM HEPES) containing 0.5 µl Klenow fragment (2 units) and incubated at room temperature for 30 min. The reaction was terminated by adding EDTA to a final concentration of 2.5 mM/50 µl reaction mix. The ds cDNA was precipitated with cold isopropanol and potassium acetate as previously described and resuspended in 0.1 x STE buffer in preparation for sizing on a sucrose gradient.

2.12. Fractionation of Double-Stranded Complementary DNA by Centrifugation.

The ds cDNA was size-fractionated by centrifugation on a 5-20% linear sucrose gradient, according to Gustafson (1982). Gradients were prepared in Sorvall TST 41.14 tubes and were set up as follows:

Sucrose %(w/v)	60% sucrose	10xSTE	H_2O (ml)	ml used
5	0.83	0.1	9.07	1.8
10	1.67	0.1	8.23	3.6
15	2.50	0.1	7.40	3.6
20	3.33	0.1	6.56	1.8

The gradient was left to diffuse for 16 h at 4°C. The sample ds cDNA pellet was resuspended in 200 µl 0.1 x STE buffer and carefully layered on to the gradient. The gradient was centrifuged at 29,000 rpm for 16 h at 6°C in the Sorvall TST 41.14 rotor.

Twenty-four fractions, each of fourteen drops, were collected from the gradient at a rate of 1.5 ml/min using an ISCO Density Gradient Fractionator, Model 185. From each, 2 µl were counted in 4 ml Unisolve Type E scintillant (Koch-Light) on the ^{3}H and ^{32}P channels (Fig.39a). Aliquots of 20 µl were removed from those fractions with the highest values (40 cpm/µl) and added to 3 ul TBE bromophenol blue marker dye (50% glycerol, 0.5 vol 10 x TBE buffer, 1% bromophenol-blue) then loaded on to a 1 % agarose Tris-borate EDTA gel as previously described. Standards of Hind III-restricted λ DNA, Hind III/Eco RI double-digested λ DNA, and Taq I-digested pHP34 DNA were

co-electrophoresed with the samples as molecular weight markers. These standards were stained by soaking the gel for 15 min in ethidium bromide (2.5 µg/ml) and located under ultraviolet light. The gel was covered with Clingfilm and a sheet of Kodak X-Omat S film was layered over it. The gel was allowed to expose for 24 hours at -70°C using Dupont Cronex Lightning-Plus intensifying screens. The fractions with the highest counts were electrophoresed as separate samples in adjoining lanes on the sizing gels. After electrophoresis and exposure to X-ray film, the sized fractions were then assessed in terms of molecular weight. Appropriate fractions were divided into 'High Molecular Weight' (HMW), 'Medium Molecular Weight' (MMW), and 'Low Molecular Weight' (LMW) DNA samples and pooled. Since each separate fraction in the gel lanes covered a range of sizes, a certain amount of overlap in molecular sizes occurred in the samples (see Fig.39b).

2.13. Synthesis of in vitro Recombinant DNA.

Fractions 4-8 from the sucrose gradient centrifugation were electrophoresed as described. The results are shown in Fig.39b. A range of sizes was observed for each fraction: fraction 4 was too faint to see; fraction 5 ranged from less than 0.56 kbp to 0.56 kbp; fraction 6 ranged between 0.56 kbp to 1.3 kbp; fraction 7 between 0.6 kbp to 2.0 kbp, and fraction 8 ranged in size from 0.9 kbp to 2.3 kbp.

After centrifugation, the ds cDNA pellets were washed twice, first with 100 µl 70% ethanol, centrifuged, then washed again with 100 µl 100% ethanol, and dried under nitrogen. Each pellet was resuspended in 20 µl 0.1 x STE buffer. Samples of 0.56 kbp and less (fractions 4 and 5) were pooled and labelled "Low Molecular Weight" DNA (LMW DNA). Samples of between 0.56-2.0 kbp (fractions 6 and 7) were labelled "Medium Molecular Weight" or MMW DNA, and samples of between 0.9-2.3 kbp (fraction 8) and greater were labelled "High Molecular Weight" or HMW DNA.

Prior to ligating the sized double-stranded cDNA (ds cDNA) in the cloning vehicle, pUC9, test ligations were prepared in order to determine optimal experimental conditions.

i) Test ligation Reaction Mix I:

The mix contained 1 µg Hae III-digested pUC 9 DNA fragments, 25 µg nuclease-free bovine serum albumin, 0.5 mM ATP, 10 mM DTT, 5 unit T_4 DNA ligase (P+S Biochemicals), 0.6 µg Hinc II-digested pUC 9, 1 µl 10 x Ligase core buffer.

The reaction mix was incubated at 20°C for 4 h, and the reaction then terminated with 0.8 µl 0.25 M Na EDTA.

ii) Test Ligation Reaction Mix II:

As above, but containing 0.45 µg Hinc II-digested pUC 9 (calf

alkaline phosphatase (CAP)-treated).

iii) Test Ligation Reaction Mix III

As above, but excluding Hae III-digested pUC9 fragments from the reaction mix.

Results from the reaction products were electrophoresed on a 1.2% agarose TBE gel, stained with ethidium bromide and examined under ultraviolet light. Once the test ligations had been completed, ligation of the ds cDNA with the CAP-treated cloning vehicle (pUC9) proceeded as follows:

i) Ligation I: 'High Molecular Weight (HMW) ds cDNA into Hinc II-digested pUC9 (CAP-treated). The reaction mix contained: 100 ng HMW ds cDNA, 2 µl Ligase core buffer, 500 ng Hinc II-digested pUC 9 (CAP-treated), 25 µg bovine serum albumin, 10 mM DTT, 0.5 mM ATP, 10 unit T4 ligase.

ii) Ligation II: 'Medium Molecular Weight (MMW) ds cDNA into Hinc II-digested pUC9 (CAP-treated). The reaction mix was similar to ligation 1 except that 100 ng MMW ds cDNA was substituted for the HMW ds cDNA.

Both reactions were incubated at 20°C for 16 h, and terminated with 0.8 µl 0.25 M EDTA. The DNA was extracted with and equal volume of phenol-chloroform, the organic phase was re-extracted with 20 µl 0.1 x STE buffer. The aqueous phases were pooled, then alcohol precipitated as previously described. The ligated DNAs (both 'high' and 'medium' molecular weights) were stored at 4°C in preparation for transformation with competent Escherichia coli cells.

2.14. Small Scale Preparation of Plasmid DNA.

Each bacterial colony was inoculated and grown overnight in at 37°C (220 rpm) in 2 ml sterile L-broth containing ampicillin (20 µg/ml). At this stage, two different protocols were carried out to extract the plasmid DNA.

Method I: This method followed the procedure of Holmes and Quigley (1981). The bacterial cells were harvested in 1.5 ml Eppendorf tubes (2 min, Eppendorf Microfuge), and resuspended in 25 µl STET buffer. To this, 5 µl freshly prepared lysosyme (10 mg/ml) in STET buffer (8% sucrose, 5% Triton-X100, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) was added and the cells then incubated for 30 min at room temperature. To completely disrupt the cells, each Eppendorf vial was placed in a boiling water bath for 2 min, then immediately centrifuged for 2 min to pellet cellular debris and denatured chromosomal DNA. The glutinous pellet was removed and the supernatant was first incubated with 2 µl Rnase A (1 mg/ml) for 30 min at 37°C, then for a further 20 min at the same temperature with 1.5 µl proteinase K (5 mg/ml).

The purified plasmid DNA was phenol-chloroform extracted and precipitated in an equal volume of isopropanol and 0.1 volume 4 M potassium acetate, pH 5.5, chilled at -20°C for 20 min, and centrifuged for 10 min. The pellet was washed twice, first in 100 µl 70% ethanol, then in 100% ethanol, dried, and resuspended in 0.1 x STE buffer (18 mM NaCl, 0.1 mM EDTA, 2 mM Tris, pH 7.5).

Method II: This method, a modification of the above from Maniatis (1982), was designed to work with all commonly-used strains of E.coli. The method is rapid and gives high yields of plasmids.

Aliquots of 1.5 ml from each overnight culture were centrifuged for 1 min in the Microfuge. The supernatant was pipetted off and the

bacterial pellet then dried under a gentle stream of nitrogen. The cell pellet was resuspended in 0.35 ml STET buffer and mixed with 25 µl freshly prepared solution of lysozyme (10 mg/ml in 10 mM Tris HCl, pH 8.0) and vortexed. The tubes were placed in a boiling water bath for 2 min and centrifuged immediately for 10 min at room temperature (Eppendorf Microfuge). Using a sterile toothpick, the pellets were removed from the vials and 40 µl 2.5 M sodium acetate with 420 ul cold isopropanol were added to the supernatant. Each vial was vortexed and the DNA then precipitated at -20°C for 15 min. The DNA was pelleted by centrifugation for 15 min at 4°C (MSE Microcentaur), then dried under nitrogen and resuspended in 50 µl TE buffer (10 mM Tris HCl, 10 mM EDTA, pH 8.0).

2.15. Large Scale Preparation of Plasmid DNA.

Preparation of Plasmid DNA.

Plasmid DNA was prepared by a modification of the method of Birnboim and Doly (1979). Cells were centrifuged at 15,500 rpm for 5 min at 6°C (MSE 18), then resuspended in 80 ml lysis solution (containing 2 mg/ml lysosyme), and left on ice (0°C) for 30 min.

To the lysis solution, 160 ml alkaline sodium dodecyl sulphate (80 ml/L cells) was added to the mixture, vortexed, and left on ice for 5 min. A further 120 ml 3 M potassium acetate, pH 4.8 was added, and the solution mixed, then left at 0°C for a 15 min.

Cellular debris, denatured chromosomal DNA, and potassium dodecyl sulphate were removed by centrifugation (8,000 rpm for 5 min at 0°C in the MSE 8 x 50 rotor). The supernatant was filtered through a layer of Kleenex tissue and the DNA was precipitated at -20°C or by adding 0.6 vol pre-chilled (-20°C) isopropanol for 30 min. The DNA was collected by centrifugation at 8,000 rpm, for 5 min at 0°C. The resultant pellet was dried by inverting the centrifuge tube over tissue paper.

Contaminating chromosomal and relaxed plasmid DNAs were removed by centrifugation in a CsCl/ethidium bromide gradient (Radloff *et al.*, 1967). The dried pellet was resuspended in 13.4 ml TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0), 14.4 g CsCl added and completely dissolved and 1.4 ml ethidium bromide (3 mg/ml) then added. The solution was divided into two Sorvall T865.1 centrifuge tubes, topped up with sterile liquid paraffin, and centrifuged at 40,000 rpm for 40 h at 20°C (Sorvall OTD 65B Ultracentrifuge in the T865.1 rotor).

After centrifugation, two bands (darker than the surrounding solution) were visible in each tube. The upper, thinner band contained both chromosomal and linearised plasmid DNA, whereas the lower, thicker band contained the covalently closed, circular plasmid DNA.

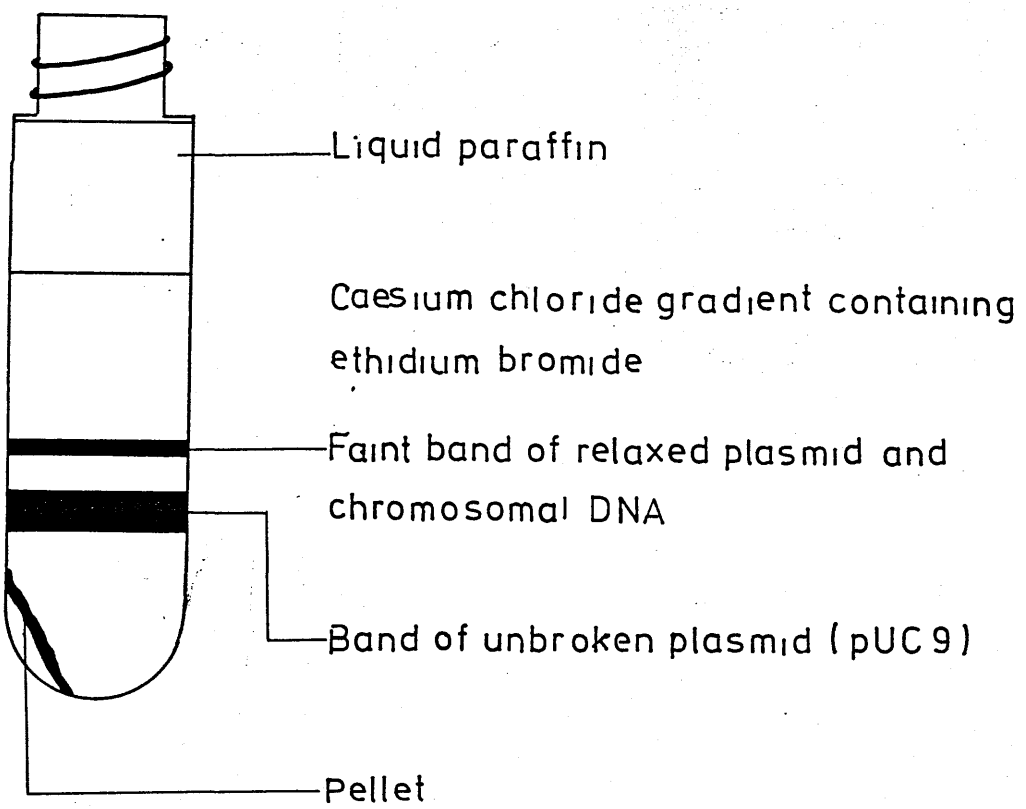


Fig.6: Purification of pUC 9 plasmid DNA by Isopycnic Centrifugation in a Caesium Chloride-Ethidium Bromide Gradient.

The ethidium bromide binds by intercalating between the DNA base pairs causing it to unwind. Covalently, closed, circular DNA such as a plasmid, possessing no free ends are limited and bind less ethidium bromide. However, a linear DNA molecule, such as fragmented chromosomal DNA, unwinds more and therefore, binds more ethidium bromide. The covalent circle has a higher density at saturating concentrations of EtBr since the density of the DNA/EtBr complex decreases as more EtBr is bound. Separation of the plasmid and the remaining DNA is therefore achieved during centrifugation in the caesium chloride gradient.

The plasmid band was collected for purification using 10 ml syringe connected to a needle, the end of which was bent at 90°. Ethidium bromide, which gave the solution a red-orange tinge, was removed by shaking with an equal volume of isopropanol saturated with caesium chloride. The upper, isopropanol phase was pipetted off and the procedure repeated six times.

The lower, partitioned phase was dialysed against 1 litre of continuously stirred 0.1 X STE buffer for 8 h at 4°C. During this period, the external buffer was changed twice to remove all traces of caesium chloride.

After dialysis, 10% SDS solution was added to the total volume to a concentration of 0.5%.

As an additional step, a 5 mg/ml solution proteinase K was freshly prepared and added to a final concentration of 100 ug/ml. The enzyme has to be freshly prepared because of auto-digestion in older solutions.

The solution was incubated at 20°C for 20 min, then centrifuged at 5,000 rpm at 18°C (MSE 18). This additional step removed any residual proteins. Half volumes of water-saturated re-distilled phenol (containing 0.1% quinolinol) and chloroform were added to the supernatant, vortex-mixed, and centrifuged at 11,700 g for 10 min (MSE Microcentaur). The solution partitioned into two phases, of which only the top aqueous phase was saved. A half volume of 0.1 X STE buffer was added to the phenol phase for re-extraction. The aqueous phases were then pooled.

The DNA in the aqueous phase was precipitated at -20°C with 0.5 vol 4 M potassium acetate,pH 5.5 and 1.3 vol 100% isopropanol for 1 h. Alternatively, a better precipitation was achieved with 0.5 vol 7.5 M ammonium acetate, pH 7.5 and 2 vol 100% ethanol.

The precipitated DNA was pelleted at 15,000 rpm for 30 min, then reprecipitated with ammonium acetate and ethanol. To shorten the time needed for precipitation of DNA at -20°C, smaller samples (i.e. less than 3 ml) were cooled in liquid nitrogen, then centrifuged at 11,700 g for 10 min (MSE Microcentaur) to pellet the DNA. The pellet was given a final wash with ethanol (1 ml), vortex-mixed, then centrifuged for 5 min and dried under a gentle stream of nitrogen. The DNA was resuspended in 0.5 ml 0.1 X STE buffer and stored at 4°C.

2.16. Preparation of Competent Cells:

Cells were prepared according to the procedure of Dagert and Ehrlich (1979). An overnight inoculum of cells, E.coli strain JM 83 (Viera and Messing, 1982), were transferred to 50 ml L-broth, grown to an absorbance value(OD_{600}) of 0.3, then immersed in ice for 30 min. The bacterial cells were harvested by centrifugation at 7,000 rpm for 5 min at 4°C, then resuspended in 20 ml ice-cold 0.1 M $CaCl_2$ and incubated at 0°C for 20 min.

The resuspended cells were centrifuged at 7,000 rpm for 5 min at 4°C. Competent cells typically gave a diffused "halo" at the base of the centrifuge tube. Cells which gave a compact pellet at this stage, however, usually gave poor transformation. Cells were suspended in 0.5 ml 0.1 M $CaCl_2$ for 24 h at 0°C in preparation for transformation. The ability of cells to become transformed is reported to be maximal after 24 h (Dagert and Erhlich, 1979) when stored on ice. Since different batches of cells showed different efficiency of transformation, a small aliquot of cells was withdrawn after 1 h and transformed with pUC 9 (see below). Only cells which produced greater than 2×10^6 transformants per µg pUC 9 DNA were considered suitable for transforming with recombinant DNA.

1) Test Transformation:

After 1 h, 5 ng pUC9 in 5 µl 0.1 x STE buffer (18 mM NaCl, 0.1 mM EDTA, 2 mM Tris, pH 7.5) was added to 50 µl competent cells, which were chilled on ice for 10 min and then incubated at 37°C for 5 min. The transformed cells were amplified by adding 2 ml L-broth and shaking at 37°C for 1 h at 220 rpm. Aliquots of 10 µl (in some cases were diluted 10-fold with sterile L-broth) were spread over L-broth agar plates containing (5-bromo-4-chloro-3-indolyl-B-D-galactoside (Xgal) at 40 µg/ml and ampicillin (100 µg/ml), incubated for 16 h at 37°C, and

examined for blue colonies.

2) Transformation by Recombinant DNA.

The bacterial cells (E.coli, strain JM 83) were prepared as previously described. Competent cells were transformed with 5 ng of ligated double-stranded cDNA as above. The transformed cells were amplified in 2 ml sterile L-broth at 220 rpm for 1 h at 37°C, then harvested in the MSE Microcentaur for 2 min (11,700 rpm).

The pellet was resuspended in 100 µl of the L-broth supernatant, and divided into 10 µl aliquots. These were spread over L-broth agar plates containing ampicillin (100 mg/L) and Xgal (40 mg/L), then incubated at 37°C for 16 h. As a control, the procedure was repeated with pUC9 described above.

Xgal is not an inducer of B-galactosidase, but is cleaved by B-galactosidase, releasing a blue indolyl derivative. E.coli, strain JM 83 is constitutive for an inactive form of the enzyme (Vierra and Messing, 1982). Only cells transformed by pUC9, which contains a fragment of the gene for B-galactosidase capable of complementing the chromosomal gene, produce blue colonies on medium containing Xgal. However, insertion of DNA fragments into the universal cloning site of pUC9 interrupts the coding sequence of this fragment. Recombinants are, therefore, lac- and give rise to white colonies. Thus, identification of plasmids with DNA inserts is simplified.

Putative recombinants, as identified by the presence of white colonies on the agar plates, were amplified in L-broth as previously described. Glycerol stocks (50% w/w glycerol/50% w/w L-broth) of each clone was prepared and stored at -20°C.

3) Screening Recombinants for PVX Sequences.

In Situ Hybridization of Bacterial Colonies:

The method used for the identification of recombinants was *in situ* hybridization of bacterial colonies. The technique was modified from that of Grunstein and Hogeness (1975). Recombinants were identified by the presence of white colonies on the agar plates. These were picked off with a sterile cocktail stick and spotted on to L-agar-ampicillin plates. Each plate was divided into a 7 x 7 grid and numbered. For screening, the end of a sterile cocktail stick was dipped into each 'positive' white colony and on to the same square on each of two replica L-broth agar/ampicillin plates. The plates were incubated upside-down at 37°C for 16 h.

Identification of Recombinant Clones:

Circular nitrocellulose filters (Schleicher and Schull nitrocellulose membrane, BA 83 or BA 85, 82 mm diameter) were pressed on to each of the first replica plates, removed and laid out, the imprinted colonies uppermost, on 3 MM paper. The second replica plates were stored at 4°C.

The filters were transferred to two sheets of 3 MM paper soaked with 0.5 M NaOH + 0.8 M NaCl for 0.5 h, then dried on dry sheets of 3 MM paper. This was repeated with sheets soaked in 1 M Tris-HCl, pH 7.5 + 1.5 M NaCl for 0.5 h and on chloroformed-soaked sheets for 20 sec each. Each filter was transferred to sheets of 3 MM paper soaked in 2 x SSC buffer for 5 min and dried at room temperature for 0.5 h. The denatured DNA was fixed by baking the filters at 80°C for 2-3 h *in vacuo*. The filters were then hybridized with cDNA probes synthesised from PVX RNA templates.

Prehybridization:

Each baked filter was sealed in a heavy gauge plastic bag with 5 ml prehybridization buffer 1 (4.5 ml 20 x SSC buffer, 0.15 ml 2 % PVP/Ficoll, 0.75 ml BSA, 0.375 ml sheared denatured DNA, 0.75 ml 10 % SDS, 8.5 ml deionised distilled water) , and incubated at 65°C for 2-3 h.

Hybridization:

After prehybridization, the buffer was removed and replaced with another 5 ml of the above buffer containing the probe at 40,000-50,000 cpm/ml and incubated at 65°C for 16 h. After incubation, the filters were washed to removed excess probe:

1) in 2 x SSC buffer + 0.1% SDS, pH 7.0, at room temperature for 15 min (two changes), and

2) in 2 x SSC + 0.1% SDS, pH 7.0, at 65°C for 1 h (two changes).

The damp filters were then set up for autoradiography at -70°C using X-Omat RP film in the presence of Dupont Cronex Lightning-Plus screens. Colonies giving a strong positive hybridisation signal were considered to contain PVX cDNA sequences.

As a precaution against false positives, a small scale preparation of DNA from each clone was carried out as previously described. Each DNA solution was diluted to 1 µg/ul and 2 µg spotted on to nitrocellulose, which was then dried and baked as previously described.

A ^{32}P-cDNA probe was synthesized from a PVX RNA template. The nitrocellulose filter was prehybridized for 3 h at 65°C and hybridized with the probe (2.0×10^6 cpm total) as previously described. Positive clones were identified from autoradiographs taken from the washed hybridized, nitrocellulose, amplified in L-broth, their DNA extracted

and labelled by nick translation.

2.17. Preparation of ATP:RNA Adenyltransferase.

i) Preparation of the Low Speed Supernatant.

The enzyme was prepared by the procedure of Sippel (1973). Ten litres of E.coli MRE 600 cells were grown in L-broth to late log phase ($O.D_{600}$ = 0.8). The cells were harvested at 15,000 rpm in the MSE 8 x 50 rotor for 5 min at 4°C and stored at -20°C.

The pellets (about 16 g) were thawed in 17 ml Buffer A (50 mM Tris-Cl, pH 7.9 at 4°C, 10mM $MgCl_2$, 0.2 M KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol) containing 0.17 mg DNase I (Sigma), and stirred on ice for 10 min with an additional 10 ml Buffer A. Cells were broken at 8,000-10,000 psi in a French pressure cell. The brei was stirred for 30 min, then centrifuged at 13,5000 rpm for 30 min at 4°C.The supernatant was decanted from the pellet and saved.

ii) Preparation of the 1M Ammonium Chloride High Speed Supernatant.

Ammonium chloride, both as a solid (1.836 g) and as a 1 M solution (8.5 ml), was added to the supernatant to give a final concentration of 1 M, which was then stirred on ice for 1 h, and centrifuged at 55,000 rpm for 2 h at 4°C in the T865 rotor. The resultant clear supernatant (33.2 ml) was decanted from the ribosomal pellet and its reddish top layer.

iii) Phase Partitioning Step.

To the supernatant: 12.28 ml of 30% w/v polyethylene glycol 600 (37 ml per 100 ml); 5.31 ml 20% w/w Dextran T500 (16 ml per 100 ml); and 12.94 g solid sodium chloride, were added. This mix was stirred for 1 h, then centrifuged at 8,000 rpm for 45 min at 6°C, separating it into two phases. The top phase was pipetted off from the glutinous bottom phase and dialysed for 15 h against 3 changes of Buffer B + 0.5 M

NaCl, pH 7.9 (25 mM Tris-Cl, pH 7.9 at 4°C, 1 mM EDTA, 5% glycerol, 0.5 M NaCl).

It was necessary to agitate the dialysis bag frequently because of the high viscosity of the content. Therefore the solution was continually stirred at 4°C. After 8 h, when tubidity appeared (caused by precipitation of residual dextran), the solution was removed from the dialysis bag and clarified by centrifugation at 10,000 rpm for 39 min at 6°C.

iv) Phosphocellulose Chromatography:

Swollen phosphocellulose (9 ml settled volume Whatman P11), equilibriated in 18 ml Buffer B + 0.5 M NaCl,pH 7.9, was added to the phase partitioning fraction and the slurry was gently stirred on ice for 3 h at 4°C. The ion-exchange resin was filtered with moderate suction in a Buchner funnel and washed with 35 ml Buffer B + 0.5 M NaCl, pH 7.9, in small portions, ensuring that the resin was kept wet at all times. A 9 mm acid-washed glass column was filled with the wet resin which was left for 5 h to settle in excess Buffer B + 0.5 M NaCl, pH 7.9. The enzyme was eluted with a linear 100 ml gradient of 0.5 M to 1.1 M NaCl in Buffer B (Fig.7). Fractions of about 2.5 ml (55 drops per tube) were collected at a flow rate of 8 ml per hour. Forty-nine fractions were collected.

v) Assay for Enzyme Activity.

To determine the specific activity of the enzyme, 10 µl from each fraction were assayed for the ability to transfer ^{3}H-ATP to tRNA. The standard assay mix used for the characterisation of ATP:RNA adenyltransferase contained: 20 mM Tris-HCl, pH 7.9; 0.1 M NaCl, 4 mM $MgCl_2$; 3.12 mM $MnCl_2$, 250 µg tRNA; 0.1 mM [^{3}H]-ATP (15-30 Ci/mol, Amersham) and 10 µl enzyme in a total volume of 50 µl. The reaction was

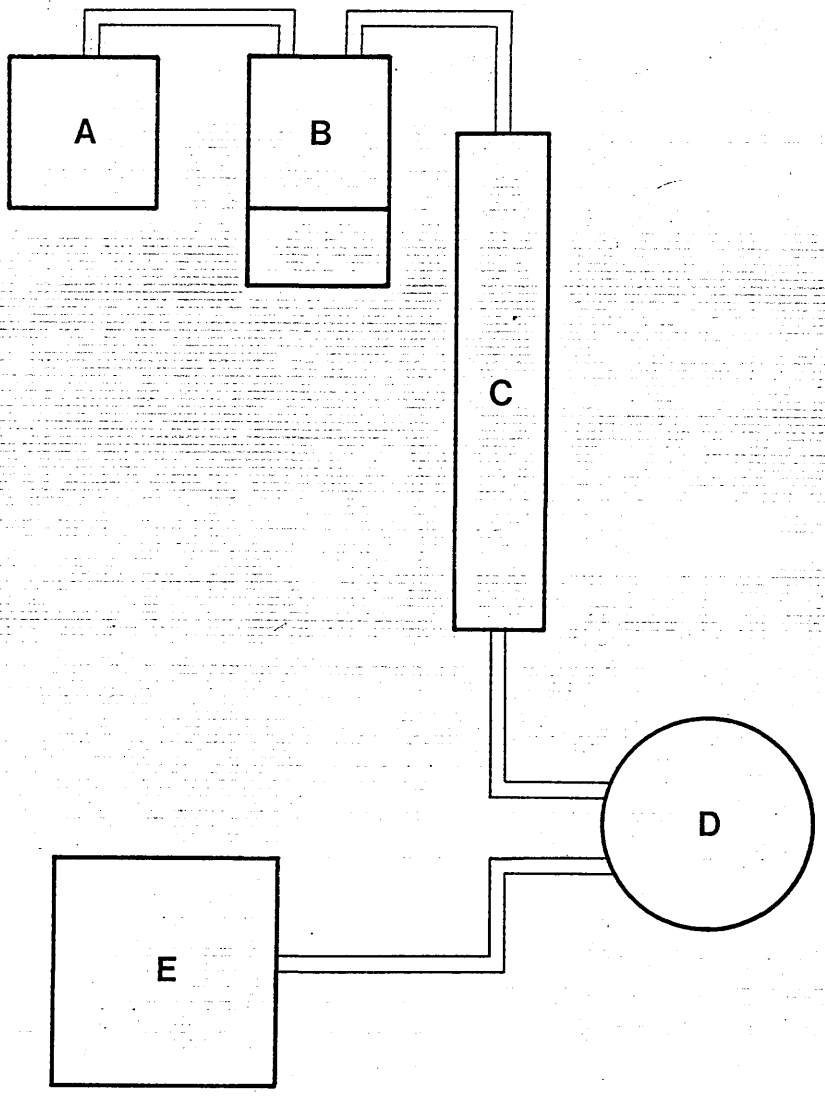


Fig.7: Phosphocellulose Chromatography Apparatus for Eluting the Enzyme, ATP:RNA Adenyltransferase.

The enzyme was eluted with a 100 ml linear gradient from 0.5 M to 1.1 M NaCl. The diagram shows: (A) 100 ml Buffer B + 1.1 M NaCl, pH 7.9; (B) 100 ml Buffer B + 0.5 M NaCl, pH 7.9; (C) phosphocellulose column; (D) EYELA microtube pump MP3; (E) fraction collector (55 drops per fraction). The extraction was carried out at 4°C and the fractions were collected at 8 ml/h.

stopped by placing on ice for 5 min, and 90 µl from each reaction then spotted on to DEAE cellulose filters (2.5 cm DE 81, Whatman).

These were air dried at 80°C for 20 min and washed to remove unreacted ATP (six changes of 100 ml 0.4 M sodium phosphate, pH 6.8, then two changes of 100 ml deionised distilled water, and two changes of 100% ethanol). ATP which has become polymerised into poly (A) tails at the 3' terminus of the RNA remains bound to the DE81 filters during washing.

The filters were counted in a scintillation counter using 0.4% PPO (diphenyloxazole) in toluene as scintillant. In addition, 90 µl of the reaction mix was spotted on to a filter, dried down at 80°C for 20 min and counted without washing in order to determine the specific activity of [3H]-ATP in each reaction. One enzyme unit is defined by the incorporation, in the assay, of 1 nmol AMP per 10 min at 37°C (Sippel, 1973).

2.18. Storage of ATP:RNA Adenyltransferase.

From the elution profile of the enzymatic activity from the phosphocellulose column, the fractions with the highest activity (465-598 units/ml) were saved and used for further experiments concerned with storage of the enzyme. To do this, the enzyme was stored in a variety of conditions which included freezing with and without glycerol (from 50% to 200% v/v).

Aliquots from the fraction with the highest activity was stored as follows:

1) 10 µl enzyme, stored at 4°C.
2) 10 µl enzyme, stored at -20°C.
3) 10 µl enzyme + 10 µl glycerol, stored at 4°C.
4) 10 µl enzyme + 10 µl glycerol, stored at -20°C.
5) 10 µl enzyme + 10 µl glycerol, quick frozen in liquid nitrogen for 15 s, then stored at -20°C.
6) 10 µl enzyme, quick frozen in liquid nitrogen for 15 s, then stored at -20°C.
7) 10 µl enzyme + 5 µl glycerol, quick frozen for 15 s, then stored at -20°C.
8) 10 µl enzyme + 15 µl glycerol, quick frozen in liquid nitrogen for 15 s, then stored at -20°C.
9) 10 µl enzyme + 20 µl glycerol, quick frozen in liquid nitrogen, then stored at -20°C.

After storage, the enzyme was characterized in the method previously described (Sippel, 1973).

Activity of Stored ATP:RNA Adenyltransferase.

Once the enzyme fraction which had the highest activity had been determined, experiments were carried out with PVX and transfer RNA to determine the optimum concentration of the enzyme to use. The protocol

for the assay was from Sippel (1973) but adjusted to 100 µl total volume.

Time Course Studies of the Enzyme.

Reaction mixes were prepared as above but without the enzyme, and vortex-mixed. After the addition of the enzyme, a series of 10 µl aliquots were removed at timed intervals to determine the time course of the addition of adenine residues to the 3' OH of tRNA. The first aliquot was taken at time (T) = 0, and directly spotted on to the DE 81 filter. This gave the background activity at time (T) = 0. Aliquots were then removed at 5, 10, 15, 20, 25, and 30 minutes, spotted on to individual filters, dried, and washed as described above to remove unreacted ATP. The filters were counted for activity as previously described and the percentage of the ATP which had polymerized was then calculated.

CHAPTER 3

RESULTS

3.1. Isolation of PVX and PVX RNA.

a) Inoculation:

Four weeks after inoculation with PVX N, the Nicotiana tabacum plants showed vein-clearing and necrotic ringspots. Typical symptoms for the N, strain-group 2 (PVB), strain-group 3 (PBI) isolates are shown in Figs.8-16.

b) Viral Yields:

Yields of viral particles and viral RNA from the three batches of PVX N infected plants are shown in Table 1 below. Yields were determined spectrophotometrically as described in section 2.1.

Table 1.

Sample Number	Weight of leaves (g)	mg vp/10 g leaves	yield of RNA/10 g leaves
1	50.0	8.3	0.48 mg
2	79.0	5.7	0.05 mg
3	94.5	4.8	0.17 mg

Viral particles were mainly obtained from the PVX N, Group 3 strain (results above) but inoculation of Nicotiana tabacum plants with two other strains of PVX, a Group 3 strain and a Group 2 (PVB) strain (in Methods and Materials 2.1c), extraction, and purification of RNAs were also carried out on these two strains. PVX-infected plants (6 weeks post-inoculated) were harvested and the viral particles extracted as previously described. The results for representative samples are given below.

Table 2.

Sample (strains)	Weight of leaves (g)	Total yield of VP	mg VP/10 g leaves leaves
Gp 3 (N)	33.3	6.66	2.3
Gp 3 (PBI)	32.7	11.50	3.9
Gp 2 (PBI)	25.4	2.68	1.3

c) Purification of PVX RNAs from Strains 2 and 3.

PVX particles are reported to contain 6 % RNA by mass (Koenig and Lesemann, 1978). Recoveries of RNA were 294 µg (average) for PVX N (4.4 % yield); 168 µg (average) for PVX (strain-group 3, PBI) (1.4 % yield); and 78 µg for PVB (strain-group 2, PBI) (2.9 % yield).

The yields from the RNA were lower than expected. The highest yield of RNA always came from the PVX Gp 3 (strain N) viral particles.

3.1.1. Symptoms of PVB (strain-Group 2)- and PVX (strain-Group 3)-infected Nicotiana tabacum plants.

Figure 8: Healthy leaf taken from a 10-week old, uninfected plant.

Figure 9: An example of a systemically infected leaf from a PVX-infected plant (strain-Group 3, PBI) taken 4 weeks after inoculation of a 6 week old Nicotiana tabacum plant. Symptoms are similar to the necrotic ringspots of PVX N-infected plants but are fainter on the leaves. In all cases, the symptoms in the inoculated leaf then spread to the younger leaves.

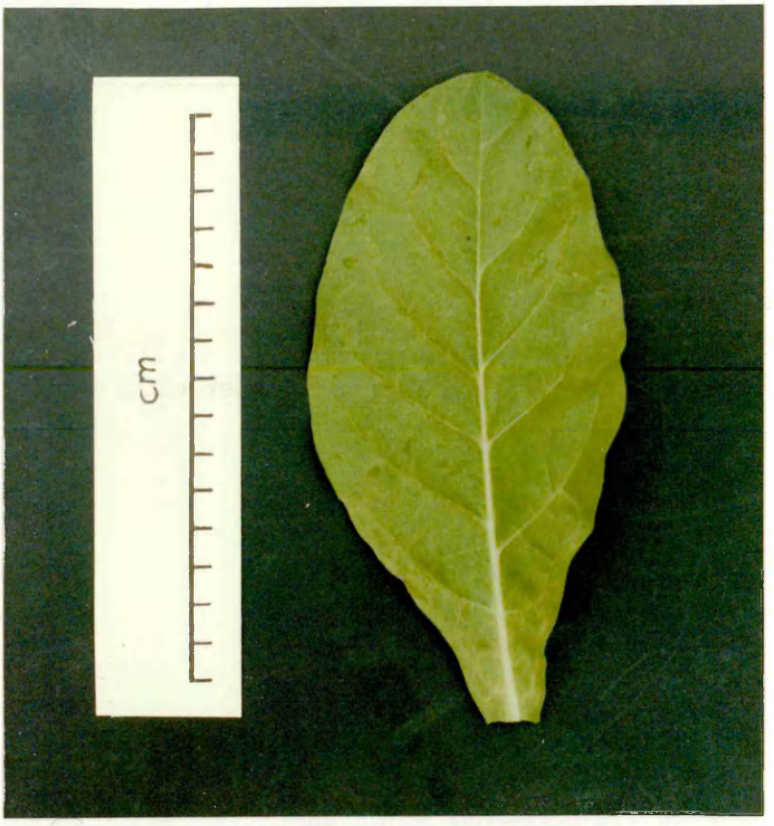


Fig.9

Figure 10: An infected, younger leaf of a Nicotiana tabacum plant showing the systemic spread of PVB (strain-Group 2) infection. The symptoms were usually seen first on these younger leaves which then spread to the older leaves below. The symptoms varied considerably from the necrotic ringspots of Group 3 infections.

Figure 11: An older PVB (strain-Group 2) systemically infected leaf showing vein-clearing symptoms and crinkling effect on Nicotiana tabacum plants. The photograph shows the effect of the virus 4 weeks after inoculation on 6 week old plants.

Fig.10

Fig.11

Figure 12: A systemically infected PVX N-infected leaf taken 4 weeks after inoculation. The photograph shows the ringspots which are characteristic of PVX N infection.

Figure 13: Healthy, uninfected, 10 week-old Nicotiana tabacum (cultivar White Burley) plant, grown under standard conditions (19°C with alternating 16 h light and 8 hour dark periods). Sap from this and similar plants were used for the nitrocellulose and ELISA assays.

Fig.12

Fig.13

Figure 14: A Nicotiana tabacum plant infected with PVX N (strain-Group 3) from which PVX N (sample 2) viral particles and RNA were extracted and purified. The photograph shows the original, inoculated leaf at the top of the photograph. In most cases, the leaf suffered 'abrasion' burns from the Celite, used during the inoculation (see Methods and Matertials 2.1). Typically, at the site of abrasion, some minor symptoms such as necrotic ringspots or vein clearing would appear. These ringspots would then spread to the remainder of the plant.

Figure 15: A PVB (strain-Group 2)-infected Nicotiana tabacum plant, showing systemically infected leaves with vein clearing and crinkling of well-infected leaves, 4 weeks after infection. The original, infected leaf is shown on the top right of the photograph and shows abrasion damage from the inoculation.

Fig.14

Fig.15

Figure 16: Nicotiana tabacum plant infected with PVX (strain-Group 3, PBI) showing systemic spread of the virus (far left leaf) from the original infected leaf (smaller, bottom leaf). Sap, purified viral particles and RNA extracte from similarly infected plants (10 week-old plants, 4 weeks post-inoculation) were used for the nitrocellulose and ELISA assays.

The yield of viral particles from the leaves varied with each extraction. However, the yields of the two PVX strains (PVX-3, PBI, and PVX N) were greater than that for the PVB strain, e.g. PVX-3, PBI gave 3.9 mg viral particles/ 10 g leaves; PVX N gave 2.3 mg viral particles/ 10 g leaves; and PVB, PBI gave 1.3 mg viral particles/ 10 g leaves (see 3.1. Table 2).

Fig.16

3.2. Enzyme-Linked Immunosorbent Assay (ELISA).

Plants infected with PVX were screened for virus using ELISA. The results of the enzyme-linked immunosorbent assays are presented in Figs.17-21. The assays were divided into two batches: 1) with serial dilutions of crude sap, and 2) purified viral particles. In the crude sap assays, sap was collected from both strain-group 2 (PVB) and strain-group 3 (PVX), serially diluted, and assayed for 1, 2, and 5 hours by ELISA. These samples were used as comparison for the sap spot assay on nitrocellulose filters.

In all cases, an increased sensitivity was noted with increased incubation times, lessening at the highest dilution. This was especially marked at 10^{-1} dilution for all samples, with the exception of the PVX-infected sap (strain-group 3, PBI), where spectrophotometric values increased up to two-fold the original readings at 5 hours incubation, e.g. PVX N-infected sap at A_{405} 1 h = 1.128 and 5 h = 2.127 (Figs.17 and 19). With all three incubations, PVX-infected sap (strain-Group 3) consistently gave the highest readings and could be clearly detected to 10^{-2} x dilution. In Fig.17, apart from the PVX-infected sap (strain-Group 3, PBI), very little difference was observed in the sensitivity of detection between the PVB (strain-Group 2) and the PVX N sap samples, although the absorbance values were higher between 10^{-2} x dilution and 10^{-5} x dilution.

The limits of detection were considered to be any values above twice that of the BCL negative control (the baseline). Therefore, positive values at 5 h for the samples can be observed easily at 10^{-4} dilution and less so at 10^{-5} dilution. The crude sap assays produced very similar results for both PVX and PVB.

Unlike the other isolates, the PVX-infected sap (strain-Group 3, PBI) consistently produced high absorbance values at high dilutions of

	Serial dilution					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Uninfected sap	+	-	-	-	-	-
PVX N sap, Sample 1	+++	++	+	+	-	-
PVX N sap, Sample 2	+++	++	+	-	-	-
PVX sap, Gp 3 (PBI)	+++	+++	+++	+++	+++	+++
PVX sap, Gp 2 (PBI)	+++	++	+	+	+	-

	2 ug	200 ng	20 ng	2 ng	200 pg	20 pg
PVX N VP, Sample 1	+++	+++	+++	+	+	-
PVX VP, Gp 3 (PBI)	+++	+++	+++	++	+	-
PVX Vp, Gp 2 (PBI)	+++	+++	+++	++	+	-

Table 3: Visual determination of ELISA assay, 3 h incubation. The substrate was allowed to react with the conjugated antibody for 5 hours. Serial dilutions of healthy and infected sap (from 10^{-1} x to 10^{-6} x dilutions) and purified viral particles (VP) from 2 µg to 20 pg were also assayed. The results showed that, for the infected sap, the PVX, strain-Group 3 (PBI) gave the strongest colour reaction in all dilutions up to 10^{-6} x dilution (2-3 logs better than the other isolates). However, results for purified viral particles (VP) gave identical results, indicating that the strong reaction in the PVX (strain-Group 3)-infected crude sap was due to a large amount of viral particles in the sample.

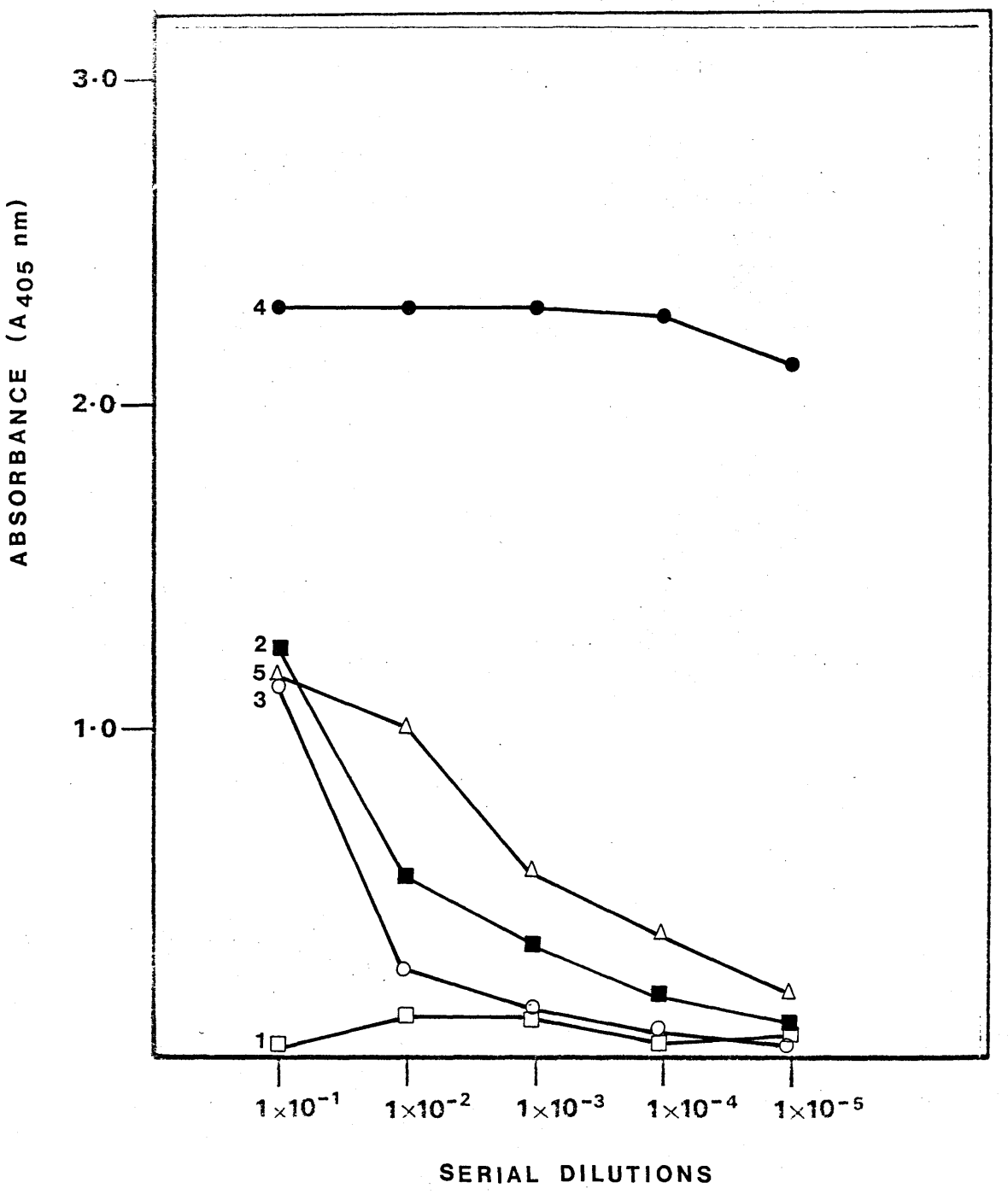


Fig.17: ELISA 1 h incubation: Samples which had been tested by dot-blot asay were tested by ELISA. Serial dilutions of healthy and infected sap samples were assayed. The graph shows serial dilutions of 1) Uninfected sap (□), 2) PVX N-infected sap (Sample 1)(■), 3) PVX N-infected sap (Sample 2)(o), 4) PVX-infected sap (Gp 3, PBI)(●), 5) PVB-infected sap (Gp 2, PBI)(Δ).

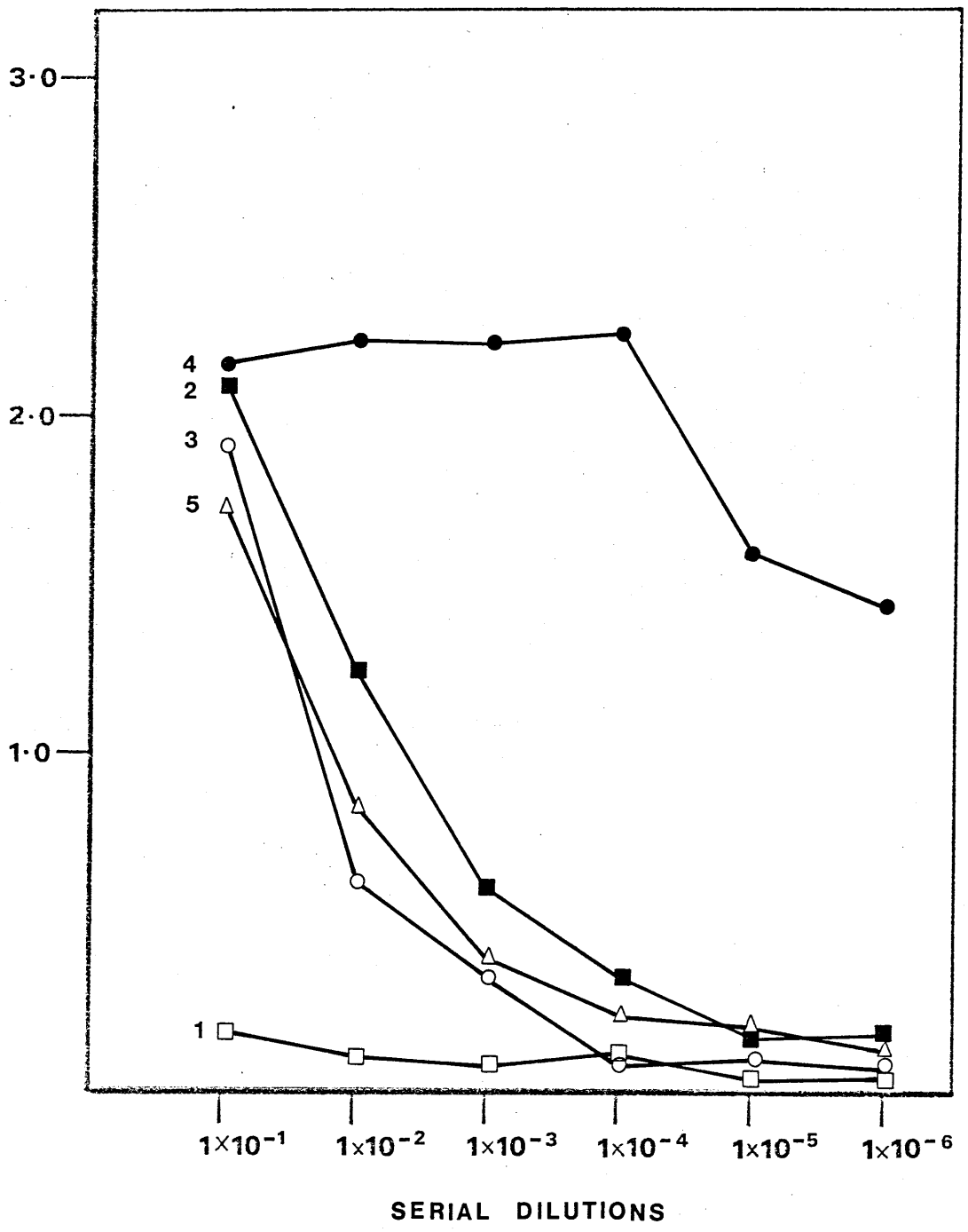


Fig.18: ELISA 2 h incubation: Results of serial dilutions of healthy and infected sap samples assayed by ELISA. The graph shows serial dilutions of 1) Uninfected sap (□), 2) PVX N-infected sap (Sample 1) (■), 3) PVX N-infected sap (Sample 2)(o), 4) PVX-infected sap (Gp 3, PBI)(●), 5) PVB-infected sap (Gp 2, PBI)(Δ).–

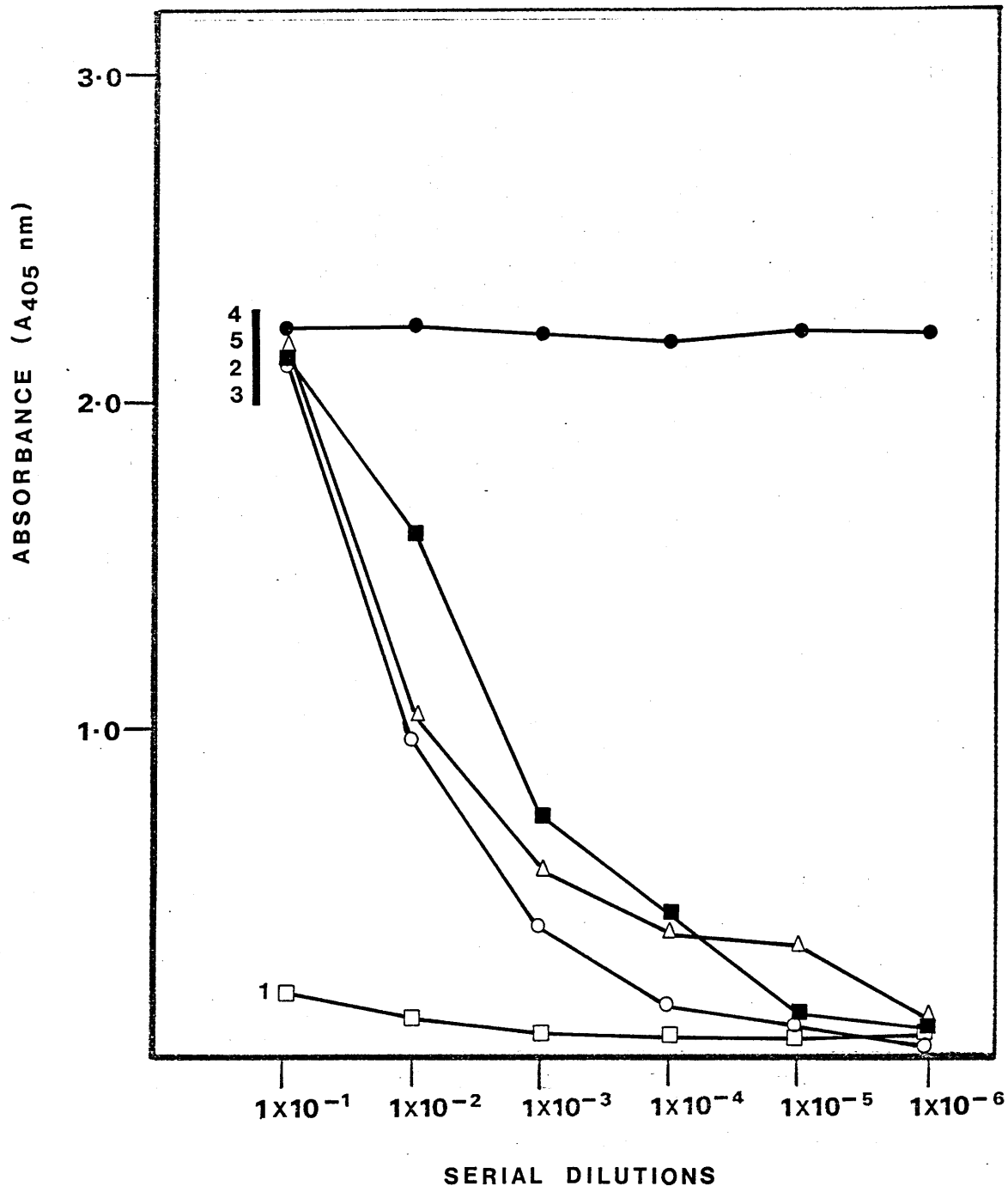


Fig.19: ELISA 5 h incubation: Serial dilutions of healthy and infected sap samples were assayed. The graph shows serial dilutions of 1) Uninfected sap (□), 2) PVX N-infected sap (Sample 1)(■), 3) PVX N-infected sap (Sample 2)(o), 4) PVX-infected sap (Gp 3, PBI)(●), 5) PVB-infected sap (Gp 2, PBI)(△).

the sap in repeat assays over the 1, 2, and 5 h incubations (Figs.17, 18, and 19). The reason for these high absorbance values was uncertain although it was postulated that the extracted sap from the infected leaves contained higher levels of viral particles than the other isolates. This possibility was backed during viral particle extractions. Of the three isolates, PVX (strain-Group 3, PBI) consistently yielded the highest levels of viral particles per 10 g leaves (e.g. 3.1.b.Table 2).

These assays (Figs.17-21) compared favourably with the results obtained by sap spot hybridisation (Fig.25) which, for a ^{32}P-labelled probe, detected to only 10^{-2} x dilution for all samples, and for a biotinylated probe, to 10^{-2} dilution for PVX-infected sap (strain-group 3, PBI) and 10^{-1} for all other samples (Fig.27). In Fig. 18, the results show an increase in sensitivity with increased incubation time and that, in certain instances, the ELISA was found to be between 2 to 3 logs more sensitive than the cDNA probes in the dot-blot assays. For example, all samples can be clearly detected at 10^{-3} x dilution but only PVB- and PVX N-, and PVX (strain-Group 3)-infected sap samples at 10^{-4} x dilution (compared with 10^{-2} x dilution with a ^{32}P-labelled probe and 10^{-1} x dilution for a biotinylated probe). PVX (strain-Group 3)-infected sap could still be clearly detected at 10^{-6} x dilution (but drops off sharply after this dilution).

The results, obtained by ELISA after a 2 h incubation (Fig.20), for purified virl particles had similar absorption values at the highest and lowest concentrations (2 µg and 20 pg). However, a wide range of readings was observed for the different isolates between 20 ng and 200 pg. Of these, PVB (strain-group 2) gave the highest readings, followed by PVX (strain-Group 3, PBI) and PVX N. These results were similar for

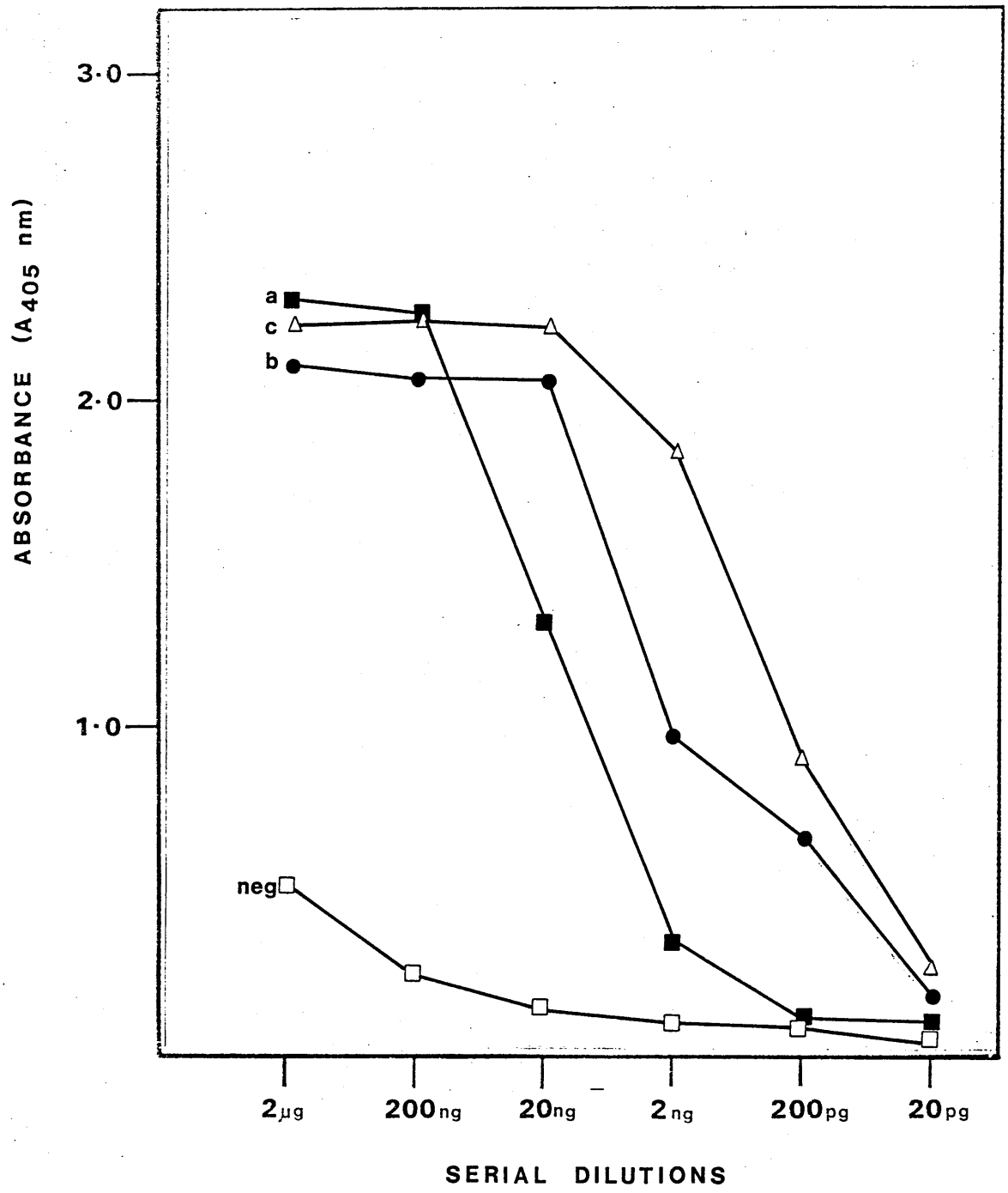


Fig.20: ELISA, 2 h incubation showing serial dilutions of viral particles (2 µg to 20 pg): 2a) PVX N (Sample 1)(■); 2b) PVX (Gp 3, PBI)(●); 2c) PVB (Gp 2, PBI) (Δ). A negative control (□) for PVX (BRL) was included.

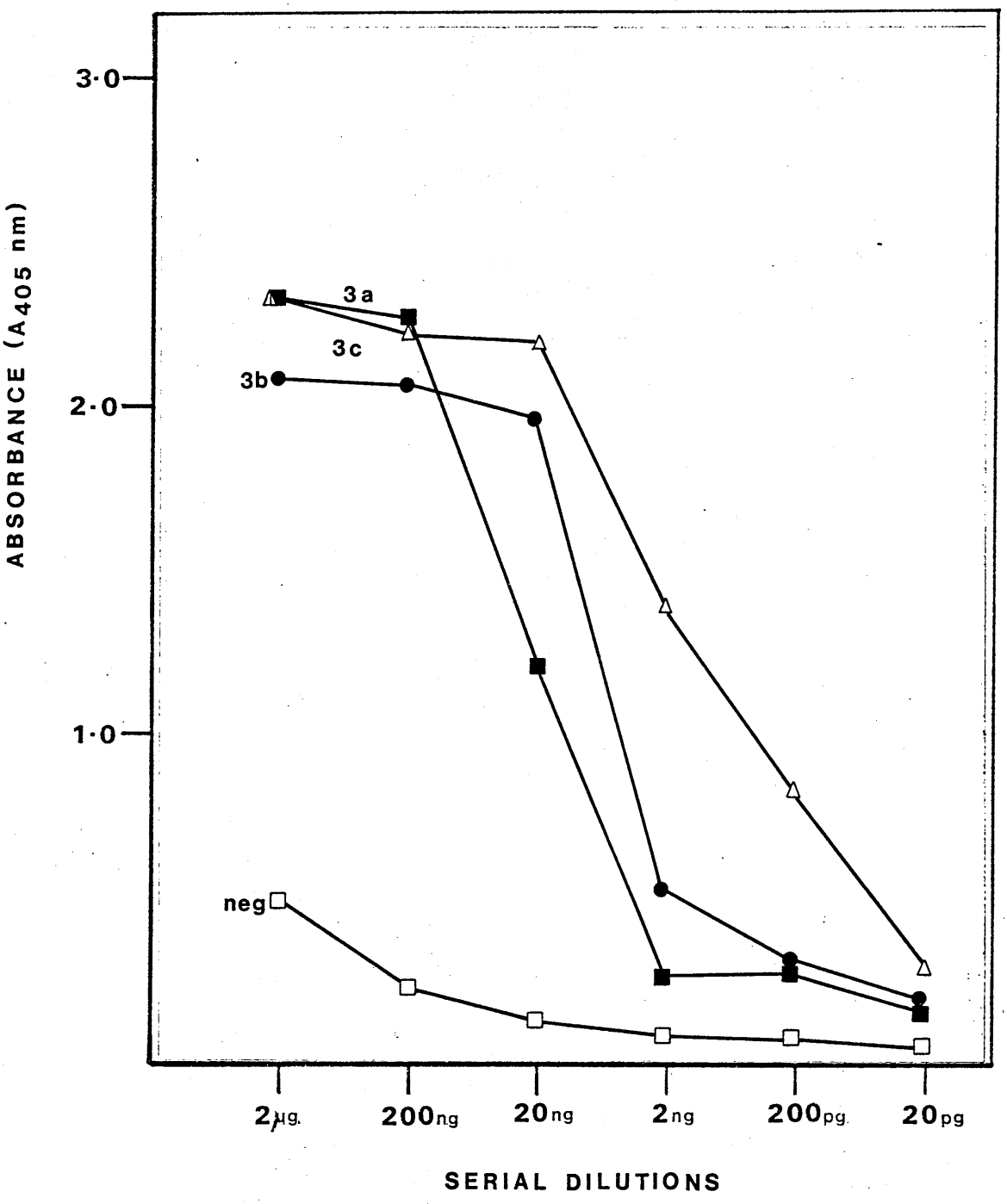


Fig.21: ELISA, 5 h incubation, showing serial dilutions of viral particles (2 µg to 20 pg): 3a) PVX N (Sample 1)(■); 3b) PVX (Gp 3, PBI)(●); 3c) PVB (Gp 2, PBI) (Δ). A negative control (□) for PVX (BRL) was included. Results were determined spectrophotometrically at A_{405} nm after 5 h incubation with substrate.

the 5 h incubation (Fig.21). As before, the baseline was twice the value of the Boehringer Mannheim negative control.

In all three samples (PVX N, PVX and PVB), the spectrophotometric values of each sample at 200 pg and at 20 pg were greater than twice that of the baseline and can be taken as positives, therefore, setting the sensitivity of the enzyme-linked immunosorbent assay for purified virus particles at between 200-20 pg for both strains (2 and 3). This sensitivity was far better than that observed for the sap spot assays (Fig.30) which detected purified viral particles to 20 ng.

An interesting reversal was observed for PVB (strain-group 2) where the spectrophotometric values for the crude sap assays at 2 and 5 h were similar to that of PVX (strain-group 3, PBI) samples. However, for the purified viral particles assays, PVB constantly gave higher readings than either of the PVX isolates. A similar reversal was also noted with PVB detected by ELISA and dot blot. In the ELISA, purified PVB could be detected with the same or greater sensitivity than the other isolates. However, with the dot-blot assay, the detection of purified PVB was consistently lower than the isolates.

When these assays for both the crude sap samples and purified viral particles (stored at -70°C) were repeated, these results were reproduced. The duplicate assays produced the same sensitivity of detection for the isolates, with high absorbance values for PVX (strain-Group 3, PBI) to 10^{-6} in the crude sap sample and gradually decreasing values for the purified viral particles for all isolates.

3.3. Sap Spot Assays with Radiolabelled and Biotinylated Probes.

Two identical filters, containing PVX N and BMV RNAs, were hybridised with either a oligo d(T)-primed radiolabelled or biotinylated probe and the sensitivities of the probes then compared. The results, shown in Fig.21, showed that both the probes detected PVX N RNA to 200 pg, equivalent to 4.3 ng (although the radiolabelled probe was more than 5 x the concentration of the biotinylated probe). The signals for the radiolabelled probe were also easier to visualise since the imprints on the X-ray film increased correspondingly with time. However, unlike the biotinylated probe, the radiolabelled probe also non-specifically hybridised with the BMV RNA.

In Fig.23, a sap spot assay was carried out to compare the detection of PVX N sap and PVX N RNA, bound to a nitrocellulose filter and hybridised with a calf thymus-primed cDNA probe at 100 ng/ml hybridisation solution. A BMV RNA control was also included to determine the level of non-specific hybridisation with the probe. The results (Fig.23) indicated that the calf thymus primer was comparable to the oligo d(T)-primed cDNA probe (Fig.22) even though the probe concentration was less (at 100 ng/ml) and the hybridisation time reduced to 4 h. Some non-specific hybridisation was still detected with the BMV RNA but the probe detected the the infected sap sample to 10^{-4} x dilution and purified PVX N RNA to 2 ng.

As a comparison to the calf thymus-primed probe, an oligo d(T)-primed cDNA probe (also at 100 ng/ml) was used to hybridise PVX N/PVX and PVB-infected sap samples as well as PVX N and BMV RNAs. The results (Fig.24) show that the oligo d(T)-primed probe was far less sensitive in detecting RNA in the crude sap samples than the calf thymus-primed probe (10^{-1} x dilution instead of 10^{-4} x dilution). The detection of purified PVX N RNA was comparable to results from the calf thymus-primed probes (2 ng) although the signal at 2 ng were much

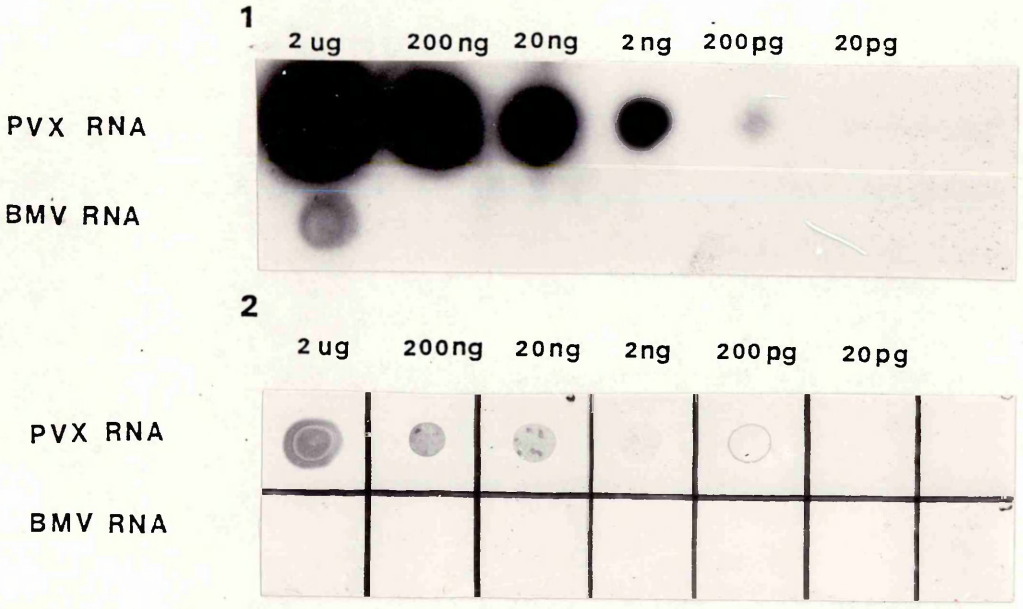
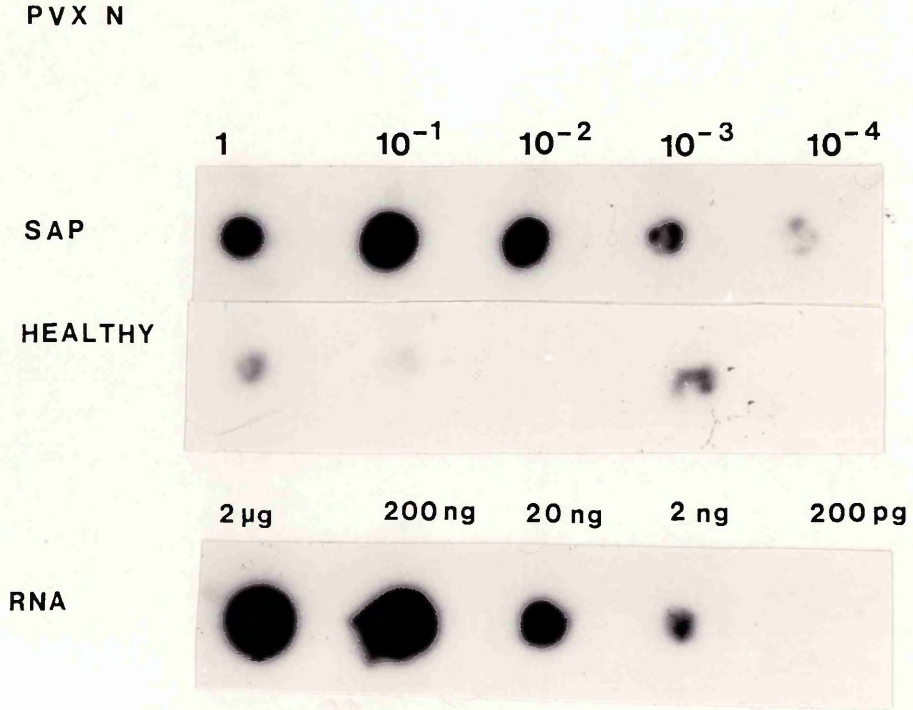


Fig.22: Comparison of two identical filters which have been hybridised with either a radiolabelled or biotinylated probe: Filter 1 has been hybridised with an oligo d(T)-primed ^{32}P-labelled probe (560 ng/ml hybridisation solution, between 1-6 kbp in size and a specific activity of 1.0×10^8 per µg DNA) while filter 2 was hybridised with a biotinylated probe (100 ng/ml hybridisation solution). Both probes were synthesised from PVX N RNA templates and the filters were hybridised for 16 h and exposed for either 4 h (^{32}P probe) or developed for 4 h (biotin probe). The filters show serial dilutions of a) PVX N RNA (2 µg to 20 pg) and b) BMV RNA control (2 µg to 20 pg).

Fig.23. Autoradiograph of a dot-blot assay on nitrocellulose using a calf thymus-primed cDNA ^{32}P-labelled probe from PVX N RNA: In order to compare the sensitivity of the radiolabelled probe for PVX-infected sap and PVX N RNA samples, a series of 10-fold dilutions were spotted and hybridised with a calf thymus-primed ^{32}P-labelled probe. The specific activity of the probe was similar to that in Fig.22. The filter shows serial dilutions of 1) PVX N-infected sap (undiluted to 10^{-4} x dilution); 2) PVX N RNA (2 µg to 200 pg); and 3) uninfected healthy sap (undiluted to 10^{-4} x dilution). The filter was prehybridised for 3 h at 65°C and hybridised for 4 h with 2.1 x 10^{6} cpm, then exposed to X-ray film for 4 h at -70°C. Some non-specific background hybridisation was also observed (spot 4, healthy sap).



weaker.

This oligo d(T)-primed probe also detected PVB-infected sap (strain-Group 2, PBI) to a lesser degree (visible only in the undiluted sample) indicating a greater specificity for strain-Group 3 isolates. These early results questioned the usefulness of using radiolabelled probes for the detection of different strains in screening large numbers of samples. In the dot-blot assay, the problem appeared to be the low levels of cross-hybridisation with healthy sap and BMV RNA. This cross-hybridisation of the PVX N RNA probe with BMV RNA and healthy sap was effectively blocked by pre-hybridising the filter with poly (U) (Fig.24: lanes 5 and 7).

A second hybridisation, using the oligo d(T)-primed probe (at 100 ng/ml) with a duplicate filter to the one used in Fig.24, was repeated but extending the exposure time of the X-ray film to 16 h from 4 h (Fig.25). The increased exposure time increased the sensitivity of detection of the probe for the PVX/PVB sap samples (detecting most samples at 10^{-2} x dilution compared to 10^{-1} x dilution at the shorter exposure time). This was even the case with the PVB-infected sap sample.

The improvement in the sensitivity of detection did not extend as far with the serial dilutions of PVX N RNA. The only improvement observed was a stronger signal at the 2 ng dilution although a faint signal could be seen at 200 pg, indicating that the sensitivity of detection could be improved with increased exposure time.

The effect of increasing the exposure time is also shown in Fig.26. The nitrocellulose filters, containing serial dilutions of just PVX N and BMV RNAs, were hybridised with a calf thymus-primed probe (at 560 ng/ml), then the film exposed for 20 min and 16 h respectively. With the shorter exposure time, the probe detected PVX N RNA to 20 ng. The

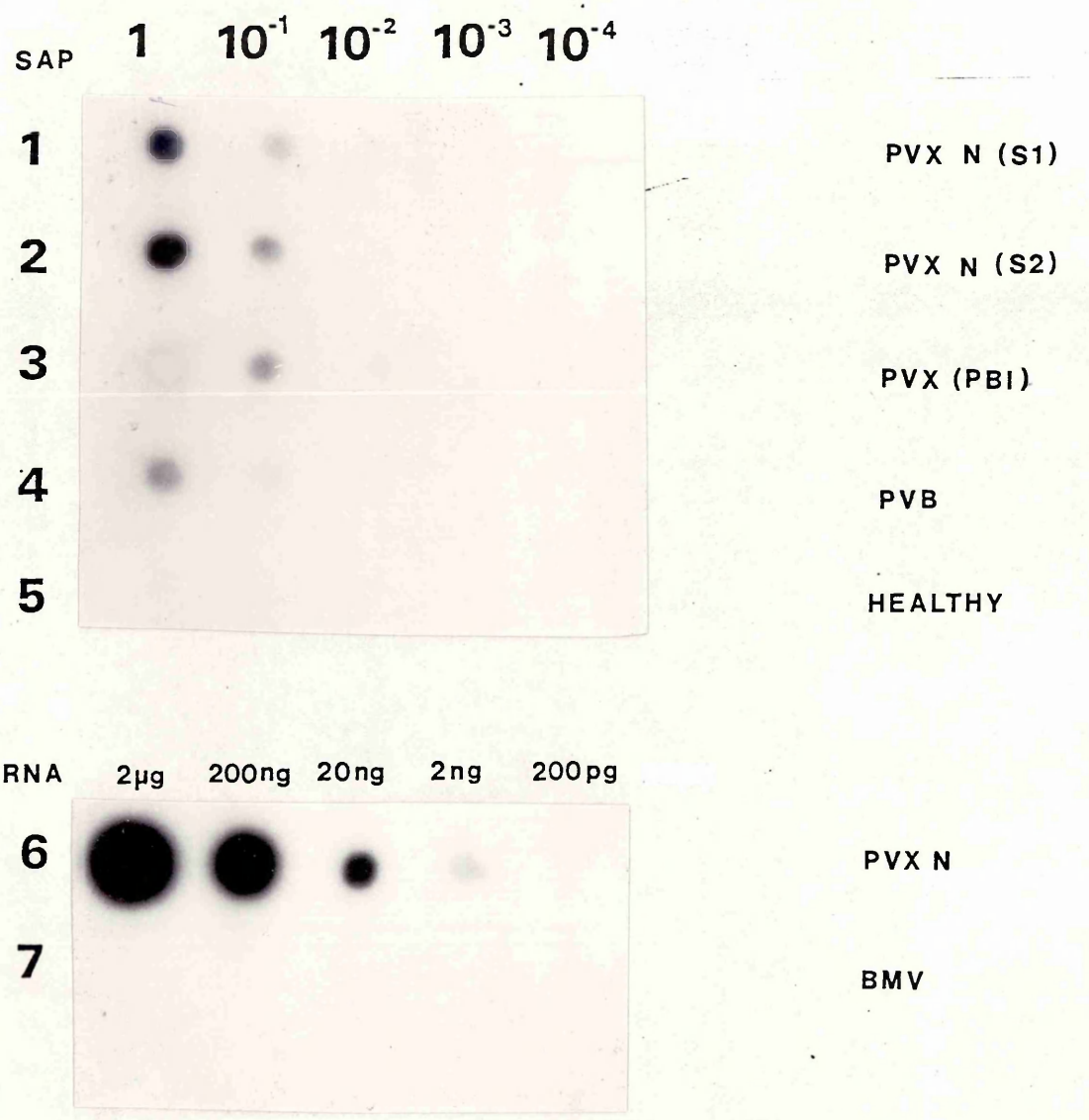


Fig.24: Autoradiograph of a dot blot assay on nitrocellulose using an oligo d(T)-primed ^{32}P-labelled cDNA probe (100 ng/ml hybridisation solution). The filter was prehybridised for 3 h with poly (U) to remove non-specific hybridisation, hybridised at 55°C for 2 h, and shows serial dilutions of (1) PVX N infected sap (sample 1); (2) PVX N infected sap (sample 2); (3) PVX infected sap (strain-group 3, PBI); (4) PVB-infected sap (strain-group 2, PBI); (5) Uninfected, healthy sap; (6) Purified PVX N RNA (2 µg to 200 pg); (7) Purified BMV RNA control (2 µg to 200 pg). The X-ray film was exposed for 4 h at -70°C before developing. This filter can be compared with a duplicate filter, hybridised with a similar oligo d(T)-primed probe but with an exposure time of 16 h.

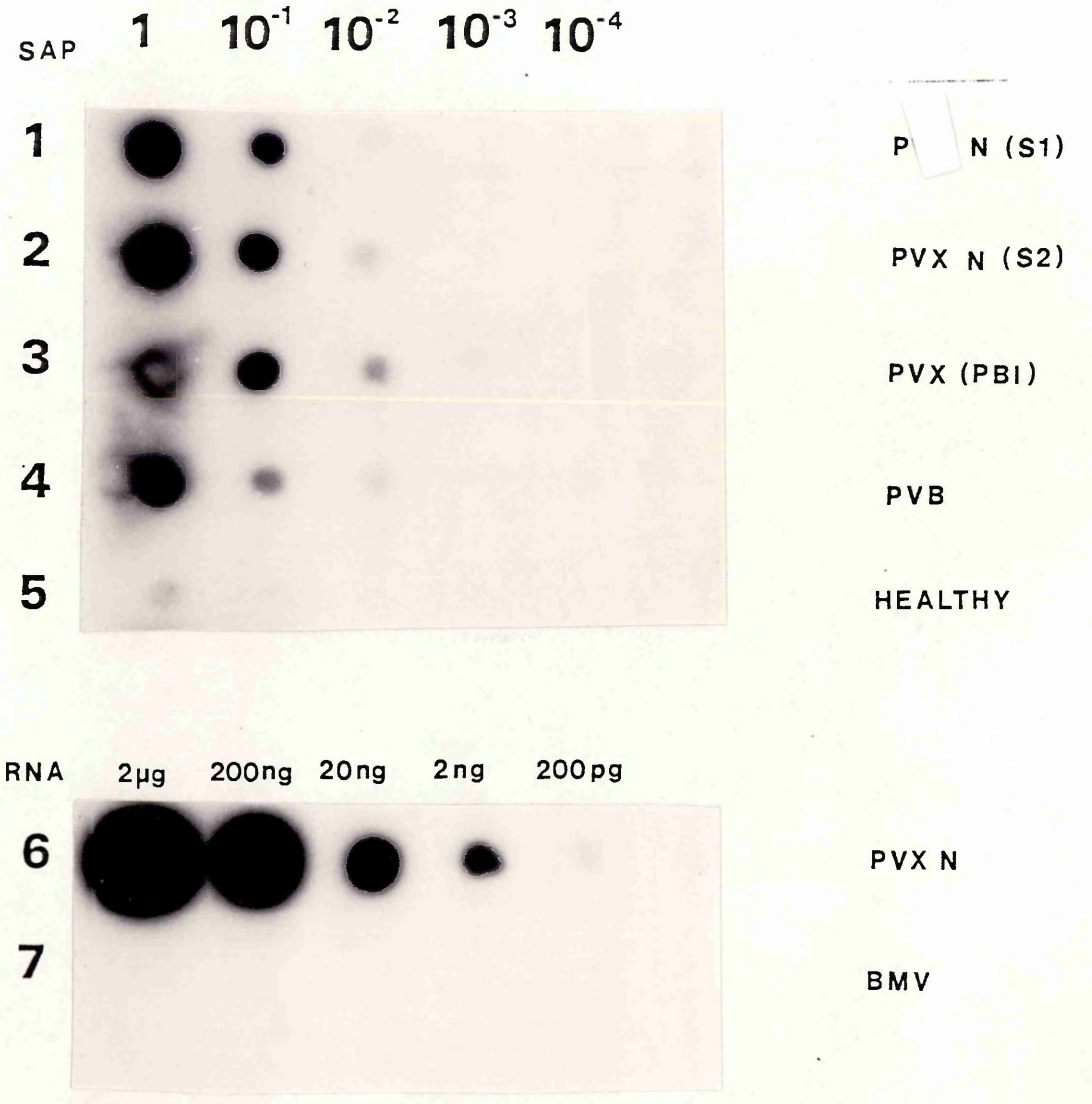


Fig.25: Sap spot assay, showing serial dilutions of PVX sap and RNA bound to a nitrocellulose filter and hybridised with a ^{32}P-labelled cDNA probe at 100 ng/ml hybridisation solution. The filter shows serial dilutions of (1) PVX N infected sap (sample 1); (2) PVX N infected sp (sample 2); (3) PVX infected sap (strain-group 3, PBI); (4) PVB-infected sap (strain-group 2, PBI); (5) Uninfected, healthy sap; (6) Purified PVX N RNA (2 µg to 200 pg); (7) Purified BMV RNA control (2 µg to 200 pg). Poly U (0.5 mg) was added to the prehybridisation solution to reduce non-specific hybridisation. The film was exposed for 16 h at -70°C before developing.

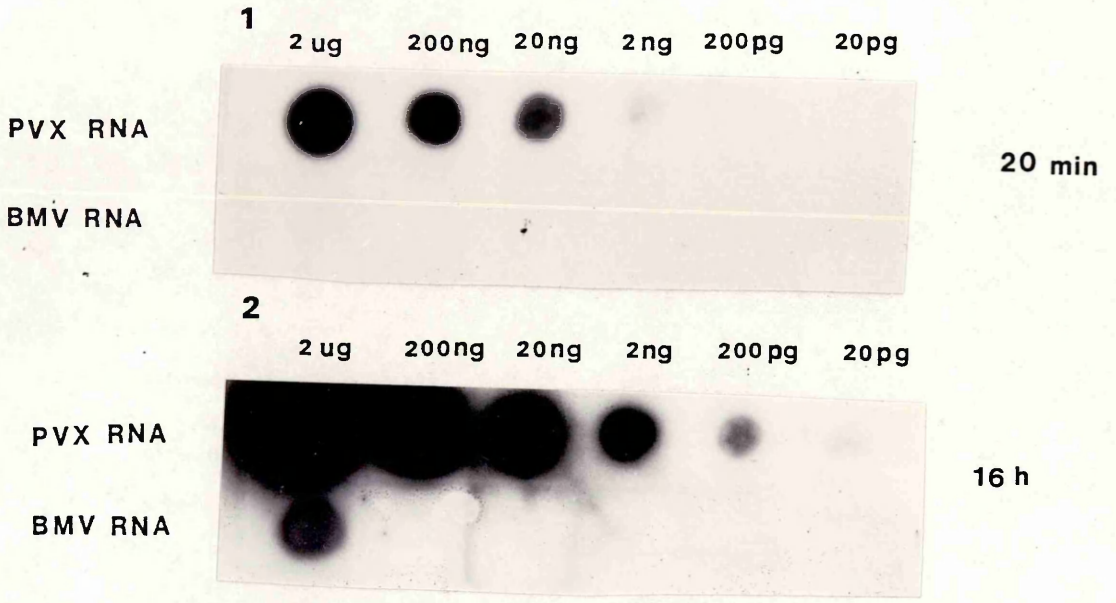


Fig.26: Autoradiographs showing the effect of increased time on the detection of PVX N RNA on nitrocellulose filters hybridised with ^{32}P-labelled cDNA probes. The filter was hybridised for 16 h with a calf thymus-primed ^{32}P-labelled cDNA probe (560 ng/ml hybridisation solution) and exposed to X-ray film for (1) 20 min or (2) 16 h. BMV RNA was also hybridised under the same conditions as the control.

sensitivity of detection increased to 200 pg (a 2 log increase) with the longer exposure time. The cross-hybridisation of the ^{32}P-labelled probe with the BMV RNA control only showed up in the 2 µg position in the 16 h exposed film and not all in the 20 min exposed film.

Filters were also hybridised with biotinylated cDNA probes. It was suggested that, for reasons of economy and the absence of an inherent half-life (unlike the radiolabelled probe), the biotinylated probe could be re-used a second and, possibly, a third time. The hybridisation was, therefore, repeated using duplicate filters with an extended incubation time for the third hybridisation (Figs.28 and 29).

In the first hybridisation (Fig.26), the infected samples from PVX N (samples 1 and 2) and PVB (strain-Group 2, PBI) were detected to 10^{-1} x dilution and PVX (strain-Group 3, PBI) to 10^{-2} x dilution with the biotinylated probe. No cross-hybridisation occurred with the healthy sap. The probe also detected PVX N RNA to 200 ng in the first hybridisation, equivalent to 40 µg virus particles. With a duplicate filter, and using the same probe at 100 ng/ml (stored for 2 months at -20°C) for a second hybridisation, the results were poorer. The positive signals on the filter was much weaker (Fig.28). The infected sap samples were detected to 10^{-1} x dilution with an increase in non-specific hybridisation.

In the third hybridisation (Fig.29), the results were similar to those of Fig.28 for the infected sap samples. The detection of PVX N RNA was hardly visible, having been greatly reduced after the first hybridisation. However, in all three cases, no cross-hybridisation occurred with either the healthy sap or BMV RNA controls.

In an alternative assay, a nitrocellulose filter was spotted with PVX/PVB-infected sap, purified PVX viral particles, and PVX N RNA and hybridised with a biotinylated probe at 30 ng/ml for 16 h (compared to

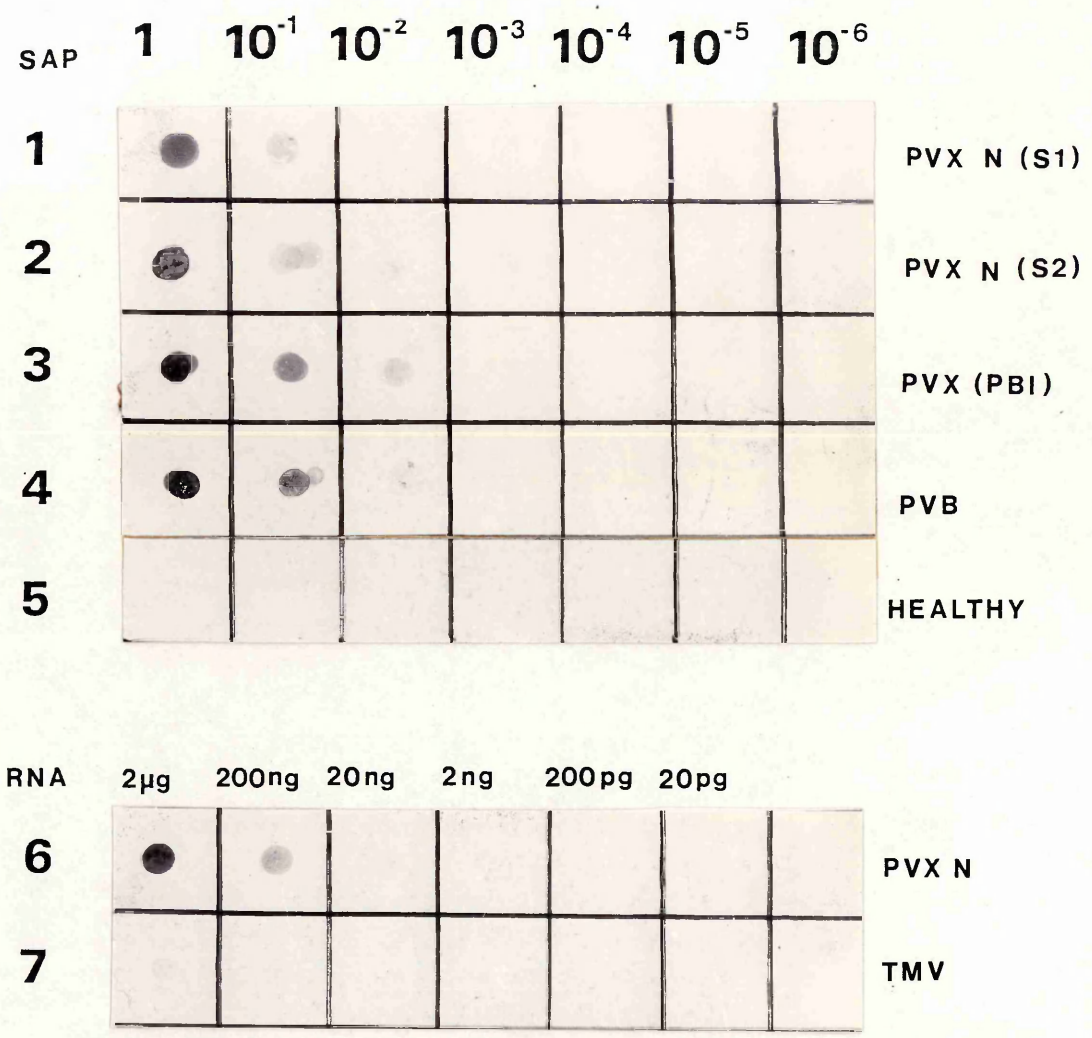


Fig.27: Bioblot of PVX-infected sap and RNA on nitrocellulose hybridised for 2 h with a biotinylated probe (100 ng/ml). The filter shows 1) PVX N-infected sap (Sample 1), 2) PVX N-infected sap (Sample 2), 3) PVX-infected sap (strain-group 3, PBI), 4) PVB-infected sap (Gp 2, PBI), 5) sap from uninfected plant, 6) PVX RNA (2 µg to 2 pg), 7) TMV RNA (2 µg to 2 pg). All sap samples were spotted in a serial dilution from undiluted sap to 10^{-6} x dilution. The filter was prehybridised for 2 h at 42°C with 5 ug poly U, and hybridised with the biotin probe for 2 h at 42°C.

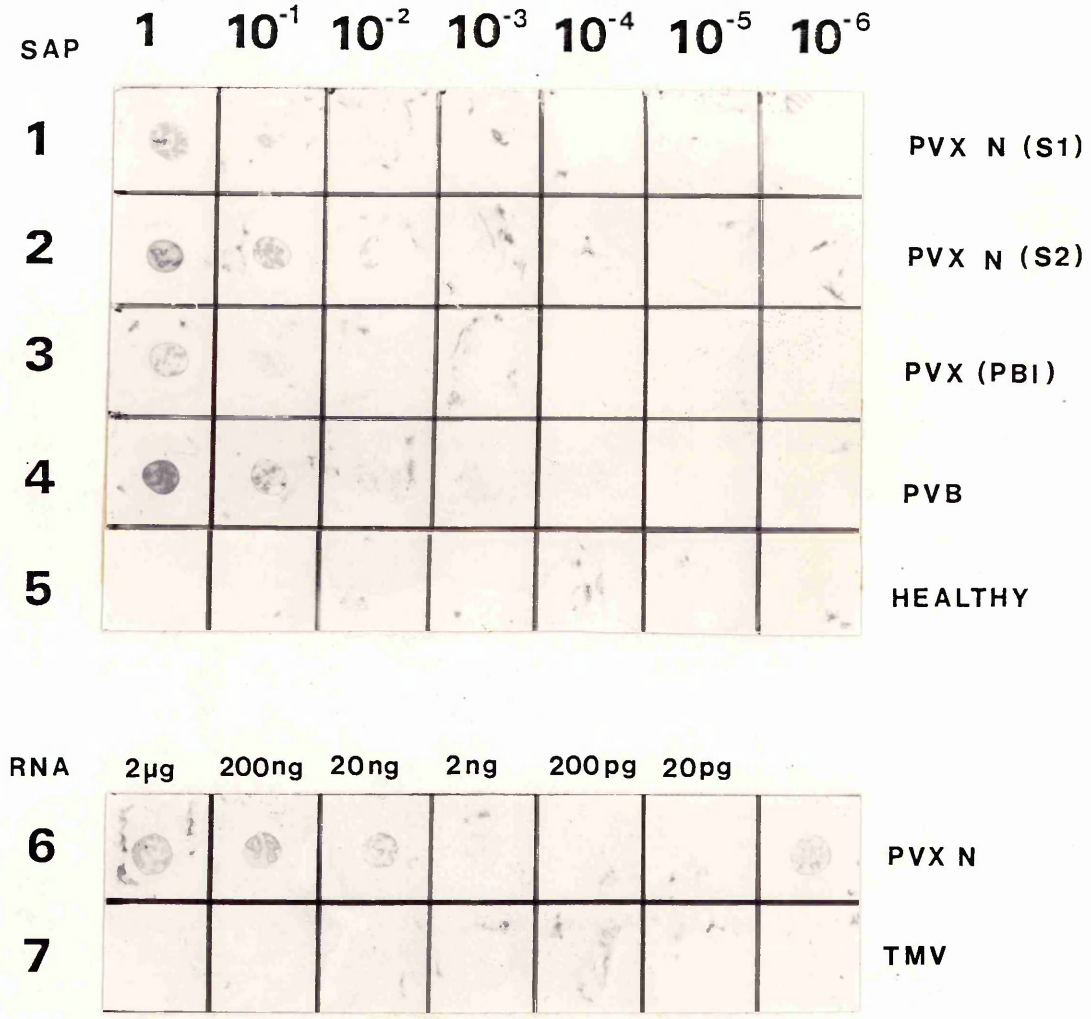


Fig.28: Sap spot assay of PVX-infected sap and RNA on nitrocellulose hybridised with a second passage biotinylated probe. The filter shows 1) PVX N-infected sap (Sample 1), 2) PVX N-infected sap (Sample 2), 3) PVX-infected sap (strain-group 3, PBI), 4) PVX-infected sap (strain-group 2, PBI), 5) sap from uninfected plant, 6) PVX RNA (2 g to 2 pg), 7) TMV RNA (2 g to 2 pg). All sap samples (in a serial dilution from undiluted sap to 10^{-6} x dilution) were spotted as two microlitre aliquots. The filter was prehybridised for 2 h at 42°C with 5 ug poly U, and hybridised with the used biotin probe (stored for 2 months at 4°C, original concentration 100 ng/ml).

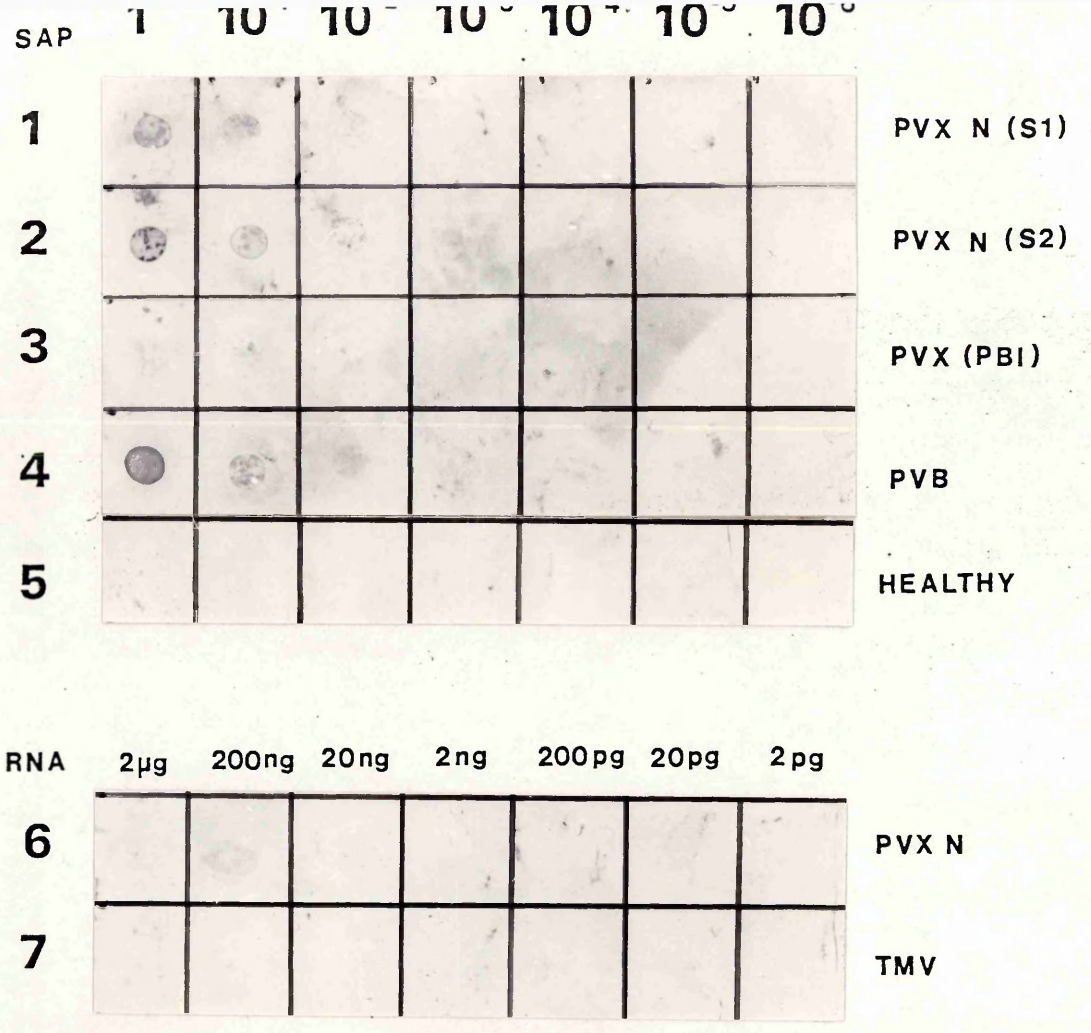


Fig.29: Sap spot assay of PVX-infected sap and RNA on nitrocellulose hybridised with a third passage biotinylated probe. The filter shows 1) PVX N-infected sap (Sample 1), 2) PVX N-infected sap (Sample 2), 3) PVX-infected sap (strain-group 3, PBI), 4) PVB-infected sap (strain-group 2, PBI), 5) sap from uninfected plant, 6) PVX RNA (2 µg to 2 pg), 7) TMV RNA (2 µg to 2 pg). All sap samples (in a serial dilution from undiluted sap to 10^{-6} x dilution) were spotted as two microlitre aliquots. The filter was prehybridised for 2 h at 42°C with 5 ug poly U, and hybridised with the twice-used biotin probe (stored for 2 months at 4°C, original concentration 100 ng/ml).

560 ng/ml previously), then incubated for 16 h with the substrates. All the isolates, except for PVX (strain-Group 3, PBI), reacted weakly with the probe to 10^{-1} x dilution on the filter (Fig.30). The PVB-infected sap (strain-Group 2, PBI) and PVX N (sample 2) were only detected in the undiluted samples. Under these conditions, the assay gave poor discrimination between healthy and PVB-infected plants and some non-specific hybridisation was observed for the healthy sap in the undiluted sample.

The signals were much stronger for the purified sap and the PVX N RNA than for crude sap, detecting the RNA in the sap samples to 20 ng (spot 3) and to 2 ng (spot 4) for the PVX N RNA. The dilutions of purified viral particles (2 µg to 20 pg), spotted on to the filter, were based on the concentration of particles and not on the RNA inside. The probe did not cross react with TMV RNA.

In a similar comparison of sap, viral particles, and RNA spots in a dot-blot assay, the nitrocellulose filter was probed with a calf thymus-primed ^{32}P-labelled cDNA probe (at 1.7 ng/ml hybridisation solution)(Fig.31). The low concentration of the probe resulted from poor incorporation of the radioisotope during cDNA synthesis. Even so, the results corresponded with those of the biotinylated probe at a higher concentraation of probe. In comparing the two probes, the PVB viral particals were detected less than the PVX isolates by 1 log from both the radilabelled and biotinylated probes. However, PVX N RNA was detected to 200 pg with this low concentration ^{32}P-labelled probe compared to the biotinylated probe which detected only 2 ng. In both cases, no cross-hybridisation with the BMV control were observed.

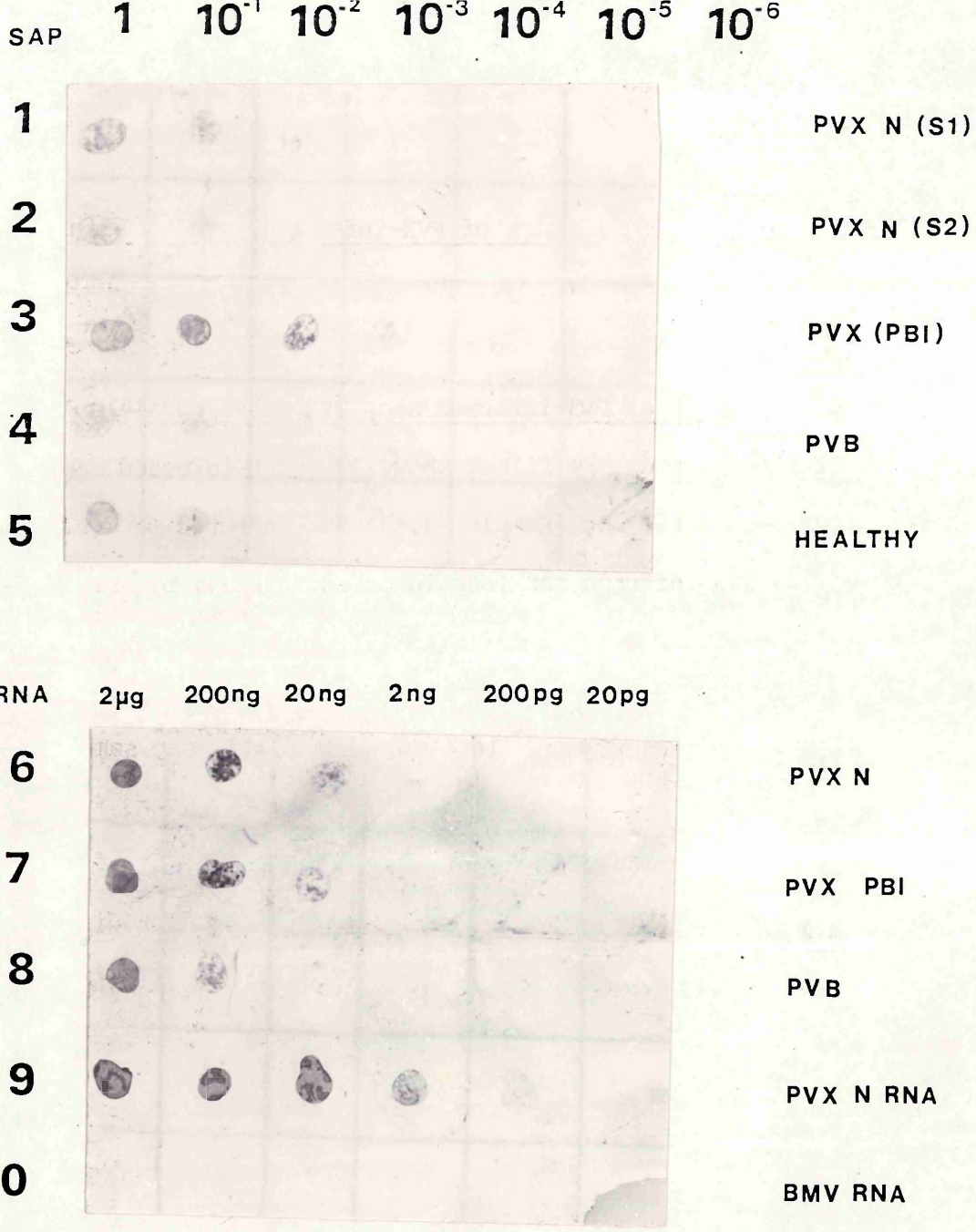
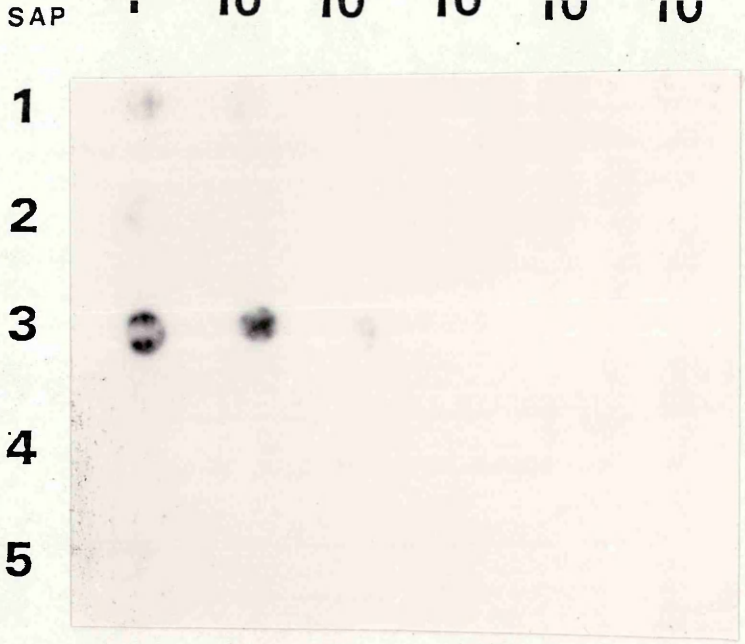

SAP
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
1
2
3
4
5
PVX N (S1)
PVX N (S2)
PVX (PBI)
PVB
HEALTHY
VP/RNA
2µg
200ng
20ng
2ng
200pg
20pg
6
7
8
9
10
PVX N
PVX PBI
PVB
PVX N RNA
BMV RNA

Fig.30: Bioblot of PVX-infected sap, PVX viral particles, and PVX RNA on nitrocellulose. The filter shows 1) PVX N-infected sap (Sample 1), 2) PVX N-infected sap (Sample 2), 3) PVX-infected sap (strain-group 3, PBI), 4) PVB-infected sap (strain-group 2, PBI), 5) uninfected sap from a healthy plant, 6) purified viral particles PVX N (Sample 1), 7) PVX viral particles (Gp 3, PBI), 8) PVB viral particles (Gp 2, PBI), 9) PVX N RNA, 10) BMV RNA. All sap samples were spotted as 2 µl aliquots in a serial dilution from undiluted sap to 10^{-6} x dilution. Purified viral particles and RNAs were spotted on in a serial dilution from 2 µg to 20 pg (the serial dilutions were of the viral particles and not of the RNA within the particles). The filter was prehybridised for 4 h with 5 µg poly U and hybridised with a biotinylated probe (at 30 ng/ml) for 16 h and reacted with substrate for colour development for 16 h. The incubation and colour development times were increased accordingly because of the reduced probe concentration (30 ng/ml instead of the original 100 ng/ml).


SAP
1
2
3
4
5
PVX N (S1)
PVX N (S2)
PVX (PBI)
PVB
HEALTHY

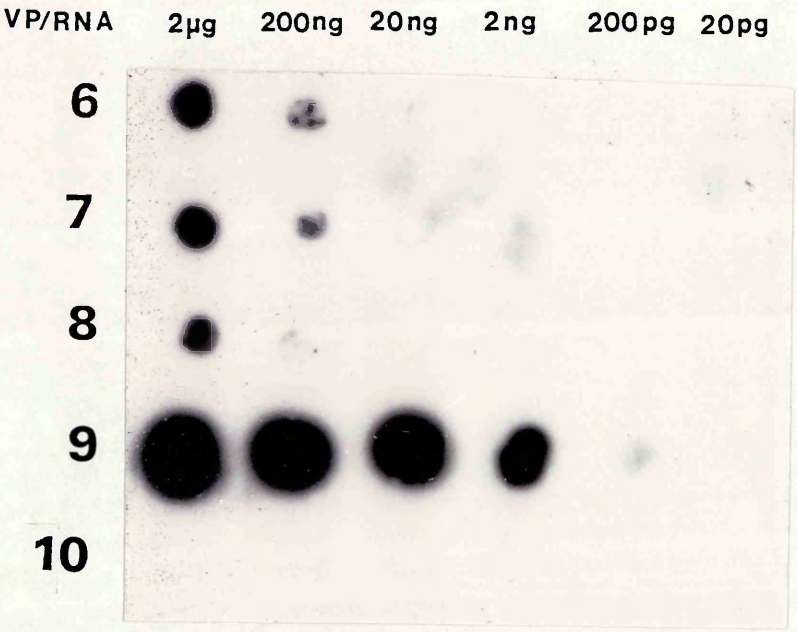

VP/RNA
2µg
200ng
20ng
2ng
200pg
20pg
6
7
8
9
10
PVX N
PVX PBI
PVB
PVX N RNA
BMV RNA

Fig.31: An alternative radiolabelled probe comparing sap, viral particle, and RNA spots in a dot blot assay, probed with calf thymus primed ^{32}P-labelled cDNA probe (1.7 ng/ml hybridisation solution). The filter shows serial dilutions of (1) PVX N infected sap (Sample 1); (2) PVX N infected sap (Sample 2); (3) PVX infected sap (strain-group 3, PBI); (4) PVB infected sap (strain-group 2, PBI); (5) Uninfected, healthy sap; (6) purified PVX N viral particles (Sample 1); (7) purified PVX viral particles (Group 3, PBI); (8) purified PVB viral particles (Group 2, PBI); (9) PVX N RNA (2 µg to 20 pg); (10) BMV RNA control (2 µg to 20 pg). The filter was prehybridised for 4 h at 65°C and hybridised with the ^{32}P-labelled probe and poly U (5 mg) for 16 h at 65°C. The low concentration of probe was not planned but resulted from the synthesis of lower than expected amounts of ^{32}P-labelled cDNA.

Summary of Optimal Conditions for the Assays.

1) The Enzyme-linked Immunosorbent Assay.

a) Crude sap: The detection of viral particle in crude sap samples by ELISA varied and was dependent on both the incubation time and the isolate. Of the three sets of incubations (1, 2, and 5 h), an increase of detection was noted with increased incubation time for all isolates except for the PVX (strain-Group 3, PBI) samples. With PVX (strain-Group 3, PBI), viral particles were detected, in repeated assays, to 10^{-6} x dilution with a 5 h incubation. With the other isolates (PVX N, samples 1 and 2) and PVB (strain-Group 2, PBI), the sensitivity of detection for all isolates increased to between 10^{-4} and 10^{-5} x dilution from 10^{-3} x dilution in a 1 h incubation. The PVB isolate was detected equally well as the PVX N samples. The ELISA, therefore, gave the most sensitive results in a 5 h incubation detecting all isolates (from both strains) to at least 10^{-5} x dilution.

b) Purified viral particles: The ELISA detected purified viral particles (from both strains) to the same sensitivity, i.e. between 20-200 pg. The level of sensitivity was similar between the 2 and 5 h incubations, both of which were better than the 1 h incubation. The optimal conditions for the detection of viral particles was, therefore, a two hour incubation with substrate, capable of detecting PVX N (strain-Group 3), PVX (strain-Group 3, PBI) and PVB (strain-Group 2, PBI) to between 20-200 pg. The use of polyclonal antibodies in the ELISA, therefore, allowed the detection of more than one strain of the virus.

2) Dot-Blot Assays.

a) Radiolabelled probes: Complementary DNA probes (synthesised from PVX N RNA) templates) were used to detect RNA in various PVX/PVB isolates during hybridisation experiments. Both oligo d(T)- and calf thymus-primed probes were used, ranging in size from 600 to about 6,000 bases.

Under the optimal conditions for the detection of RNA in crude sap samples by the oligo d(T)-primed probes detected the PVB and two of the three PVX isolates (except for PVX N sample 1) to 10^{-2} x dilution. These results, using a probe at 100 ng/ml (1.0×10^{8} dpm/ml) and a 16 h exposure, were a log better than the results of the same probe with a 4 h exposure. It was likely that a further increase in the exposure time of the filter to the X-ray film would have improved the signals and improved the sensitivity of the detection.

A similar probe, synthesised from calf-thymus-primed PVX N RNA, detected the PVX N isolate to 10^{-4} x dilution with a 4 h exposure. Compared to the oligo d(T)-primed probe in a 4 h hybridisation and 4 h exposure, this was a 3 log increase in sensitivity of detection. This was due to a higher probable complexity of the calf thymus-primed probe compared to the oligo d(T)-primed probe which is restricted to the 3' sequence.

In the detection of purified PVX N RNA, both the probes detected the RNA to 2 ng with a 4 h exposure to film.

Therefore, this comparison between the two probes at 100 ng/ml with a 4 h hybridisation and 4 h exposure for crude sap samples proved that the calf thymus-primed probe was the more sensitive. The sensitivities of the probes were also increased when the exposure time was extended, e.g. from 4 h to 16 h. With the extended exposure time, an improvement

of 1 log for the crude sap samples and to 200 pg for purified PVX N RNA, was noted for both probes (calf thymus- and oligo d(T)-primed). From this, it was concluded that optimal conditions for dot-blot hybridisations with radiolabelled probes would involve a highly concentrated probe (at 500 ng/ml or more) in a 4 h hybridisation and a 16 h or greater exposure.

b) Biotinylated probes: The optimal conditions for hybridisations with biotinylated probes were also assessed. In the detection of RNA in crude sap samples of the two strains, a biotinylated probe at 100 ng/ml in a 4 h hybridisation and 4 h colour development (synthesised from a PVX N RNA template) detected PVX (strain-Group 3, PBI) to 10^{-2} x dilution and to 10^{-1} x dilution for all other isolates including PVB (strain-Group 2, PBI). A similar probe but at a lower concentration (30 ng/ml) and in a 16 h hybridisation, detected the samples to the same degree although the signals were much weaker. Non-specific hybridisation was also more pronounced for the healthy sap sample.

For the detection of PVX N RNA, the same probe detected RNA to 200 pg in a 4 h hybridisation and 4 h colour development, comparable to the radiolabelled probes.

c) Choice of the best assay: In the choice of the best assay, several criteria have to be considered. These include the speed of the assay; the possibility for detection of a large number of samples; the ease of detecting more than one strain; detecting infectious particles; and the ease of handling. The ELISA was very sensitive for crude sap samples, detecting virus particles in all isolates to at least 10^{-4} x dilution in a 5 h incubation. The advantages of this assay include the ease of handling large numbers of samples, and the capability of

detecting purified viral particles of both strains (Groups 2 and 3) equally well. The results can be obtained within a day. However, varying amounts of the viral antigen from the leaf extracts may give extremely high readings which are not representative of the infectivity of the particles, e.g. PVX (strain-Group 3, PBI) which could be detected to 10^{-6} x dilution but which, in the dot-blot assays, was detected to the same sensitivity as the other isolates.

Therefore, despite some of the disadvantages of the dot-blot assays such as the use of radiolabelled isotopes with the need for containment facilities and its natural decay, the dot blot assay was considered more representative of the infective nature of the virus.

Within the dot-blot assay, the radiolabelled probes were compared with the non-radioactive alternative, the interaction of biotin and streptavidin using an enzyme as the detection system. Despite the fact that the biotinylated probe was non-hazardous, relatively easy to set up, and could be stored for periods of time which would be normally too long for ^{32}P-labelled probes, the eventual results showed that the radiolabelled probes produced clearer signals on autoradiograms than comparative filters hybridised with biotinylated probes. In addition, the sensitivity of the radiolabelled probe could be increased with an increase in the exposure time on X-ray film. The use of a calf thymus-primed probe, at 100 ng/ml in a 4 h hybridisation and 4 h exposure, detected PVX N RNA in crude sap samples to 10^{-4} x dilution, a comparable result to those obtained by the ELISA.

In addition, the radiolabelled probes were also capable of detecting both PVX (strain-Group 3, PBI) and PVB (strain-Group 2, PBI) RNAs in crude sap samples and as purified viral particle with equal sensivity. In conclusion, therefore, the radiolabelled complementary DNA probe (primed by calf thymus DNA) was chosen as the overall best

assay, able to detect RNA in crude sap samples to 10^{-4} x dilution depending on the exposure time, with a sensitivity of 20 ng for the purified viral particles and 200 pg for purified PVX N RNA, a sensitivity which may be improved with increased exposure time.

A comparison of two probes, a radiolabelled and a biotinylated probe, in the detection of PVX N RNA in crude sp samples.

Crude sap was collected from 20 PVX N-infected Nicotianatabacum plants. Two microlitre aliquots of each sample was spotted on to 2 sheets of nitrocellulose filters (samples 1-13 and 15-21). An additional sap spot from a healthy, uninfected plant was included in each filter (spot 14) as control.

In the first filter (Fig.32), the filter was hybridised with a calf thymus-primed, ^{32}P-labelled cDNA probe (560 ng/ml hybridisation solution) for 16 h. The filter was exposed for increasing periods of time in order to determine if the extended exposures increased the sensitivity (Filters 1, 2, and 3).

The second, duplicate filter (Fig.33) was hybridised with a biotinylated probe (100 ng/ml hybridisation solution)for 16 h and develped as previously described for 4 h. The "exposure" time of the filter was equivalent to filter 2 of the ^{32}P-labelled probe. Extension of the colour development time does not improve the sensitivity of the probe, and maximum colour development is usually achieved by 4 h as previously described.

With the radiolabelled probe, the three filters show that the strongest signals with a 4 h exposure were sap spots 7 and 9. However, with a longer exposure (16 h), signals from the remaining infected plants started to appear. No cross-hybridisation with the healthy sap sample occurred.

With the biotinylated probe, most of the sap spots appeared to show up quite strongly. However, there was a

exposure

2. 4h exposure

3. 16 h exposure

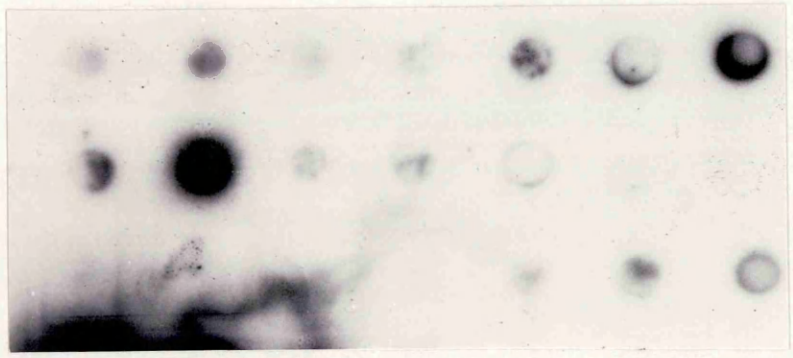


Fig 32: A comparison of the sensitivities of two probes: 1) radiolabelled and 2) biotinylated. The filter was hybridised with calf thymus-primed, ^{32}P-labelled cDNA probe (560 ng cDNA/ml hybridisation solution) for 16 h. The three autoradiographs show film which had been exposed to the filter for (1) 20 min; (2) 4 h; and (3) 16 h respectively. Sap from spot 9 was purified for future use as PVX N infected sap/RNA (sample 1), and from spot 7 as PVX N infected sap/RNA (sample 2).

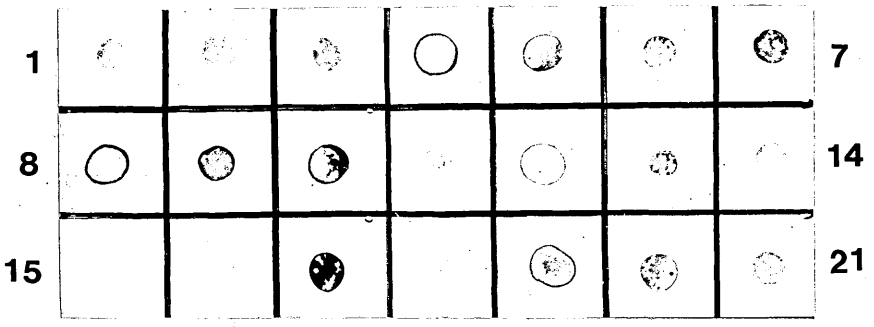


Fig.33: Nitrocellulose filter showing sap spots from 21 PVX-infected Nicotiana tabacum plants. The filter was hybridised with biotinylated cDNA (100 ng cDNA/ml hybridisation solution) for 4 h and developed as decribed.

high level of cross hybridisation with the healthy sap sample (Fig.33:sap spot 14) making identification of the infected samles difficult. The biotinylated probe appeared to be hybridising with the particulate matter in the samples. Since this is the sort of assay which might be expected to be used in screening programmes, the tendency for healthy sap to cross-hybridise at low dilution must cast doubt on the applicability of the assay.

The Synthesis of PVX N, PVX, and PVB complementary DNA from their Respective RNAs.

In the initial experiments, native PVX N RNA had polyadenylate residues added to the 3' terminus by the action of poly (A) polymerase, based on the reported data of Sonenberg et al. (1978).

In the experiments, in vitro polyadenylated PVX N RNA (Table 4:1) was used as a template for the synthesis of cDNA, in addition to native PVX N RNA and BMV RNA (Table 4:2 and 3), both of which were included as controls. Results from these experiments showed that native PVX N RNA was capable of synthesising cDNA without prior addition of poly (A) residues. This suggested that the native PVX N RNA was either already polyadenylated and of a sufficient length to initiate cDNA synthesis with an oligo d(T) primer, or that the RNA possessed a 3' tRNA-like hairpin structure which was also capable of priming a complementary DNA strand. The second possibility can be discounted with the absence of any synthesised cDNA when oligo d(T) was excluded from the reaction mix (Table 4:4).

Following these results, attempts at synthesising cDNA with native PVX (strain-Group 3, PBI) and PVB (strain-Group 2, PBI) RNAs were carried out. The results are shown in Table 5. In the case of all 3 strains of PVX, oligo d(T)-primed cDNA synthesis was significantly greater than that with TMV. All three isolates were able to initiate cDNA synthesis using oligo d(T) as primer without the addition of poly (A) tracts, although the best results were consistently obtained from PVX (strain-Group 3, PBI), followed by PVX N, and lastly by PVB (Table 5).

After the synthesised cDNAs had been purified (from the initial PVX N, PVX and PVB RNA templates), they were electrophoresed on a alkaline agarose gel (Fig.34). After electrophoresis, ^{32}P-labelled tracts were set up for autoradiography and exposed as described. The strongest

RNA Sample	Fractions containing ^{32}P-cDNA (total cpm)	Unincorporated ^{32}P nucleotides	% incorporation of nucleotides	Synthesized cDNA (g)	Product/RNA template ratio
1	5325	160382	3.32	43.8×10^{-6}	21.0%
2	13980	681133	2.05	27.1×10^{-6}	13.5%
3	935	157579	0.59	7.78×10^{-6}	3.8%
4	0	78999	0	0	0

Table 4: As part of the cloning experiments, the initial aim was to produce full length cDNA by the addition of polyadenylates to the RNA. As control, in vitro polyadenylated PVX N RNA RNA was included without primer. The table shows results of cDNA synthesis with RNA templates of native PVX, in vitro polyadenylated PVX, and BMV. Sample (1) contained in vitro polyadenylated PVX RNA with oligo d(T) as primer; (2) contained native PVX RNA, oligo d(T) primed; (3) BMV RNA with oligo d(T); (4) in vitro polyadenylated PVX RNA; no primer. Reactions were carried out at 42°C for 1.5 h and terminated with EDTA.

signal came from the PVX N (sample 2) isolate, followed by the PVX (strain-Group 3, PBI), the PVX N (sample 1), and PVB (strain-Group 2, PBI) isolates. The PVX N (sample 2) cDNA also appeared to be almost full-length (about 6.5 kb) but varying in size to less than 0.6 kbp. The PVX (strain-Group 3) and PVB also gave large products but these were shorter than those obtained with PVX N.

The synthesis of a complementary strand with PVB RNA as a template resulted in the lowest cDNA/RNA template ratio (Table 5:3) and correspondingly showed up on the gel as the weakest tract, as compared to the PVX isolates.

Table 5:

RNA Sample	Fractions containing ^{32}P-cDNA (total cpm)	Unincorporated ^{32}P nucleotides	% incorporation of nucleotides	Synthesized cDNA (g)	Product/RNA template ratio
1 (PVX N)	5737	248887	2.3	0.30×10^{-6}	15%
2 (PVX 3, PBI)	2865	77599	3.6	0.48×10^{-6}	24%
3 (PVB)	1196	79554	1.5	0.19×10^{-6}	10%
4 (TMV)	512	73602	0.69	0.09×10^{-6}	4.0%
5 (PVX N)	257	135100	0.19	0.02×10^{-6}	0.9%
6 (PVX 3, PBI)	201	200368	0.1	0.01×10^{-6}	0.6%
7 (PVB)	215	188560	0.11	0.01×10^{-6}	0.7%
8 (TMV)	282	240830	0.11	0.15×10^{-6}	0.1%

Table 5: Complementary first-strand synthesis using PVX strain-Group 2 and 3 RNA templates. The results from the previous experiment indicated the possibility of cDNA synthesis with native PVX N RNA. The experiment was repeated with strains 3 (PBI) and 2 (PBI) and appeared to be successful. The table shows a comparison of cDNA synthesis with RNA templates from PVX strains from Groups 2 and 3. Samples 1-4 contained oligo d(T) as primer in the reaction mix, while samples 5-8 were duplicate reactions but without oligo d(T). (1) PVX N RNA, Group 3; (2) PVX RNA (strain-Group 3, PBI); (3) PVB RNA, Group 2 (PBI); (4) TMV RNA; (5-8) As above. Reactions were carried out at 42°C for 1.5 h and terminated with EDTA. The results showed a small background with TMV RNA (4 % product/template ratio) while the absence of oligo d(T) primer (samples 5-8) gave virtually no synthesis of cDNA at all (0.1-0.9 % product/template ratio).

3.6. Determination of the Poly (A) Region of PVX N RNA and its Location on the Genomic RNA.

From the results of the cDNA synthesis experiments, the presence of natural poly (A) tracts on the PVX N RNA was indicated. To confirm this, a series of experiments was designed not only to size this poly (A) region but also to determine its location within the RNA genome.

In the first set of experiments, PVX N RNA was degraded by the combined action of RNases A and T1 which digested all the RNA with the exception of the poly (A) sequences. These were then sized by polyacrylamide gel electrophoresis (Fig.35). The autoradiograph shows a nested series of poly (A) fragments of between 7-12 nucleotides in length. Athough these fragments are smaller than those reported by Morozov et al. (1981), they represent polyadenylate sequences which are of sufficient length to prime with oligo d(T). Several bands, representing varying polyadenylate lengths, can be seen. An indication of the fragment sizes was given by the relative positions of the xylene cyanol (XC) and bromophenol blue marker dyes (BPB). In a 20% acrylamide gel, RNA fragments which are 25 nucleotides in length co-migrate with the xylene cyanol dye front, while shorter fragments of 8 nucleotides in length co-migrate with the bromophenol blue marker dye front (Maniatis, 1982).

A second experiment involved the action of another RNase, RNase H, which digested the poly (A) sequences after hybridisation with oligo d(T) DNA. The digested PVX N RNA was electrophoresed together with native PVX N RNA, and their sizes then compared (Fig.36). After digestion, the RNA (lane 2) migrated slightly but reproducibly faster than the undigested controls. This indicates that the poly (A) region is located at or close to one end of the RNA (an internal poly (A) would have resulted in the RNA being cleaved into two fragments). The small difference in the size of the PVX RNA after digestion also

indicates that it is probably fairly short.

In the last experiment, PVX N RNA was 3' terminally radiolabelled and directly sequenced by the procedure of Donis Keller et al. In the first step, the labelled RNA was purified and separated from the excess radiolabel by a primary polyacrylamide gel electrophoresis (Fig.37). The major band of labelled RNA was determined after autoradiography, then excised and eluded from the gel.

The purified, labelled RNA was next divided into aliquots which were then individually partially digested with one of the five RNases (specific for different bases).

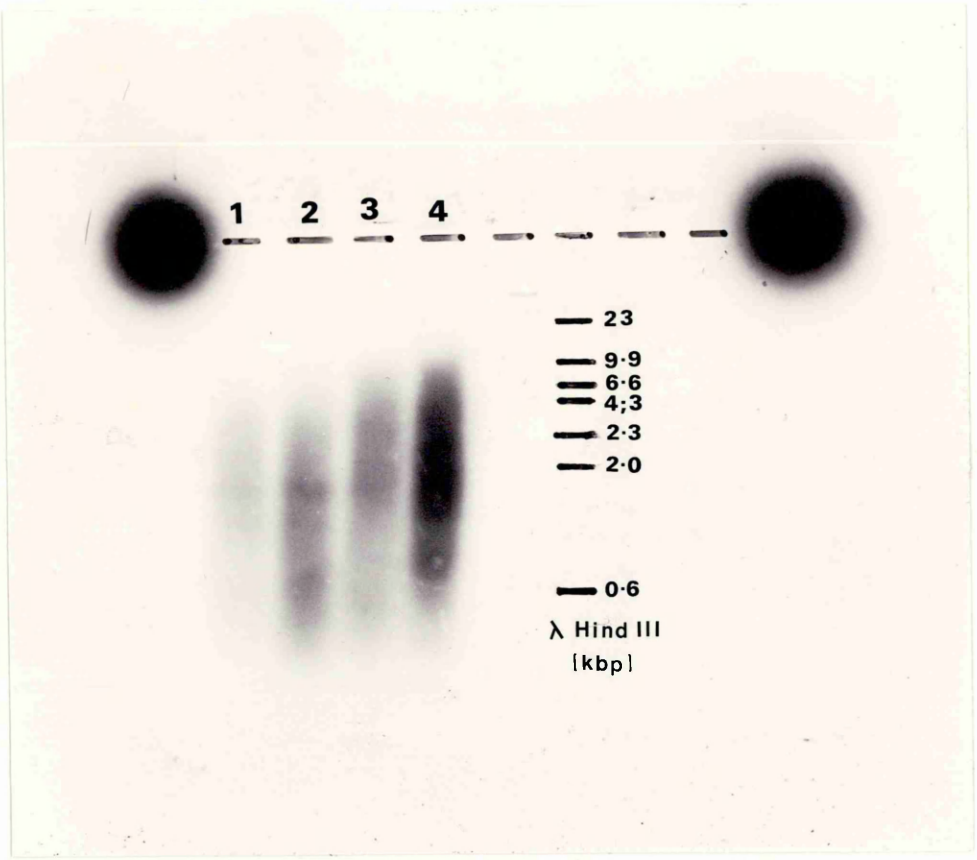


Fig.34: Autoradiograph showing relative sizes of cDNA from 3 PVX strains: 1) PVB (strain-Group 2, PBI), 2) PVX (strain-Group 3, PBI), 3) PVX N, 4) PVX N, sample 2. Aliquots of ^{32}P-labelled cDNA, containing 40,000 cpm were loaded on to the gel for electrophoresis on a TBE gel and set up for autoradiography at -70°C for 48 h. A control sample of unlabelled Hind III was also electrophoresed with the labelled samples. The sizing bands were detected by staining in 1 % toluidine blue.

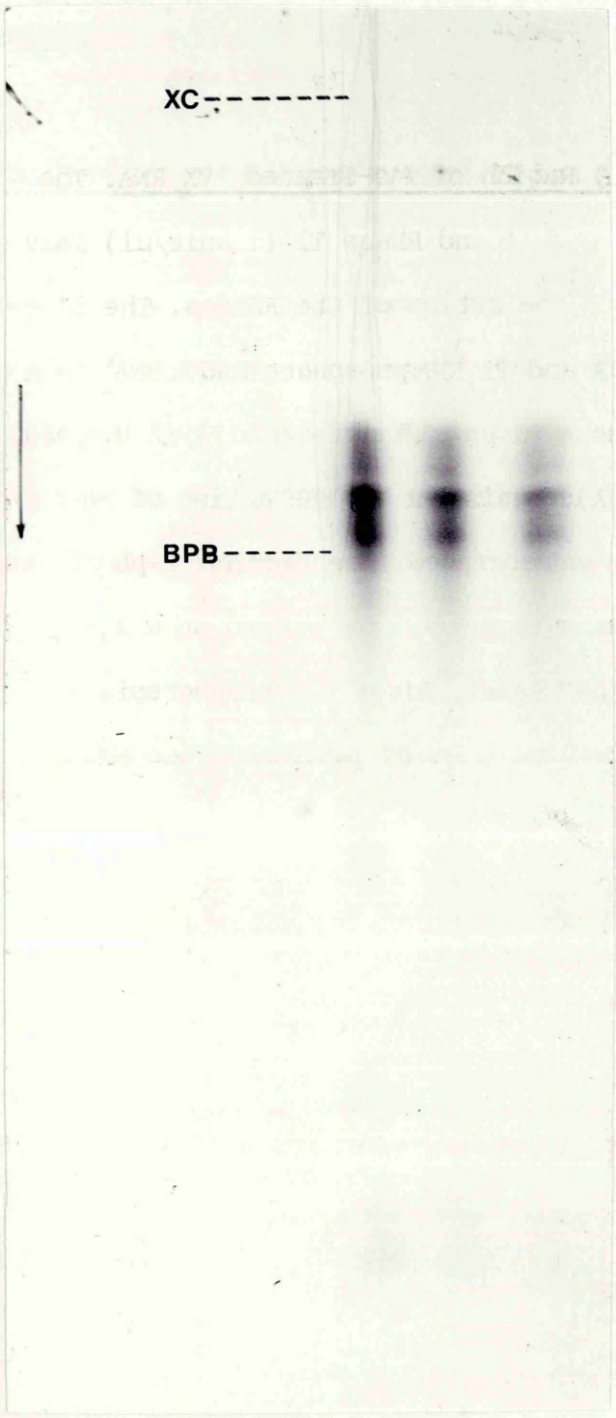

ORIGIN
≡25 nucleotides
XC
BPB
≡8 nucleotides
direction of
migration

Fig. 35

Fig 35. The Poly (A) Region of PVX RNA and the Determination of its Size.

Results of the A and T1 RNAses-treated PVX RNA. The RNA was incubated with RNase A (1 μg/μl) and RNase T1 (1 unit/μl) leaving stretches of poly (A), resistant to the action of the RNases. The 5' terminal of the RNA was phosphorylated with ^{32}P-phosphate and the ^{32}P-labelled fragments then electrophoresed on a 20% acrylamide gel (50,000 cpm loaded per lane). After electrophoresis at 1000V, the gel was set up for autoradiography as described (see Section 2.8).

Chromatography of PVX RNA by Oligo d(T) Cellulose.

A solution containing 50 µg PVX RNA in Buffer A was loaded on to an oligo d(T) column and re-circulated for 1 h at 20 ml/h. Unbound RNA ("poly A-") was then pumped off and stored. Bound RNA ("poly A+") was eluted in 10 mM HEPES (total 5 ml). The concentration of both fractions were assayed spectrophotometrically. The results are presented in Table 4.

Table 6:

	µg RNA	µg bound	µg unbound
Experiment 1	50 µg	28.0 µg	22.0 µg
Experiment 2	28.0 µg	10 µg	18 µg

In Experiment 1, the proportion bound was 56 % and the proportion unbound was therefore 44 %.

In order to determine the percentage of the poly (A)+ RNA which would bind again to the oligo d(T) cellulose, the bound RNA (i.e. 28µg - the poly A+ fraction) was passaged through the column a second time under the same conditions and the fractions then assayed spectrophotometrically.

After the second passage through the oligo d(T) column, the bound poly A+ PVX RNA was alcohol precipitated and resuspended in 50 ul 0.1 x STE buffer. 10 ul was diluted to 1 ml and a reading taken at 260 and 280 nm.

In Experiment 2, only 43 % of the poly (A)+ fraction bound to the oligo d(T) cellulose with a second passage and the proportion unbound was 57 %.

Despite care to exclude RNases, the recovery of RNA after two passages through the column was only 62 % (in duplicate experiments, this value varied between 46-57 % in the first passage through the

column as bound RNA, and between 37-43 % with the second passage). A high percentage of the original poly (A)+ RNA was lost, in duplicate experiments, to the column on its second passage which was probably due to the non-stringent binding conditions used to bind the RNA, or to RNase activity, irreversible binding, or failure to ethanol precipitate. Nevertheless, 43 % of the PVX RNA bound to the column after two passages, suggesting that this fraction must contain poly (A) or oligo (A) tracts of sufficient length to bind to the complementary oligo d(T) cellulose. Besides loss of RNA due to RNases, and failure to precipitate, the stretch of poly (A) tract in the RNA may have been of a length (possibly between 10-20 bases in length) to only weakly bind to the column with each passage, therefore, producing percentages of between 40-50 % binding with each passage.

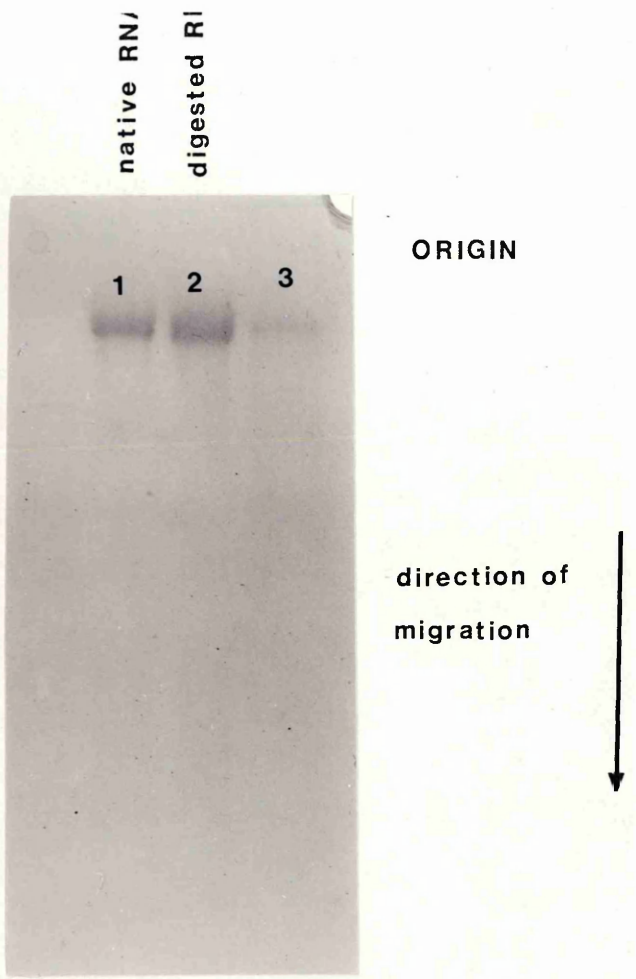


Fig.36: Results of Digestion by RNase H on PVX N RNA.

Purified native PVX N RNA was loaded into wells 1 and 3, while RNase H-digested PVX N RNA was loaded into well 2 (2 unit/µl). The samples were electrophoresed on a 5 % polyacrylamide gel at 60-70 mA.

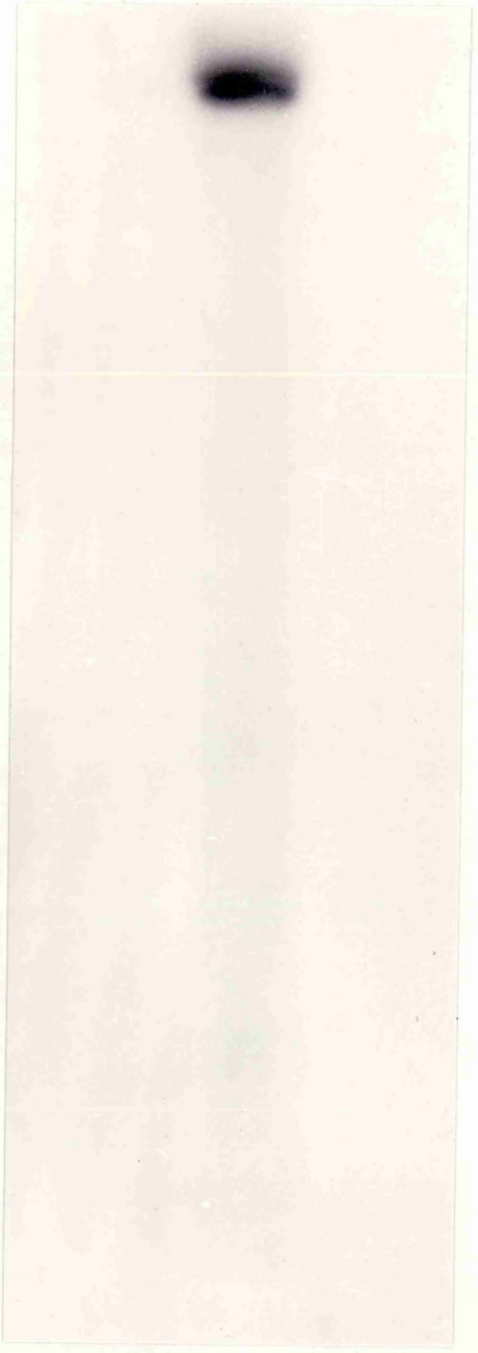


Fig.37. Purification of 3'-labelled RNA by Polyacrylamide Gel Electrophoresis: ^{32}P-labelled PVX RNA was denatured in 7 M urea and loaded on to a 4 % acrylamide gel cast in 50 mM Tris-borate, pH 8.3/1 mM EDTA prior to electrophoresis at 125 V for 5 h. After electrophoresis, the gel was set up for autoradiography. The exposed X-ray film above shows the heavy band of labelled RNA at the top of the gel. The excess label has migrated at a faster rate during electrophoresis and has run off the bottom of the gel.

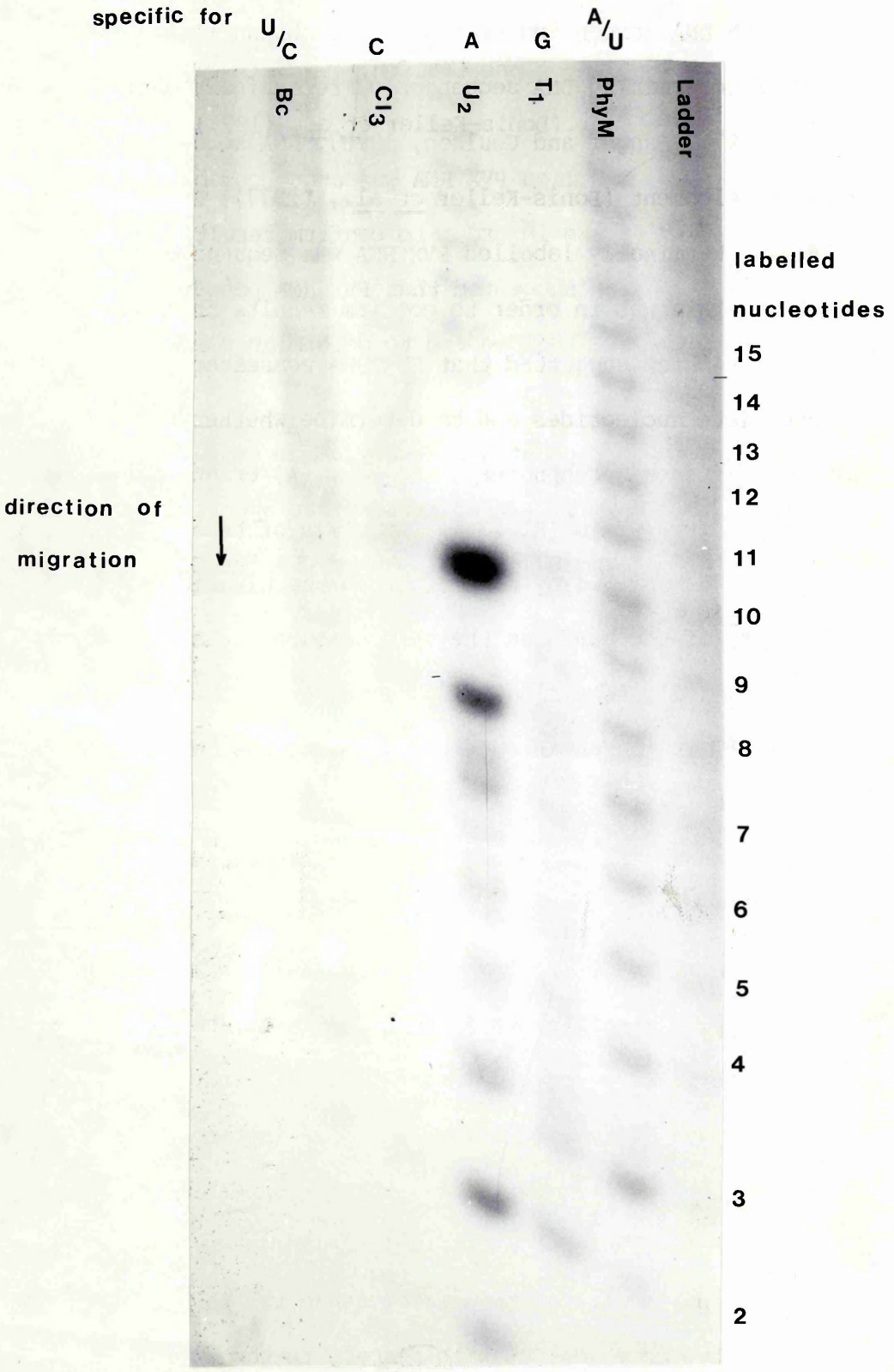

specific for
U/C
C
A
G
A/U
Bc
Cl3
U2
T1
PhyM
Ladder
direction of
migration
labelled
nucleotides
15
14
13
12
11
10
9
8
7
6
5
4
3
2

Fig.38. 3' Terminal RNA Sequencing of PVX RNA.

Although DNA sequencing of cloned recombinants is a fairly common method of determining DNA sequences of recombinant clones (Maxam and Gilbert, 1977, Sanger and Coulson, 1978), RNA sequencing is a more recent development (Donis-Keller et al., (1977); Lockard et al., (1978). 3' terminally labelled PVX RNA was sequenced by limited enzyme digestion. This was in order to confirm results from earlier experiments which suggested that PVX RNA possessed a region of polyadenylate nucleotides and to determine whether this was at its 3' terminus. After electrophoresis the poly (A) tracts were exposed by exposure to X-ray film (Fig.38). The sizes of these heterologous tracts were determined by comparison to the alkali digested "ladder". The clarity of the bands on the autoradiograph was poor, resulting from the use of intensification screens during the exposure of the gel to the film. In the absence of the screens, the bands were too faint to see on the film.

An autoradiograph of a 20% acrylamide sequencing gel is shown in Fig.38. The RNase T1 was specific for G residues, RNase B.cereus for C and U residues, RNase CL_3 for C rsidues, while RNAse U_2 was A specific and RNAse Phy M specific for A and U residues. These last two RNases show ladders from the bottom of the gel. This indicates a tract of A residues at the 3' terminus. Ladders begin in the other lanes somewhat higher up (from about residue 10). These results are consistent with the presence of a heterodisperse poly (A) tract at the 3' terminus. Variations in the size of this tract cause the beginning of the "true" 3' sequence to be staggered with respect to the 3' ^{32}P label. The superimposition of the staggered sequences on the gel result in the laddering. From the point at which the laddering in these lanes begin, the minimum distance from the 3' end at which residues other than A are found (i.e. the shortest length of poly (A) tail) is estimated at

10 bases. The enzyme RNase CL_3 gave poor digestion of the RNA. Levy and Karpetsky (1980) report that this enzyme is inhibited by polyadenylate and digests poly (A)+ RNA partially.

3.7. Synthesis of Complementary DNA (cDNA) Clones, Containing PVX Sequences.

cDNA clones were prepared as described in Materials and Methods.

1) Synthesis of cDNA.

The amount of first strand cDNA synthesised was estimated by gel filtration on Sephadex G100 of a 2 µl sample. Twenty-five fractions (approximately 0.5 ml per fraction) were collected and 22 ul aliquots were counted on the ^{32}P channel (Packard Liquid Scintillation Spectrometer, Model 3380).

Yields of cDNA for this reaction varied considerably, ranging from 5% to 25% cDNA/RNA template ratio, from 0.1 µg to 0.56 µg cDNA synthesised. A typical synthesis of first strand complementary DNA is shown in Section 3.7.2. Values ranged from 4-30 % cDNA/RNA template ratio where 2.0 µg PVX RNA was the standard amount of RNA used as the template in the majority of first strand reactions. For the construction of the recombinants, a total of 1.8 µg of single-stranded cDNA (1st strand) was prepared from 30 µg PVX RNA.

2) Synthesis of Second-Strand cDNA.

Double-stranded cDNA was synthesised as described in Section 2.11. Yields of second-strand were calculated as shown in Section 3.7.2.

The yield of double-stranded cDNA was 0.25 µg from 0.4 µg single-stranded cDNA. This was a 63 % product/cDNA template ratio and in duplicate experiments, ranged from 17-30 % on average.

3) Second Strand Completion Reaction.

In the second-strand reactions, the maximum yield of second-strand was 63 %. Failure to complete the second-strand synthesis was overcome by the use of reverse transcriptase (Maniatis, 1982). The results

from 63.0% to 74.25% product/template ratio.

4) Sl Nuclease Reaction.

In order to confirm the extent of synthesis of second-strand and to remove single-stranded regions and the hairpin region corresponding to the 5' direction of the original RNA template, the ds cDNA was treated with Sl nuclease. The digestion was again analysed by gel filtration on Sephadex Gl00. A typical result is shown in Section 3.7.2. Results indicated that the double-stranded cDNA was 91% resistant to the action of the Sl nuclease.

3.7.2: Calculations for the Synthesis of Double-Stranded Complementary DNA:

a) Synthesis of cDNA:

In the synthesis of cDNA, yields varied considerably. However, a typical calculation is given below:

Fractions containing ^{32}P-cDNA (total cpm)	=	1328
Unincorporated ^{32}P nucleotides (total cpm)	=	108866
% incorporation of nucleotides	=	1.2 %
Amount of cDNA synthesised (g)	=	0.16 µg
cDNA/RNA template ratio	=	7.9

b) Synthesis of Second-Strand cDNA:

Fractions containing ^{3}H-labelled cDNA (total cpm)	=	1201
Unincorporated ^{3}H-nucleotides (total cpm)	=	156917
% incorporation of nucleotides	=	0.76 %
Amount of ds cDNA synthesized (g)	=	0.25 µg
Amount of ss cDNA template	=	0.4 µg
Product/cDNA template ratio (%)	=	63%

c) Second-Strand Completion Reaction:

A typical result of carrying out a second-strand completion reaction on the ds cDNA is shown below.

Fractions containing ^{3}H-labelled ds cDNA (total cpm)	=	2342
Unincorporated ^{3}H-nucleotides	=	12856

% incorporation of nucleotides		
+ second-strand ds cDNA	=	1.8%
Amount of ds cDNA synthesized (g)	=	0.29µg
Product/template ratio (%)	=	74.25

d) Sl Nuclease Reaction:

Calculations for the Sl nuclease reaction were based on the difference for ^{3}H values of the sample before and after the reaction.

a) Sample before Sl Nuclease.

^{3}H-labelled ds cDNA (total cpm)	=	307
^{3}H in remaining fractions (total cpm)	=	742

Therefore, in the pre-Sl nuclease reaction, ^{3}H-labelled ds cDNA represented 41.3% of the total sample.

b) Sample after Sl Nuclease.

^{3}H-labelled ds cDNA (total cpm)	=	252
^{3}H in remaining fractions (total cpm)	=	669

Therefore, in the post-Sl nuclease reaction, ^{3}H-labelled ds cDNA represented 37.6% of the total sample.

Since the original second-strand is a very small count compared to the ^{3}H added and since the specific activities of the second-strand synthesised from reverse transcriptase was the same as that synthesised with Klenow fragment, then the percentage resistance of

the ds cDNA is:

$$^{3}H\text{: S1 resistance} = \frac{37.6}{41.3} \times 100$$

$$= 91\ \%$$

A certain amount of ^{3}H-dCTP apparently co-precipitated with the ds cDNA following the second-strand completion reaction. This resulted in a significant amount of ^{3}H appearing in the position of unincorporated nucleotides even before S1 treatment. This was corrected for when calculating for S1 sensitivity.

Fractionation of Double-Stranded cDNA after Sucrose Gradient Centrifugation.

The synthesised ds CDNA was sized fractionated on a 5-20 % sucrose gradient as previously described (Section 2.12). After centrifugation, twenty-four fractions were collected. Aliquots from each fraction were then counted to determine those with the highest values. A graph showing the results can be seen in Fig.39a. The aliquots with the highest ^{32}P values (over 40 cpm/µl) came from fractions 4,5,6,7, and 8.

Further aliquots were then taken from these fractions and electrophoresed on a 1 % agarose Tris-borate/EDTA gel (Fig.39b). After exposure to Kodak X-Omat S film, the sized fractions were revealed as smears of varying molecular sizes. Of these fractions, 4 and 5 were combined, giving the "Low Molecular Weight" (MMW) DNA (with 0.56 kbp and less); fractions 6 and 7 were combined, giving the "Medium Molecular Weight" (MMW) DNA (ranging in size from 0.56 to 2.0 kbp); fraction 8 was labelled "High Molecular Weight" (HMW) DNA (from 0.9 to 2.3 kbp). An additional sample, B (taken from the base of the base of the gradient) was also electrophoresed, showing up as a dark smear of widely varying sizes.

The medium and high molecular weight ds cDNAs were then used in the transformation experiments.

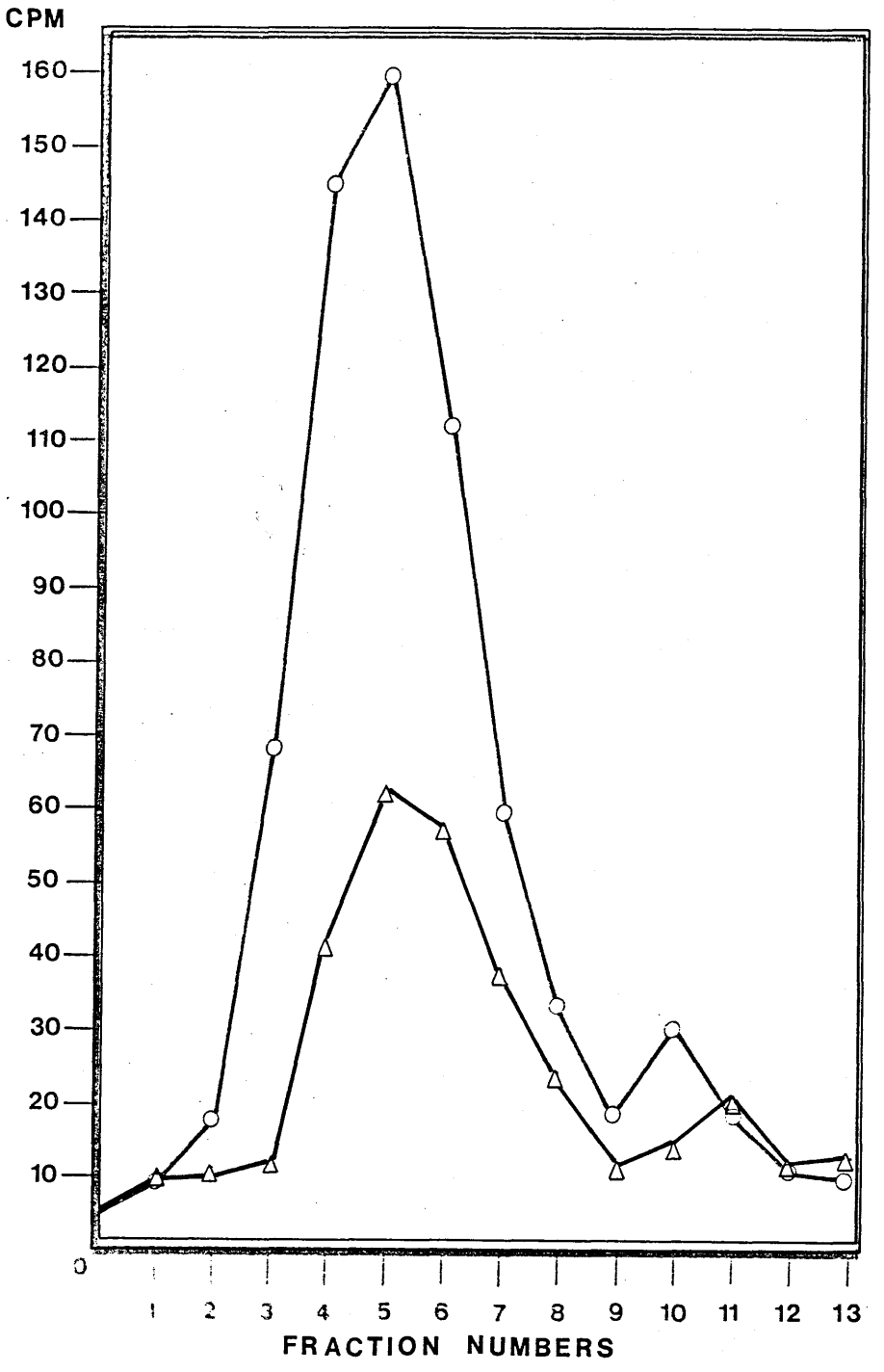


Fig.39a: Fractionation of double-stranded complementary DNA after sucrose gradient centrifugation, showing the incorporation of ^{32}P-dCTP (o) and ^{3}H-dCTP (Δ) into the ds cDNA. The highest incorporation of both ^{32}P-dCTP and ^{32}H occurred in fractions 4, 5, 6, and 7.

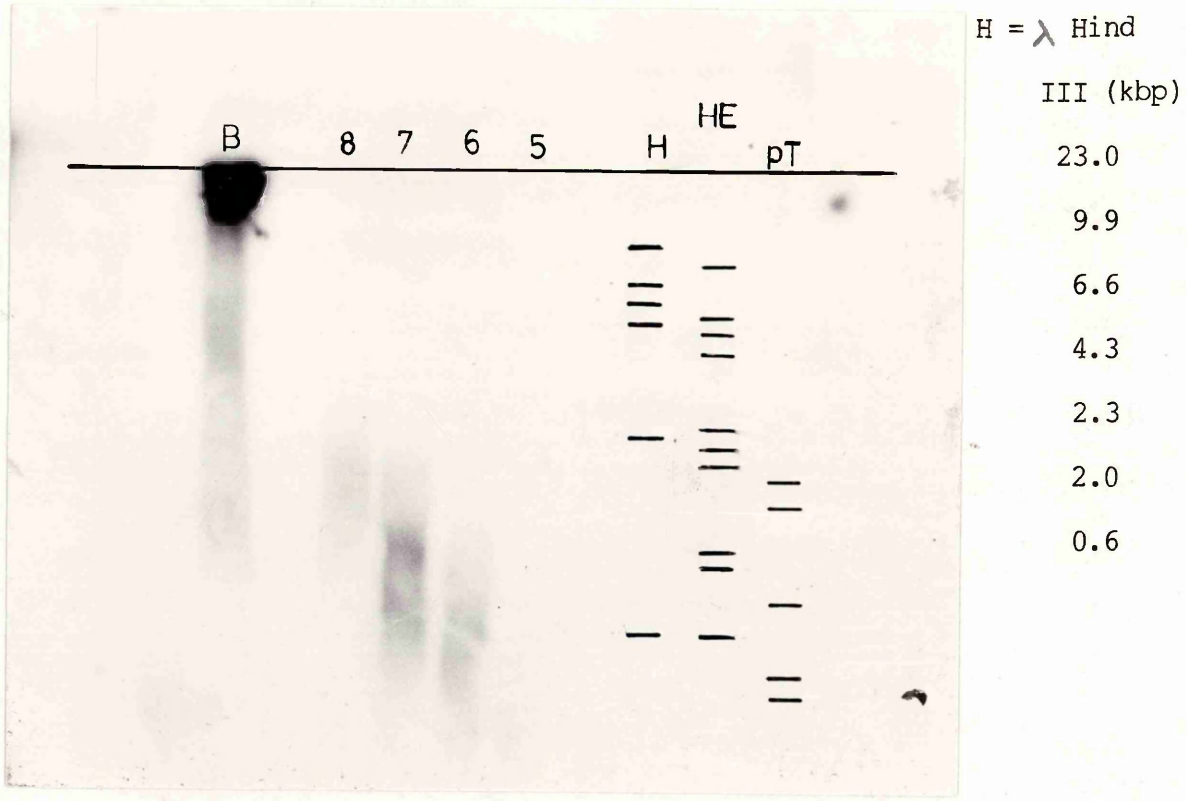


Fig.39b: Autoradiograph showing relative sizes of fractions 4-8 on a TBE gel after electrophoresis following centrifugation of dscDNA in a sucrose gradient. Fractions 4 and 5 were pooled giving the Low Molecular Weight (LMW) ds cDNA; fractions 6 and 7 were pooled giving the Medium Molecular Weight (MMW) ds cDNA, while Fraction 8 was kept as the High Molecular Weight (HMW) ds cDNA. A sample, B, was also removed from the base of the sucrose gradient and electrophoresed with the others.

3.9. Transformation of Escherichia coli cells with ds cDNA and Screening of the Positive Recombinants.

The fractionated and sized ds cDNA fractions were pooled into three lots according to size as described. Only the High Molecular and Middle Molecular weight DNA samples were used for transformation in E.coli cells.

3.9.2 Transformation of Escherichia coli cells with "High Molecular Weight" and "Medium Molecular Weight" ds cDNAs.

Competent JM83 cells were transformed with in vitro recombinants containing HMW ds cDNA or MMW ds cDNA ligated with pUC9 (see Section 2.15). As a control, cells were transformed with native pUC9 DNA. Fifty µl aliquots of cells were incubated with 100 ng HMW/pUC9 DNA, 100 ng MMW/pUC9 DNA or 100 ng native pUC9 DNA. The cells were plated as 10 µl aliquots on X-gal ampicillin plates and incubated for 16 h at 37°C. Control plates of cells transformed with native pUC 9 were incubated under the same conditions. Positive colonies after transformation were identified from X-gal ampicillin plates. Those colonies containing DNA inserts were expected to be white as opposed to those without inserts which were blue. Not all the white colonies were assumed to be positive since exonuclease action followed by re-circularisation of pUC 9 also produced white colonies. Each of the white colonies were picked off the X-gal ampicillin plates and grown as colonies on secondary L-broth agar/ampicillin plates (Fig.40).

The number of white colonies (those containing inserts) and blue colonies (colonies containing native pUC 9) were counted. The results were as follows:

Table 7:

	High Molecular Weight ds cDNA

Plate No.	White	Blue
1	6	0
2	6	0
3	20	0
4	13	0
5	9	0
6	11	0
7	23	0
8	7	0
9	12	0
10	15	0
Total	122	0

Medium Molecular Weight ds cDNA

Plate No.	White	Blue
1	23	9
2	10	10
3	12	6
4	9	4
5	2	5
6	8	5
7	19	8
8	21	10
9	24	18
10	24	9
Total	152	84

The ten plates with the cells containing native pUC 9 as controls all contained more than 500 blue colonies with no white colonies.

Correcting for the dilution of the samples (see Section 2.16), these represent transformation efficiencies of

2.3×10^5 colonies/μg HMW/pUC9 DNA.

3.2×10^5 colonies/μg MMW/pUC9 DNA.

The control native pUC9 DNA gave greater than 10.6×10^6 colonies/μg DNA. Although yields were only 2.1 and 3.0 % of pUC9 controls, a large number of putative recombinants were obtained.

3.10. Identification of Colonies containing cDNA inserts to PVX RNA.

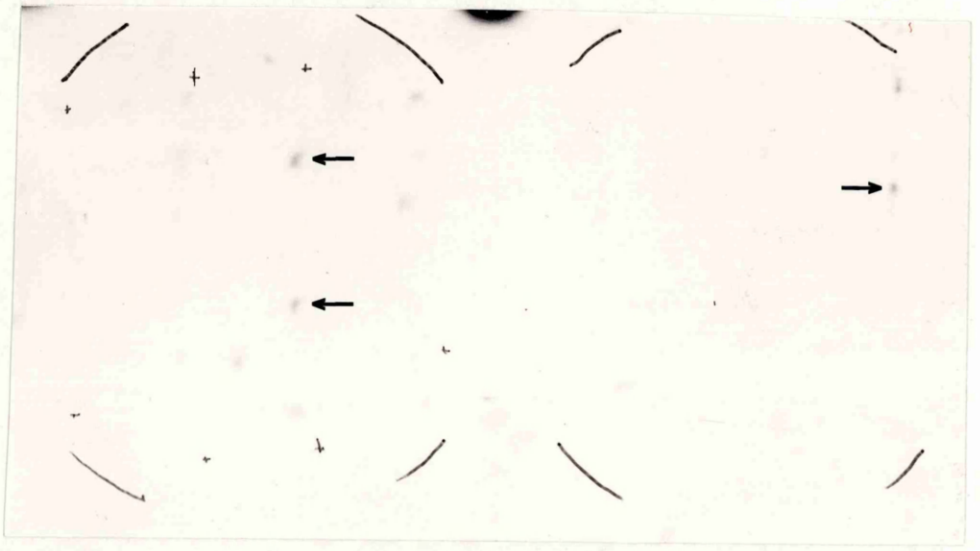
Of 10 possible clones screeened for complementary sequences to PVX N RNA (Section 2.16.3), only one (pPVX21 N) produced a strong visible signal during hybridisation with RNA on filters. The insert size of pPVX21 N was determined by digestion with restriction enzymes and agarose gel electrophoresis.

The clone could not be digested with Eco RI and in a double digestion with Hind III and Eco RI, the plasmid was linearised. This result was repeated in a double digest with Bam HI and Eco RI, suggesting that a non-specific nucleases had deleted the Eco RI site from the plasmid which was then ligated to the insert.

The sizing and visualisation of clone pPVX N 21 (used for the synthesis of the nick-translated probe) is shown in Fig.41. On the basis of the size of the linearised pPVX21 N DNA, the insert size is 500-600 bp. pPVX21 N digested with EcoRI + Hind III (lane 2) was electrophoresed on a TBE gel with EcoRI + Hind III pUC 9 (lane 3) and DNA digested with Hind III and Eco RI restriction enzymes (lane 1).

Positive colonies were picked off agar plates and spotted in rows on L-broth agar/ampicillin plates and grown at 37°C for 16 h. The plates were imprinted on nitrocellulose, the colonies lysed (the DNA denatured and bound by baking), and dried prior to hybridisation with ^{32}P-labelled probe (approximately 50,000 cpm/ml hybridisation solution) at 65°C for 16 h. The arrows in Fig.40 show the signals on the autoradiograph of positive colonies. On average, 4.5 % of the colonies were positive on the first hybridisation. This value fell to less than 1 % when the positive colonies were re-grown and hybridised once more showing a high percentage of false positives present on the first hybridisation.

Fig.40: Autoradiograph of nitrocellulose showing positive recombinant clones hybridised with a radiolabelled probe. The hybridised colonies on the nitrocellulose filters were then exposed to X-ray film for between 4-7 days.



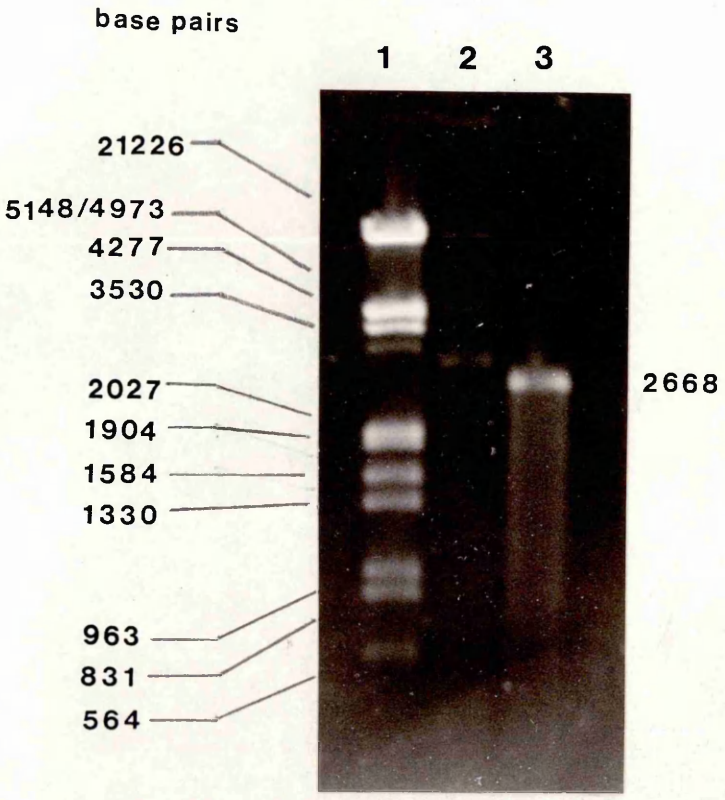


Fig.41: Determination of the size of pPVX21 N DNA.

The sizing and visualisation of clone pPVX N 21 (used for the synthesis of the cnick-translated probe) is shown below. The clone (lane 2) was electrophoresed on a TBE gel with native pUC 9 (lane 3) and DNA digested with Hind III and Eco RI restriction enzymes (lane 1).

Nick Translated probes in Hybridisation Experiments.

The protocol for the nick-translation is described in Section 2.4.4. The DNA was next purified and nick-translated as described. The specific activity of the probe was 1.2×10^8 dpm/µg DNA.

The nick-translated probe was hybridised with filters containing serial dilutions of infected crude sap, purified viral particles (both PVX N, PVX, as well as PVB), and purified PVX N RNA, with BMV RNA as a control.

The results, shown in Figs.42 and 43, show that the nick-translated probe was far less sensitive during hybridisation experiments than either of the two cDNA probes. Whereas the probes (oligo d(T)- or calf thymus-primed) detected infected sap, purified viral particles and purified PVX N RNA, the nick-translated probe detected only the PVX N RNA to 200 ng after a 2 day exposure (Fig.42).

This sensitivity of detection was only slightly improved when the filter was left to expose for 7 days. The sensitivity of detection for PVX N RNA increased by a log to 20 ng (Fig.43), compared with 200 pg for both the radiolabelled and biotinylated probes but this was offset by an increase in non-specific signals on the filter. No cross-hybridisation was observed with the BMV RNA.

The probe was also very insensitive in the detection of the purified viral particles and crude sap samples. Only the PVX (strain-Group 3, PBI) crude sap sample, out of all the isolates, was detected in the 7 day exposed film, and then only to 10^{-2}. The purified viral particles isolates were not detected by the probe. The detection of only the PVX (strain-Group 3, PBI) out of all the isolates may have been due to the abnormally high levels of virus particles and, therefore, RNA in the infected leaf samples (Table 2 and Figs.17,18 and 19).

The nick-translated probe was, therefore, far less sensitive in the

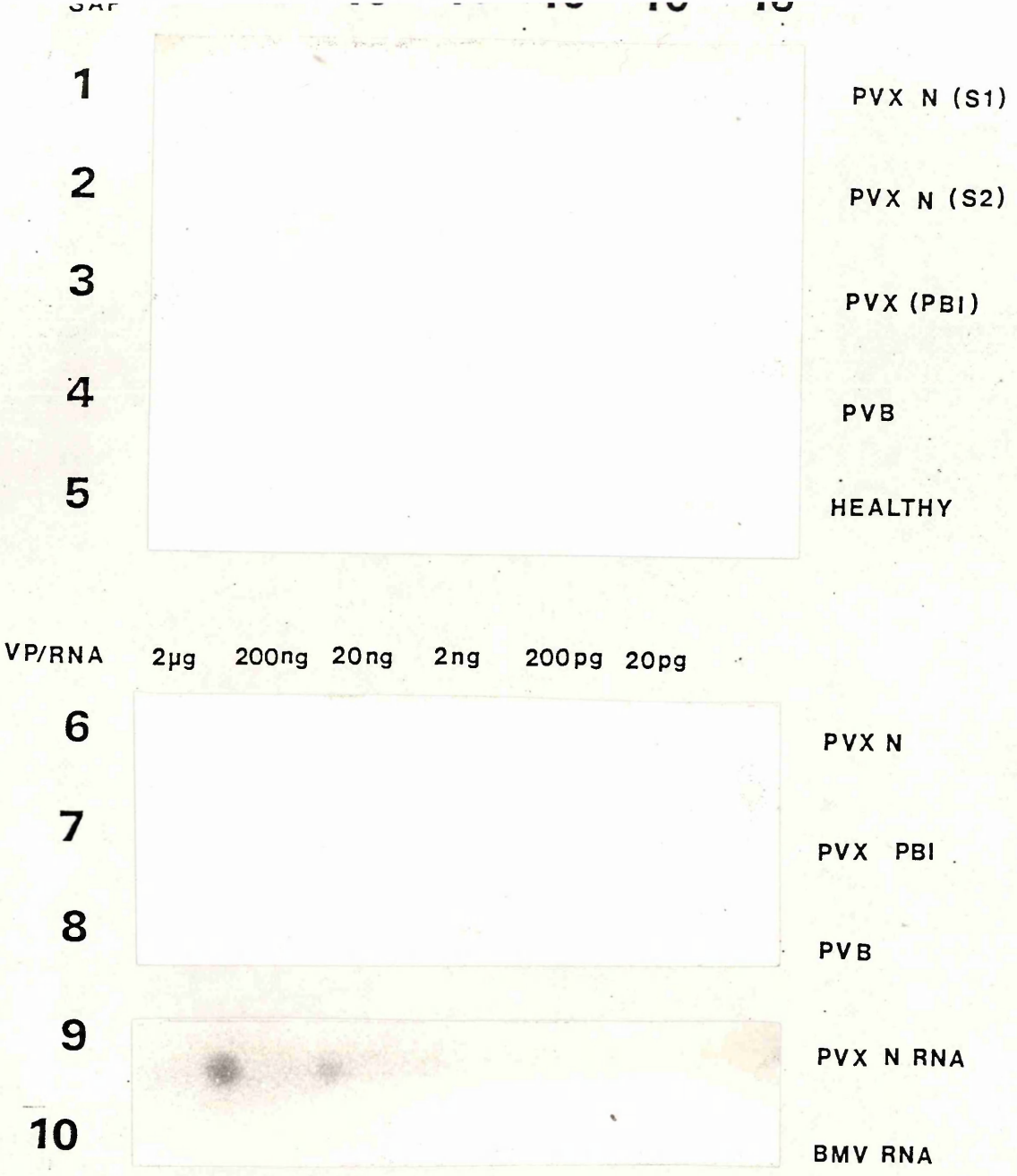


Fig.42. Sap spot assay using pPVX21 N (a nick-translated probe) as a probe in the detection of crude sap, purified viral particles, and PVX N RNA. The probe had a specific activity of 1.2×10^8 dpm/µg DNA. The filter shows: a) Sap samples 1) PVB, 2) PVX (PBI), 3) PVX (S1), 4) PVX (S2), 5) healthy; b) Viral particles 6) PVX N, 7) PVX (PBI), 8) PVB; c) purified RNA 9) PVX N, 10) BMV. The filter was exposed for two days at -70°C before developing.

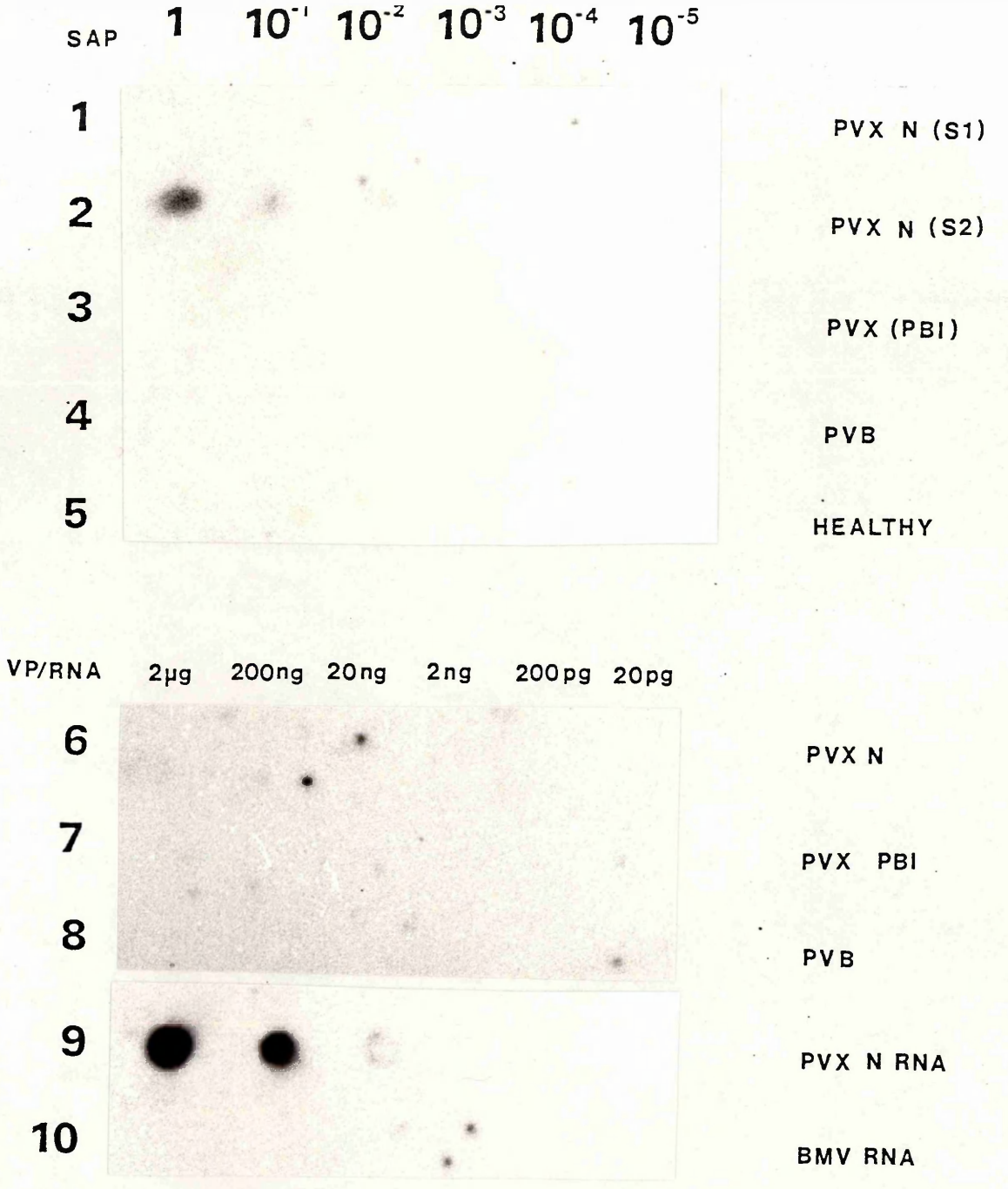


Fig.43. Sap spot assay using a nick translated probe (pPVX21 N) as probe. An identical filter was hybridised with the probe and exposed to X-ray film for 7 days. The filter shows: a) Sap samples 1) PVB, 2) PVX (PBI), 3) PVX (S1), 4) PVX (S2), 5) healthy; b) Viral particles 6) PVX N, 7) PVX (PBI), 8) PVB; c) purified RNA 9) PVX N, 10) BMV

detection of PVX or PVB than either of the radiolabelled or biotinylated probes by at least 2 logs for the PVX N RNA and, with the exception of PVX (strain-Group 3, PBI), totally insensitive to PVX N and PVB sap and purified viral particles. The probe also required extended exposure time in order to detect the PVX (strain-Group 3, PBI) isolate.

The Preparation and Fractionation of ATP:RNA Adenyltransferase.

ATP:RNA adenyltransferase (otherwise known as "poly (A) polymerase") is an enzyme capable of adding polyadenylate nucleotides to the 3' end of RNA chains. The enzyme was purified as described in Section 2.17. Prior to assaying for activity, the enzyme was eluted with a linear gradient of 0.5 M to 1 M NaCl in Buffer B. Fractions of 2.5 ml were collected at a flow rate of 8 ml per hour. These fractions were assayed for enzyme activity as described (section 2.17.v).

Results for the assay of enzyme activity are shown on Table 8 and Fig.44. The dried down filters in the standard reaction mix indicated that enzyme activity was greatest in fractions 16, 17, and 18. These fractions were stored as glycerol stocks at -70°C and thawed when needed.

The stored, purified enzyme was used in preliminary experiments with tRNA to determine the optimal conditions for the addition of poly (A) tracts (Tables 9-12). From these tailing experiments, it was determined that the synthesis of poly (A) tracts was significantly better with 0.1 mM ^{3}H-ATP than with 1.0 mM ^{3}H-ATP using the standard reaction mix (Tables 9 and 10). When the enzyme was used on PVX N RNA, the length of the poly (A) tract increased substantially with incubation time (Table 12). The calculations of the synthesis of poly (A) tracts to the RNA templates were as follows: e.g. for 0.3 µg tRNA at T_{60},

Total incorporated in reaction mix (100 µl)	=	6480 cpm
Total number of counts (100 µl)	=	320,000 cpm
Therefore, incorporation of ATP	=	2.02×10^{-2}
Final concentration ATP	=	0.1 mM

Incorporation $= (2.02 \times 10^{-2}) \times (1 \times 10^{-8})$

$= 2.02 \times 10^{-10}$ moles

0.3 µl tRNA used $= 1.2 \times 10^{-11}$ moles

Therefore, the number of residues added

$$= \frac{2.02 \times 10^{-10}}{1.2 \times 10^{-11}} = 17 \text{ poly (A) residues}$$

Vial No.	Fraction No.	Average Total count	Average cpm
1	13	191.0	38.2
2	14	250.5	50.1
3	15	262.5	52.5
4	16	6715.5	1343.1
5	17	8495.0	1699.0
6	18	4697.5	939.5
7	19	1503.5	300.7
8	20	866.0	173.2
9	21	783.0	156.6
10	22	633.5	126.7
11	23	431.5	86.3
12	24	293.0	58.6
13	25	270.5	54.1
14	^{3}H-ATP	35469.5	7093.9
15	Blank	53.5	10.7

Table 8: Results for the assay of ATP:RNA adenyltransferase enzyme activity after preparation: 10 µl aliquots from each fraction were assayed for the ability to transfer ^{3}H-ATP to tRNA. Filters containing the dried-down aliquots were counted in 0.4% PPO (diphenyloxazole) in toluene.

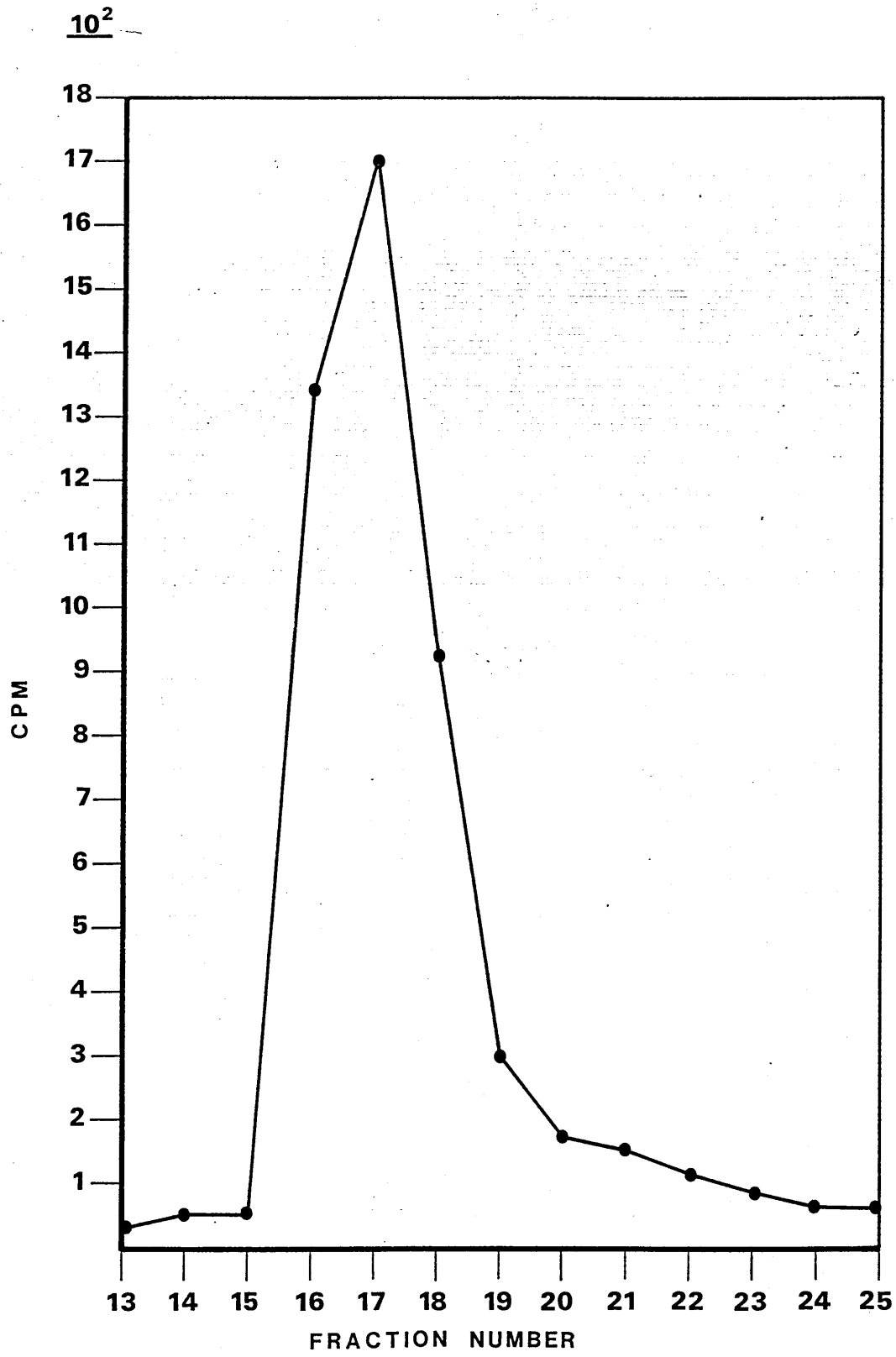


Fig.44: ATP:RNA adenyltransferase activity after preparation. Each fraction was assayed for ability to transfer ^{3}H-ATP to tRNA. Filters containing the dried-down aliquots were counted in 0.4% PPO (diphenyloxazole) in toluene. The graph shows peak activity in fractions 16, 17, and 18.

Vial No.	Sample Incubation Time	Cpm Count (10 min)	Length of Poly (A) Nucleotides
1	0	125	-
2	5	184	2
3	10	269	4
4	15	290	5
5	20	493	11
6	30	522	12
7	60	565	13
8	^{3}H-ATP	28591	-

Table 9: Effect of incubation time on the synthesis of polyadenylate tracts on to tRNA using 0.1 mM ^{3}H-ATP and 5.0 µg tRNA.

Vial No.	Sample Incubation Time	Cpm Count (10 min)	Length of Poly (A) Nucleotides
1	0	256	-
2	5	371	1
3	10	264	-
4	15	292	-
5	20	279	-
6	30	424	2
7	60	525	2
8	^{3}H-ATP	91791	-

Table 10: Effect of incubation time on the synthesis of polyadenylate tracts on to tRNA using 1.0 mM ^{3}H-ATP with 5 µg tRNA.

Vial No.	Sample Incubation Time	Cpm Count (10 min)	Length of Poly (A) Nucleotides
1	0	221	-
2	5	346	1
3	10	396	3
4	15	419	5
5	20	552	9
6	30	730	13
7	60	869	17
8	^{3}H-ATP	32090	-

Table 11: The effect of incubation time on the synthesis of polyadenylate tracts on to tRNA using 0.1 mM ^{3}H-ATP with 0.3 µg tRNA.

Vial No.	Sample Incubation Time	Cpm Count (10 min)	Length of Poly (A) Nucleotides
1	0	79	-
2	10	287	24
3	20	464	46
4	30	746	80
5	40	1000	110
6	50	1196	134
7	60	1324	149
8	80	1422	161
9	^{3}H-ATP	35019	-

Table 12: The effect of incubation time on the synthesis of polyadenylate tracts on to PVX RNA using 0.1 mM ^{2}H-ATP with 5 µg RNA.

CHAPTER 4

DISCUSSION

A wide range of plant diseases are caused by viruses. Many of these are of economic importance in relation to food production and the decorative plant industries. In addition, plant viruses have to be diagnosed in other situations, such as i) when plant samples have to be analysed for quarantine reasons; ii) for disease surveys in crops or weeds; and iii) the need for testing large numbers of samples such as propagating material for the absence of one or more viruses. The need for detecting these economically important viruses is, therefore, vital. However, difficulties often arise since certain symptoms caused by plant viruses sometimes resemble those caused by nutrient deficiencies, toxicities, insect secretions, mutations, or other factors. The diagnostic method eventually chosen will be dependent on a number of factors such as the availability of time, space and materials, the experience of the investigator, and the importance of the problem.

In general, when results for large numbers of samples are required, such as for the mass testing of propagating material, only one method is usually chosen. The method must, therefore, be easy to handle and relatively sensitive. Serological methods have been used extensively for mass testing and indexing potato stocks for the presence of PVX and other viruses (de Bokx, 1972; Shepard and Claflin, 1975).

Experimentally, three systems for the detection of potato virus X (PVX) were developed and compared, the first of which was a serological assay designed for assaying large numbers of samples. This system was compared with the development of two alternative systems utilising techniques from molecular biology.

4.1. Enzyme-linked Immunosorbent Assay (ELISA).

A serological method of virus detection was used for PVX as with other viruses, since the specificity of the antigen/antibody

relationship provided a versatile tool which was dependent on the simple nature of the proteins in the viral coat. In addition to their specificity, serological techniques have been used for their speed and the scope they provide for standardisation. However, in some cases, conventional serological techniques cannot be used because of limitations such as low virus concentrations, unsuitable particle homology, or the presence in plant extracts of virus inactivators or inhibitors. With the use of the enzyme-linked immunosorbent assay (ELISA), many of these limitations have been overcome.

Enzyme-labelled antibodies had been used for the detection of virus antigens in tissue sections prior to the development of the ELISA (Nakane and Pierce, 1966; Wicker and Arameas, 1969) but the "double antibody sandwich" for plant viruses was only developed by Voller, Bidwell, and Bartlett (1976b) relatively recently. The virus in the test sample is selectively trapped and immobilised by specific antibody adsorbed on to a solid surface such as polystyrene microtitre plates (Voller et al, 1974). The trapped virus is then reacted further with specific antibody to which an enzyme, such as alkaline phosphatase, has been linked. After washing, to remove unbound antibody, the enzyme-labelled antibody that has complexed with the trapped virus is detected spectrophotometrically after adding a suitable enzyme substrate. (Fig.2). The use of the ELISA has several advantages over alternative radiolabelled immunoassays without the disadvantages of an inherent rate of decay and being potentially hazardous to health.

In the assay, experiments designed to compare the detection of PVX N viral particles with the presence of the virus in crude plant sap from infected Nicotiana tabacum were carried out. The sensitivity of

the system was determined by assaying serial dilutions of two isolates of PVX (strain-Group 3) and one from strain-Group 2 (PVB).

In the enzyme-linked immunosorbent assay, serial dilutions of purified viral particles (2 µg to 20 pg) were assayed and directly compared with 10-fold serial dilutions of crude plant sap from healthy and infected Nicotiana tabacum plants. Colour development in the initial assays were carried out for 1 hour and, to determine whether the sensitivity of the system improved with time, then repeated for extended periods of time. Results of these assays are presented in Table 3 and Figs.17-21. All viral isolates (PVX N, PVX (strain-Group 3), and PVB (strain-Group 2) were assayed together with positive and negative controls. The results for the assays were determined both visually and spectrophotometrically.

The visual determination of the colour produced was divided subjectively into strong (+++), medium (++), or weak (+). The spectrophotometric readings were taken at A_{405} nm.

From the results of the assays with purified virus particles, very little difference was observed between the three isolates at the highest and lowest concentrations although these absorbance values varied widely between 20 ng to 200 pg (Figs.20 and 21). This indicated that the polyclonal antibody used for the ELISAs reacted equally well with all isolates of purified viral particles and could be used to detect the virus in crude sap samples from different isolates. Increasing the incubation period did not markedly change the level of sensitivity, either visually or spectrophotometrically.

The sensitivity for the detection of virus particles in crude sap in infected plants was then compared. Results from the spectrophotometric determination of positive wells are shown in

Fig.17-19. Of these, PVX-infected sap (strain-Group 3, PBI) gave the strongest reactions throughout the dilutions for 1, 2, and 5 hours incubations (Fig.17). The other samples, however, showed substantial decreases in absorption at A_{405} nm with each dilution. Additionally, unlike the PVX (strain-Group 3)-infected isolate, an increase in the incubation time between the antibody-enzyme conjugate and the substrate, substantially increased the colour production and spectrophotometric readings (Fig.17-19). As an example, at a 10^{-2} x dilution, the spectrophotometric reading (A_{405} nm) for PVX N-infected sap (sample 1) rose from 0.540 at 1 hour, to 1.239 at 2 hours, to 1.589 at 5 hours incubation with substrate (or visually, from ++ at 1 hour to +++ at 5 hours). Overall, at 10^{-1} x dilution, the spectrophotometric readings of all samples increased with an increase in substrate incubation time. At 5 hours incubation, all PVX-infected samples at 10^{-1} x dilution produced readings of 2.0 or above (A_{405} nm) or +++ visually. These results were reproduced in repeat assays with stored (-70°C) crude sap and viral particle samples. The high absorbances obtained with sap from PVX (strain-Group 3, PBI)-infected plants probably effects a genuinely higher level of virus within plants compared to PVX N and PVB since dot blot hybridisation gave quantatively similar results (see examples, Figs.27 and 30).

The sensitivity of the assay for virus in crude sap, therefore, substantially increased with increased incubation time for all isolates except PVX (strain-Group 3, PBI). However, this increase in sensitivity of detection was not reflected for purified viral particles to any great extent. A possible explanation for this may be that the detection of purified viral particles was at its lower limit, i.e. the assay detected the minimum amounts of virus possible.

Depending on the isolate, readings for the samples containing 2 ng viral particles did not change significantly in colour with increased incubation time, suggesting that the amount of virus present was not sufficient to affect the substrate concentration. This was not the case at 200 ng, where spectrophotometric readings approached the maximum values over time. In the case of the crude sap samples, the readings were not quantitative and concentrations of the virus would have varied in the extracted crude sap. Therefore, the increase in colour over time suggests that the virus was present at concentrations above 1 ng/µl. Negative controls of healthy, uninfected sap and one supplied by Boehringer Mannheim, Biochemica, were also included in the assay. Values for infected sap of greater than 2 x the readings for these uninfected controls were taken to be positive. Values below these cut-off points for crude sap samples or for purified viral particles were taken as negatives.

From these assays, the enzyme-linked immunosorbent assay was found to be sensitive for viral particles to at least 2 ng in all isolates but, depending on the isolate (strain-Groups 3 and 2, PBI), could detect virus to 200 pg. With the crude sap samples, the detection of viral particles was sensitive to 10^{-2} for 1 hour, 10^{-3} for 2 hours, and 10^{-4} for 5 hours, depending on the virus/sap concentrations.

The PVX (strain-Group 3, PBI) isolate gave the strongest reaction in the assays with spectrophotometric readings on average, above 2.0 (or +++ visually) for all dilutions suggesting a very high concentration of viral particles in the crude sap sample. Even at 10^{-6} the reading was not markedly different from the 10^{-1} reading. The readings for the viral particles of PVX N and PVB isolates (strain-Groups 3 and 2) decreased with increasing dilution (Figs.20

and 21). These results indicated that the sensitivity of the method for the three strains did not vary greatly, presumably reflecting the equal affinity of the polyvalent antibodies for the various strains.

Voller et al., (1976a) applied the method to the two viruses: the arabis mosaic virus (AMV - an isometric virus) and plum pox virus (PPV - a filamentous virus) which are important pathogens. It was found that the viruses could be detected in both purified preparations and in unclarified plant homogenates. A virus titre of 1 ng/ml could be detected by the assay for PPV as compared to 20-200 pg for PVX. This sensitivity for PPV compared compared very well with other methods for the detection of PPV such as radial diffusion (1000 ng/ml); tube precipitation (500 ng/ml); electron microscopy (100 ng/ml); infectivity tests (100 ng/ml - representing less than 1 lesion per inoculated leaf for Chenopodium foetidum. The limits of visual detection for both viruses of colour corresponded approximately to an extinction value of A_{405} nm = 0.1, which is similar to the results obtained for PVX. It was also noted that photometric measurement enabled the detection of virus at a dilution up to 10-fold compared with visual detection.

These results show that the advantages of the ELISA over other labelled-antibody methods such as those which employ latex particles, fluorescent compounds, and ferritin, are its combination of economy of reactants, extreme sensitivity and potential for measurement. It is a highly versatile method, which as shown (Voller et al., 1976a), can detect both isometric and filamentous viruses in both purified preparations and untreated extracts of herbaceous hosts and infected crop plants. The difference in the reaction principle, i.e. which relies upon the detection of non-precipitating reactions using

enzyme-labelled antibodies rather than those based on the formation and detection of immune precipitates, means that the efficiency of the ELISA technique is independent of the ratio of antibody to antigen. Therefore, the virus can be detected at all concentrations once the appropriate concentrations for the antibody preparation are determined. Secondly, the method is good for quantitative measurements since the retention of the enzyme-labelled antibody is a function of and proportional to virus concentration.

Although the enzyme-linked immunosorbent assay is relatively sensitive in detecting virus in infected sap, it is limited by the fact that only viral antigens or coat proteins could be detected. The assay does not indicate whether the particles are infectious or even if the particles are complete. Tomlinson and Walkey (1967) found that fragmented, sonicated PVX resulted in products which were serologically indistinguishable from the intact virus.

Therefore, alternatives were sought which were at least as sensitive as the ELISA, and which could give an indication of the viral particles' potential infectivity. In addition, the new assays had to be relatively rapid and able to handle large numbers of samples for assaying. This was achieved by the hybridisation of complementary probes to plant viral nucleic acids immobilised on a solid support such as a nitrocellulose membrane (see 1.3.10 for details).

4.2.1. Detection of Viral RNA using Probes.

PVX RNA, bound to the filters, was detected by hybridisation to labelled, PVX-complementary DNA. These probes, either labelled with ^{32}P-dCTP or biotin-dUTP, were synthesised from PVX RNA templates.

These assays, unlike the ELISA, used the complementary DNA probes

to detect viral RNA, not viral protein, and were synthesised from RNA or from nick translation of a clone. The first of these two assays used radiolabelled sequences which, after hybridisation, could be detected by autoradiography. The second assay was a non-radiolabelled alternative which overcame some of the disadvantages of the first assay which included i) a defined shelf-life; ii) health hazards during handling; and iii) the use of -70°C freezers and dark rooms for the development of autoradiographs.

The eventual candidate chosen for this alternative probe utilised the strong binding ability of biotin (a small, water-soluble vitamin) to streptavidin (an avidin analogue which has four high affinity sites for biotin). In the process, biotinylated cDNA was bound to complementary PVX RNA sequences during hybridisation and the excess washed off. Streptavidin, then a biotinylated enzyme polymer were then bound to the biotinylated probe/target hybrid. When the substrate dyes (NBT and BCIP) were added, the target DNA was visualised as a purplish spot on the nitrocellulose filter.

Both of these probes (radiolabelled and non-radiolabelled) were assayed on PVX RNA-bound filters and their sensitivity then compared (Fig.22). In the first assay, two prepared filters of serially diluted PVX and BMV RNAs, were hybridised in the presence of i) a ^{32}P-labelled cDNA probe and ii) a biotinylated cDNA probe for 16 h. The result of these hybridisations (Fig.21) showed that the filter which had been hybridised with the ^{32}P-labelled probe (Filter 1) gave much clearer positive signals for PVX RNA from 2 µg to 200 pg. However, hybridisation with the biotinylated probe under the same hybridisation conditions detected PVX RNA to 200 pg (Filter 2). These signals, however, were much weaker and did not decrease from 2 µg to 200 pg in

size like the radiolabelled probe. With the biotinylated probe, the detection of signals resulted from the precipitation of a coloured product and was, therefore, restricted by the size of the aliquoted samples on the filter. Estimation of concentration of RNA using the bioblot is, therefore, more difficult than with labelled probe.

The use of autoradiography for the detection of the signals from radiolabelled probes gave a series of spots on the X-ray film with time. Therefore, even with very low levels of probe, signals from hybridisations with PVX could be picked up if the X-ray film was left in contact with the filter over an extended period of time. This increased the sensitivity of the assay by at least a log. With the biotinylated probe, however, the signals produced on the filter were determined by the precipitation of the dyes on to each site and were not amplified with time. Colour production on the filters reached a maximum after a four hour incubation with the substrate dyes.

The level of non-specific binding was determined by the addition of serially diluted BMV RNA to the filters. Non-specific hybridisation only occurred with the ^{32}P-labelled probe at 2 μg (Fig 22: Filter 1). The biotinylated probe did not cross-hybridise with the BMV RNA at any dilution.

4.2.2. Alternative Radiolabelled Probes.

Further assays were carried out with alternative radiolabelled probes in order to determine if these probes were just as sensitive or more so than the oligo d(T)-primed cDNA probe. The first of these alternative probes was a cDNA probe which had been primed with calf thymus DNA. With a 4 hour hybridisation and a 4 hour exposure, the

probe detected PVX RNA to 200 pg (Fig.23). This sensitivity was comparable to an oligo d(T) probe which had been hybridised to a filter for 2 hours and exposed for 4 hours (Fig.24). The calf thymus-primed cDNA probe, unlike the terminally primed oligo d(T) probe, generated random sequences along the PVX RNA template which were more representative of the entire RNA, and appeared to be as sensitive as the oligo d(T)-primed probe.

To improve the sensitivity of detection, filters were hybridised with radiolabelled probes at the same concentrations (560 ng/ml) but the length of exposure of the film was increased (Fig.26). A 100-fold improvement was observed when exposure of the filter to X-ray film was extended from 20 min to 16 hours (= 48 x)(Fig.26: Filters 1 and 2). This improvement of signal detection increased steadily with increased exposure time.

As previously observed, the radiolabelled probes were cross-hybridising with the BMV RNA control, probably with poly (A) tracts present on the BMV RNA. To confirm this, the filter was prehybridised with 5 mg poly (U) which bound to poly (A) regions. The result of adding poly (U) can be seen in Fig.25. Non-specific cross-hybridisation of the probe with BMV RNA was blocked on the filter with a 4 h hybridisation (100 ng/ml) and an extended exposure time of 16 h at -70°C.

4.2.3. Detection of PVX RNA in Crude Sap.

The aim of these assays was to develop a easy-to-handle protocol which would be sensitive and be able to detect small amounts of PVX RNA directly in samples of crude sap. Therefore, stocks of well-infected plant sap had to be obtained. PVX N was inoculated into Nicotiana tabacum plants and the sap was then collected from 21 plants. Two microlitre aliquots from each plant were then spotted on

to nitrocelluose, fixed and hybridised with ^{32}P-labelled and biotinylated probes as described. The two strongest positives which were detected on the filter hybridised with ^{32}P-labelled probe were spots 7 and 9 (Fig.32).

When the assay was repeated with a biotinylated probe (100 ng/ml)(Fig.33), similar results were obtained after a 4 hour colour development with strong positives for plants 9 (sample 1), 7 (sample 2) and 17 (Fig.33). However, the biotinylated probe showed a greater degree of hybridisation with the remaining sap samples than the radiolabelled probe. This may be due to particulate matter in the crude sap by the biotinylated probe, unlike the ^{32}P-labelled probe. The sensitivity of these two methods was difficult to gauge in comparison with each other. Overall, however, the signals from the autoradiographs with a radiolabelled probe were easier to detect than those from the filters hybridised with a biotinylated probe. The contrast between the signals and the background for the radiolabelled probe was greater and was, in general, usually less than that for the biotinylated probe. With the biotinylated probe, a constant low background of non-specific hybridisation was observed although with the radiolabelled probe, some smearing did occur with a couple of filters which had not been thoroughly washed after hybridisation, e.g. Fig.32, filter 3. The appearance of non-specific signals from traces of radiolabelled probe on the filters also complicated the assessment of signals, e.g. Fig.23, row 3 at 10^{-3}. However, the most disturbing feature of the bioblot was the tendency to give positive signals with high concentration of sap from healthy controls (see Fig.23 but also, by contrast, Fig.27). The reason for this is not clear but may result from trapping of the biotin-AP by particulate matter.

4.2.4. The Detection of RNA with Increased Probe Concentration.

In the dot blot assays, the radiolabelled probe (at 100 ng/ml) hybridised with all the infected sap samples (PVX N (samples 1 and 2); PVX (strain-group 3) and PVB (strain-Group 2)) to 10^{-1} x dilution, although a weaker signal was produced from PVB. Only a slight cross-hybridisation signal was detected with the healthy, uninfected sap (Fig.24).

A duplicate bioblot filter was also hybridised under comparable conditions with a biotinylated probe at 100 ng/ml. With a 2 hour hybridisation and a 4 hour incubation with the substrate, the probe only detected PVX N RNA to 200 ng (compared with 2 ng to 200 pg with an oligo d(T)-primed radiolabelled probe). Clear signals with very little background were noted for the crude sap samples (Fig.27). PVX (strain-Group 3, PBI) gave the strongest reaction where the probe hybridised to 10^{-2} x dilution and to 10^{-1} x dilution with the remaining samples. Using both biotinylated and ^{32}P probes, the sensitivity of detection of purified PVB was one log less than PVX N or PVX (strain-Group 3, PBI)(see Figs.30 and 31). This presumably results from sequence heterogeneity since the two group 3 strains would be expected to be closely related but to differ more from PVB. The sap spot hybridisation is thus more strain dependent than ELISA.

4.2.5. Re-use of Biotinylated Probes: Second and Third Passage.

A reported additional advantage of the biotinylated probes (DNA Detection System Manual: Bethesda Research Laboratories) was the ability of the hybridisation mixture, containing the biotin-labelled probe, to be re-used a second and possibly a third time if stored at 4°C. This possibility of a second or third usage after long term storage (greater than 2 months) was an obvious advantage over the

^{32}P-labelled probe where there was an inherent halflife of 2 weeks.

Assays were set up in which the biotinylated probes, at 100 ng/ml, were used for a second and third passage after 2 months storage when comparable radiolabelled probes had decayed to an extent where products could not be detected by autoradiography (Figs.27-29). The biotinylated probe used (original concentration 100 ng/ml), had been hybridised once before with a filter. After storage and before re-use, the probe was denatured in a boiling bath and cooled on ice just prior to use. Conditions for prehybridisation and hybridisation were repeated as described. A further assay was then carried out using the same probe (which had been already used twice) for a third passage. After the second hybridisation, a duplicate filter was used for the third hybridisation with the probe. It was assumed that the biotinylated probe would have been at a much lower concentration at this stage, having 1) already gone through two hybridisations with a corresponding reduction in probe concentration, 2) the probe would have possibly undergone some degradation, and 3) possible non-specific hybridisation of the probe to the nitrocellulose filters. Taking these into account, the hybridisation time was lengthened to 4 hours to compensate for the reduction of the complementary probe after its two previous hybridisations.

The results of these repeated hybridisations can be seen in Figs.27-29. Overall, the signals are much fainter with a greatly increased level of background non-specific hybridisation. Surprisingly, the detection of PVX (strain-Group 3, PBI), which had given the best results (to 10^{-2}) in the first hybridisation, decreased sharply in the second hybridisation where only the undiluted sample could be detected. In the third and final hybridisation, this hybridisation of the probe with PVX (strain-Group 3, PBI) decreased

even further, resulting in a faint signal in the undiluted sample (Fig.29).

For the other sap samples in the third hybridisation, the probe detected RNA in both the PVX N samples (samples 1 and 2) and PVB (strain-Group 2) to 10^{-2} x dilution, although these signals were very faint. These results represented a 10-fold increase in sensitivity detection over the results from the second passaged filter when the incubation time was increased. The signals for PVB-infected sap (a group 2 strain) were stronger than those for PVX N (sample 1) and PVX (PBI) and equal to those for PVX N (sample 2) in both the second and third hybridisations and indicated that biotinylated probes which were specific for strain-Group 3 could also be used for strain-Group 2.

With the samples of purified PVX RNA spotted on the filter, the probe, in both cases (second and third passage) gave very weak signals, especially for the third hybridisation.

When a biotinylated probe was re-used for a second and third hybridisation, slightly better results were obtained for infected sap (for certain isolates from both strains) in that the signals, though fainter, could still be detected. These alterations in sensitivity to various strains on re-use of probe indicate that the re-use of probes is unlikely to be a practical possibility in screening applications.

Baulcombe et al. (1984) detected purified virus particles to 1 ng and 50 pg purified RNA from samples spotted as 1 µl aliquots on nitrocellulose, both of which were slightly more sensitive than for the radiolabelled or biotinylated probes, possibly because a nick-translated probe was used in their assays cloned from a longer sequence of the RNA template. Baulcombe et al. (1984), however, did not compare the sensitivity of either the probe or their ELISA for PVB, strain-Group 2 and, therefore, a direct comparison of results is

not possible.

4.2.6. Assays with Reduced Probe Concentrations.

Sap spot assays were also carried out in order to determine the amount of reduction in sensitivity in both probes at reduced concentrations. In the first assay, the concentration of the biotinylated probe was reduced to 30 ng/ml. The filter was prehybridised with poly (U), hybridisation was extended for 16 hours, and the colour development allowed to develop for 16 hours. The filter (Fig.30) was divided into three: the first section was for infected and uninfected crude plant sap samples; the second section contained purified viral particles from all isolates; and lastly, a section for PVX N and BMV RNAs.

With the infected crude sap samples (Fig.30: rows 1-5), faint signals were picked up for all samples to 10^{-1} x dilution, except for the PVX (strain-Group 3, PBI) isolate which was detected to 10^{-3} x dilution. The PVX N-infected sap (strain-Group 3, sample 2) hybridised less than the others and was only detected in the undiluted aliquot. However, with this extended hybridisation of the filter with probe, the healthy, uninfected control was also non-specifically hybridised (Fig.25). Therefore, using reduced probe concentrations only produced clear signals on short hybridisations. Trying to improve the sensitivity by increasing the hybridisation time failed because of non-specific hybridisation with the control.

The probe was sensitive to PVX N RNA to 200 pg although the signal was very faint. With both filters, the addition of poly (U) cancelled non-specific cross-hybridisation with BMV RNA.

4.2.7. Sap Spot Hybridisation using cDNA Clones as Probes.

Duplicate filters, spotted with PVX isolates were hybridised with cloned probes (Methods 2.4.4). The nick-translated probe was highly specific for PVX N RNA but was only capable of detecting the RNA to 20 ng with a reduced sensitivity when compared to results obtained with ^{32}P-labelled or biotinylated cDNA probes. In addition, there was very little cross-hybridisation with PVB (strain-Group 2).

Baulcombe et al. (1984) reported the detection of 50 pg bound PVX on nitrocellulose, using a cDNA copy of the common European strain (strain-group 3) potato virus X RNA and amplified by cloning into E.coli plasmid pBR322 at the Pstl site. Initially, 24 clones were screened by electrophoresis of colony lysates in agarose gels to determine the size of the inserts. Plasmid DNA was isolated from the clone with the largest insert. The size of the largest clone pPVX13 was 1100 bases long. By using this insert as a probe, Baulcombe et al. (1984) detected down to 1 ng of purified virus or 50 pg of purified RNA. They inferred that, since the virus is 5 % RNA, these values indicated that the sensitivity of detection was unaffected by encapsidation of the virus and confirmed that pretreatment to disrupt the virus particle was unnecessary. The increased sensitivity reported by Baulcombe et al. (1984) can be accounted for by the larger size of their insert used for the synthesis of the nick translated probe (1100 bp compared to 550 bp for pPVX N 21). The distribution of sequence heterogeneity between PVX group 2 and 3 strains is not known. However, clones containing short cDNA sequences, primed by oligo d(T) may well be biased towards 3' terminal regions of the RNA. These contain the gene for the coat protein (Morozov et al., 1983) which is likely to show heterogeneity between strains. It may be significant that cDNA primed by calf thymus oligonucleotides also hybridise more strongly to PVB than cDNA primed with oligo d(T).

Baulcombe et al. (1984) also reported the use of ELISA as a comparison between the two methods. Their results detected 4.3 ng of virus at 1000 ng/ml with the sap spot hybridisation method. Compared with their results, results from the ELISA (Fig.20) for PVX N (sample 1) and PVX (strain-Group 3, PBI) were more sensitive, detecting to between 20 pg and 200 pg with a 2 h incubation with substrate. Waterhouse et al. (1986) carried out dot-blot assays using ^{32}P-labelled plasmid probes which detected barley yellow dwarf virus (BYDV) in crude sap extracts from infected plants which were at least as sensitive as ELISA (to 10^{-3} in crude sap extracts and 1 ng for viral particles). Depending on the clone, the probe was specific for one or both serotypes of the virus. Other cloned probes hybridised with both serotypes as well as with 3 other luteoviruses. Their probes detected less than 10 ng for RNA and between 10-100 ng for viral particles.

The practical application of this dot-blot method was first described by Brandsma and Miller (1980) which was applied to the detection of Epstein-Barr virus (EBV) sequences in lymphoblastoid cells. The cells to be tested were immobilised on a nitrocellulose membrane. The DNA in the immobilised sample was denatured in situ and hybridised to a probe consisting of radioactively labelled EBV DNA.

Other DNA viruses which can be detected using this method include herpes simplex virus (HSV) in infected cells (Stalhandskke and Pettersson, 1982) and human cytomegalovirus (CMV) in urine specimens (Chou and Merigan, 1983; Spector et al., 1984; Virtanen et al., 1984). The detection level of CMV is approximately 5 pg of viral DNA.

The first practical application of the spot hybridisation method for the detection of RNA viruses was the detection of the potato spindle tuber viroid (Owens and Diener, 1981). The method, based on

the enzyme-linked immunosorbent assay, permitted the sampling of 300 tubers per hour. PSTV was covalently bound to both diazobenzyloxymethyl (DBM) paper (which proved to be difficult to handle and expensive) and also to nitrocellulose (simpler to use, inexpensive and producible). Hybridisation was carried out with a clone (pDC-29) which contained almost the entire 359-nucleotide sequence. PSTV bound stably to both the DBM paper and the nitrocellulose and that, after autoradiography, the virus could be detected between 250 pg and 83 pg. This was comparable to the results with the radiolabelled cDNA probe for PVX N RNA which could be detected to 200 pg (Fig. 22). In a similar assay, ^{32}P-labelled cloned cDNA probes of the simian rotavirus SA11 specifically detected rotaviral RNA sequences and were more sensitive for detecting SA11 than the commercial enzyme-linked immunosorbent assay test (Dimitrov et al., 1985). A full-length probe of the SA11 gene 6 detected about 270 pg of purified SA11 ds RNA. Under the conditions for PVX, maximum sensitivity of detection of PVX was 200 pg. The system thus appears to be comparable with that used to detect DNA viruses. These results with PVX compare well with those for RNA viruses and viroids. However, the cloned probe detected PVX N RNA to only 20 ng.

The use of the spot hybridisation for the detection of RNA is, in general, more complicated, since RNA binds less well to nitrocellulose and is more sensitive to high pH and to nucleases than DNA. The construction of the probe also involves more work since cDNA copies have to be cloned rather than DNA fragments.

Alternative probes incorporating non-radioactive nucleotides were also assayed together with the ^{32}P-probes. The description of the synthesis of analogs of dUTP and UTP that contain a biotin molecule covalently bound to the C-5 position of the pyrimidine ring through an

allylamine linker arm has been described by Langer et al. (1981) (Fig.3). They found that polynucleotides containing low levels of biotin substitution (50 molecules or less per kilobase) have denaturation, reassociation, and hybridisation characteristics similar to those of unsubstituted controls. The method used for the visualisation of the biotin-labelled probe hybridised to DNA or RNA immobilised to nitrocellulose involved incubating the probe with the preformed complex made with avidin-DH (or streptavidin) and biotinylated polymers of intestinal alkaline phosphatase. The filters were then incubated with a mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT). The sensitivity of the system was claimed to be in the region of 20-30 pg of target DNA. These reported values are much more sensitive than the results obtained with PVX. However, it has been suggested that the method may be more sensitive for the detection of DNA than for RNA bound to nitrocellulose. The levels of detection for potato virus X in the assays using biotin-streptavidin was sensitive to 200 pg (Fig.22) with a PVX N cDNA probe at 100 ng/ml and a four hour colour development. This was equivalent to the sensitivity obtained with ^{32}P cDNA at 560 ng/ml and exposed to film for 4 hours. The problem arising from the transfer and covalent coupling of RNA to activated cellulose paper (DBM paper) according to the methods of Alwine et al. (1977) and Alwine et al. (1980) has been reported by Thomas (1980). The use of activated paper for coupling RNA was found to present two main problems: that only 500 pg of specific RNA per band was just detectable (after several days exposure) after hybridistion using high activity probes prepared by nick translation (55,000 cpm/µg) after the technique for the separation of DNA fragments by electrophoresis (Southern, 1975). Although this sensitivity was similar to other

reported values (Alwine et al., 1977; Alwine et al., 1980), it was estimated that this was only 1-10 % of that for detecting specific DNA sequences on nitrocellulose by using similar probes. It was concluded that the increased sensitivity of the nitrocellulose paper probably reflected that the nitrocellulose has a higher binding capacity for DNA (about 80 $\mu g/cm^2$) compared to the capacity of most preparations of activated paper for binding RNA or DNA (1-2 $\mu g/cm^2$). Secondly, the preparation and activation of DBM paper was expensive, time consuming, and most importantly, variable.

As an additional step in the two phase hybridisation procedure, dextran sulphate was included according to the technique by Wahl et al. (1979). Wetmur (1975) observed that anionic dextran polymers accelerate reannealing of DNA in solution. Therefore, such compounds should also accelerate hybridisation of probes to immobilised DNA. The use of dextran sulphate was tested by Wahl et al. (1979). In hybridisation experiments with DNA from a hamster cell mutant, they found that the signal obtained after a fixed time of hybridisation increased dramatically with increasing concentration of the polymer. After 2 hours of hybridisation in the presence of 10 % dextran sulphate the signal obtained was 3-4 times greater than that after 72 hours in its absence. Wetmur (1975) has attributed the 10-fold increase in the reassociation rate caused by 10 % dextran sulphate in homogenous solution to exclusion of DNA from the volume occupied by the polymer - i.e. by concentrating the DNA. In a two-phase hybridisation, only the soluble component can be concentrated, resulting in a 3-4 fold increase with the single-stranded probe as observed.

4.2.8. Summary and Comparison of Screening Methods.

The different methods discussed above were found to vary in sensitivity when used in the detection of virus particles, both in crude sap and purified, and in the detection of RNA. In the enzyme-linked immunosorbent assays (ELISA's), isolates from the two strains of PVX were compared using both purified virus and virus in crude sap. The assay on purified virus showed very little difference in sensitivity of detection between the three isolates (PVX - samples 1 and 2, and PVB - strain-Group 2) at any dilution. The assay detected virus particles to 20 pg and lengthening the incubation time with the dye did not affect the sensitivity.

In assays for infected crude sap, PVX (strain-Group 3) gave the strongest reactions in all dilutions at 1, 2, and 5 hour incubations. The positive signals were easily distinguishable, even at 10^{-6} x dilution. This was probably due to the greater virus levels in the plant and was supported by the results of viral particle extractions. However, the remaining two isolates (PVB and PVX N) showed substantial decreases in absorption at A_{405} nm with each log dilution. With these two isolates the virus particles could still be clearly detected at 10^{-4} x dilution.

The sensitivity of the assay was also increased by lengthening the incubation time for PVB and PVX N (Samples 1 and 2) where colour production was enhanced, especially in the 20 ng - 200 pg range. Increased incubation times had no effect on PVX (strain-Group 3, PBI). The assay was, therefore, sensitive for purified viral particles to 200 pg for strains 2 and 3 (PBI), but only to 2 ng with PVX N (strain-Group 3, samples 1 and 2). With crude sap, the assay was sensitive to 10^{-4} x dilution for PVX N (sample 1) after 1 and 2 hour incubations, and to 10^{-5} x dilution for a 5 hour incubation. The assay was slightly less sensitive for PVX N (sample 2), detecting the virus

to 10^{-2} x dilution after a 1 hour incubation, 10^{-3} x dilution after 2 hours and 10^{-4} x dilution after 5 hours. These discrepancies presumably reflected different levels of virus in different plants. Indeed, when 21 sample plants were grown under similar conditions, major differences were observed in the hybridisation signals (Figs. 32 and 33). The reason for these apparent differences in virus spread is not clear.

PVX (strain-Group 3) was easily detectable at 10^{-6} x dilution at all three incubation times. Therefore, with the enzyme-linked immunosorbent assay, the method using polyvalent antibodies, detected more than one strain of PVX and was, therefore, more useful in the detection of various PVX strains in possible field trials.

As a contrast to the ELISAs which only detected viral particles, complementary probes (which could directly detect infectious RNA) were also compared. In a direct comparison between a biotinylated probe and a radiolabelled probe, both detected PVX RNA to 200 pg although the positive signals were easier to see with the radiolabelled probe, mainly due to the amplification of signals during autoradiography. There was also some slight non-specific binding at the higher concentrations of RNA with the ^{32}P-labelled probe although this was cancelled with the addition of poly U to the hybridisation mixture. The biotinylated probe did not react non-specifically at any dilution.

An alternative calf thymus-primed, ^{32}P-labelled probe to PVX N RNA (a homologous probe) detected PVX N RNA to 2 ng (10-fold less than the oligo d(T)-primed probe at the same incubtion time). Its sensitivity was comparable to the oligo d(T) probe. This was expected since the calf thymus-primed probe qas more likely to contain a greater variety of the PVX RNA sequence. The sensitivity of detection using the ^{32}P-labelled probe was improved up to 100-fold by extending the

exposure time of the filter to the X-ray film, e.g. in Fig.26, the ^{32}P-labelled probe detected 20 ng PVX N RNA with a 20 min exposure but increased its sensitivity of detection to 200 pg with a 16 h exposure.

After testing the probe's sensitivity on RNA, the ^{32}P probes were next tested on crude sap, directly obtained from infected plants. At the optimal concentration of probe and a 4 h exposure, PVX N (samples 1 and 2), PVX (strain-Group 3, PBI) and PVB (strain-Group 2, PBI) were all detected to 10^{-1} x dilution. With a biotinylated probe of the same concentration, the sensitivity for PVX (strain-Group 3, PBI) was improved over the ^{32}P probe, detecting RNA in crude sap to 10^{-2} x dilution (PVX strain-Group 3, PBI) and to 10^{-1} x dilution for all other samples. The positive signals with the biotinylated probe could all be easily identified, unlike the ^{32}P-labelled probe where the PVB signals were weaker than the rest. These results, however, were less sensitive than the ELISA which detected isolates to at least 10^{-4}.

Since the biotinylated probe did not have an inherent half life of two weeks unlike the radiolabelled probe, the probe (with a reduced concentration after the first hybridisation) could be re-used a second and third time after long term storage at -20°C. In the original hybridisation with a 100 ng/ml PVX N cDNA probe in a 2 h hybridisation, the probe detected all isolates in crude sap to 10^{-1} x dilution except for PVX (strain-Group 3, PBI) which it detected to 10^{-2} x dilution.

After storage, and with an extended hybridisation (4 h), the probe only slightly decreased its sensitivity for all isolates (10^{-2} x dilution) with the exception of PVX (strain-Group 3, PBI) which was only detected in the undiluted sap. This is a log decrease for all samples but a 2 log decrease in sensitivity for PVX (strain-Group 3, PBI). However, all signals were faint and a noticeable increase in

background hybridisation was observed.

Although the probe successfully detected RNA in crude sap, the signals were much weaker for purified RNA in the second and third hybridisations, only detecting between 2 µg and 200 ng. Re-using the probes is, therefore, unlikely to be a practical possibility.

The sensitivity of the probes was also determined at reduced concentrations. A biotinylated probe (at 30 ng/ml) was used but with the hybridisation time and incubation time/exposure time extended to 16 h. The results were only slightly better than the original results for the first hybridisation. However, an increase of non-specific hybridisation was also noted throughout the filter, making identification of some of the more dilute samples difficult. This suggested that the use of probes at lower concentration could only be used with greatly extended incubation times and with a corresponding increase in non-specific hybridisation.

With purified viral particles, the low concentration biotinylated probe detected the viral particles to 20 ng for PVX N (sample 1) and PVX (strain-Group 3, PBI) and to 200 ng for PVB (strain-Group 2, PBI).

A low concentration radiolabelled probe (1.7 ng/ml) was also used. At this concentration, however, the probe only detected PVX (strain-Group 3, PBI)-infected sap to 10^{-1} x dilution and only in the undiluted sap for PVX N (sample 1). The probe did not detect PVB (strain-Group 2) in the crude sap sample at all at this concentration although it did detect PVB at 2 µg of viral particles (a log less than for the PVX isolates). Non-specific hybridisation was blocked by the addition of poly (U) to the pre-hybridisation solution.

These results show the versatility of the ELISA and dot blot screening methods. Variations on these two basic methods have not been

developed for use in possible future screening for plant viruses. Hibi and Saito (1985) based their assay on the ELISA but bound the antigen/antibody to nitrocellulose. Their method was sensitive to 20 pg for purified TMV antigen which is comparable to results for the isolates of PVX antigens using the ELISA. However, one reported disadvantage was non-specific reactions with the filter but it was suggested that these could be minimised by using antibodies purified through affinity chromatography columns or monoclonal antibodies.

Another, newer ELISA-based assay has been developed by Pugh et al. (1985) which uses two separate enzyme systems to amplify signals (Fig.45). In this method, one molecule of NAD triggers the production of many molecules of the dye formazan which greatly increases the sensitivity of the test.

The biotin-avidin interaction has also been used in recent immunoassays (Guesdon et al., 1979; Barnard et al., 1985). Guesdon et al. (1979) developed two methods: the Bridged Avidin-Biotin (BRAB) and the Avidin-Biotin (LAB) techniques (Figs.46 and 47) to detect bound antigen. The method was improved by Hsu et al. (1981). The applicatin of the biotin-labelled secondary antibody is followed by the addition of avidin-biotin-peroxidase complex. The avidin bridges biotin-labelled peroxidase molecules and therefore, a lattice of several peroxidase molecules is formed. When this is bound to the biotin associated with the secondary antibodies, a high staining intensity results. The applicability of these methods, however, will eventually be dependent upon the suitability of each method for large scale screening, as well as the cost and the time needed to obtain results.

	ELISA	^{32}P-blot	Bioblot
PVX N sap	10^{-5}	10^{-4}	10^{-1}
PVB sap	10^{-5}	10^{-2}	10^{-1}
PVX-3 sap	10^{-6}	10^{-2}	10^{-2}
PVX N VP	20-200 pg	200 ng	20 ng
PVB VP	20-200 pg	20-200 ng	20-200 ng
PVX-3 VP	20-200 pg	20 ng	20 ng
PVX N RNA	-	200 pg	200 pg

A table showing the maximum sensitivities of the various assays (ELISA, ^{32}P-blot and the bioblot) under optimal conditions in the detection of virus in crude sap, purified viral particles, and PVX N RNA.

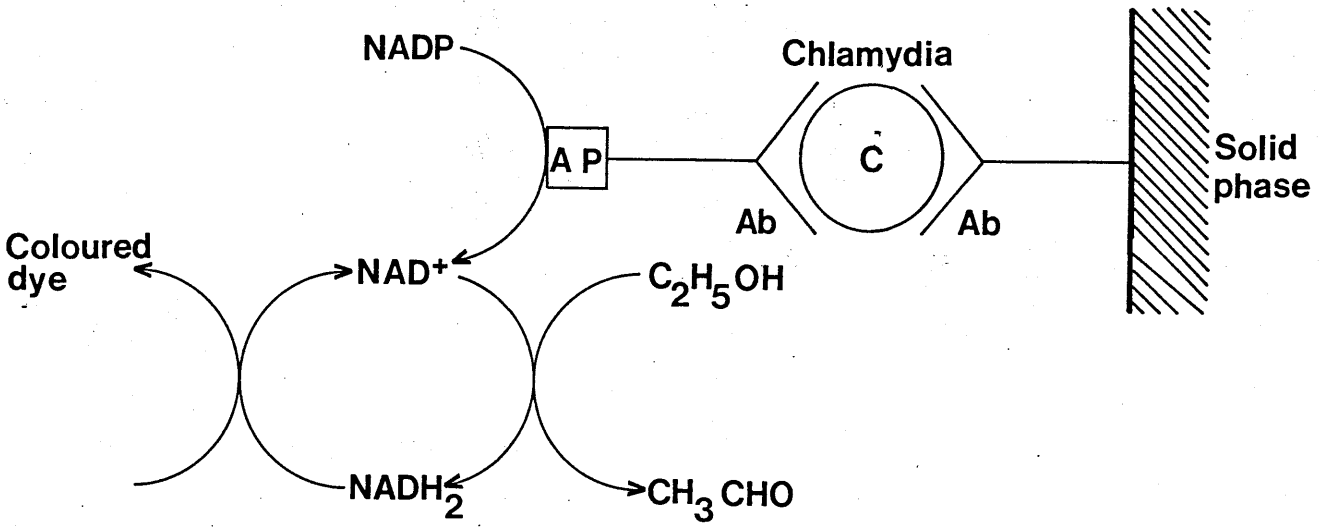


Fig.45: Enzyme amplified immunoassay with amplification process in two stages (Pugh et al., 1985).

AP Alakaline phosphatase

C Chlamydia polysaccharide

NADP Nicotinamide adenine dinucleotide phosphate

NAD Nicotinamide adenine dinucleotide

Fig. 46.

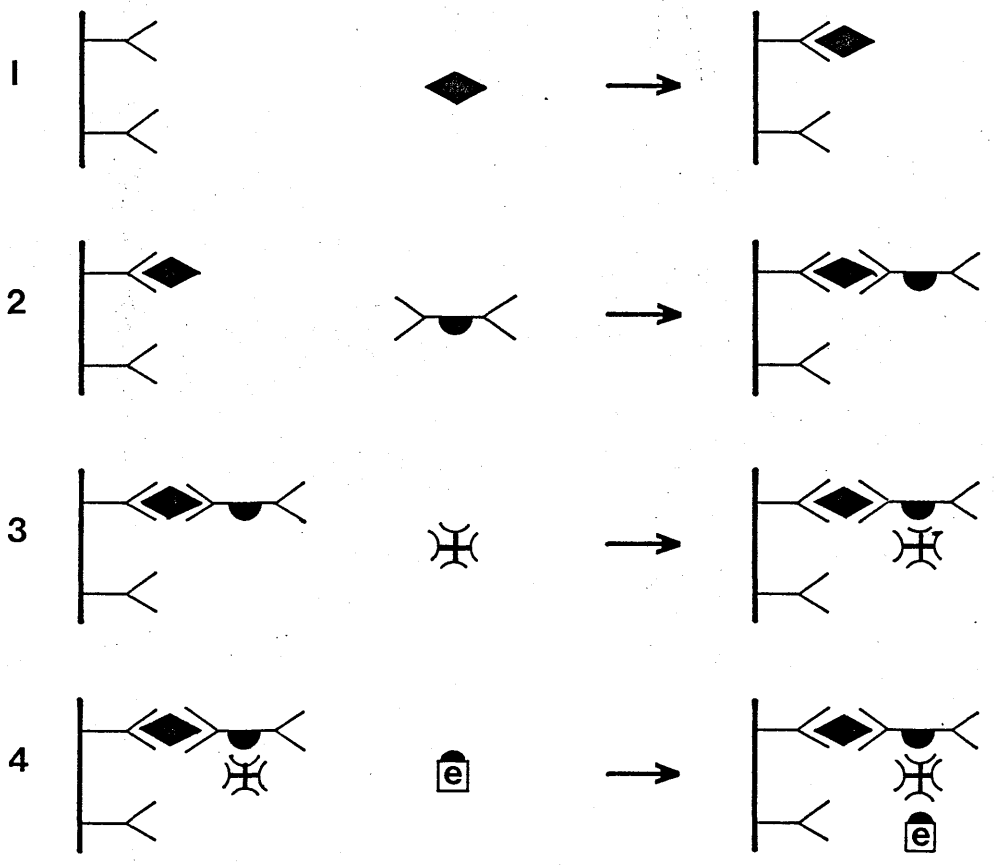


Fig.47

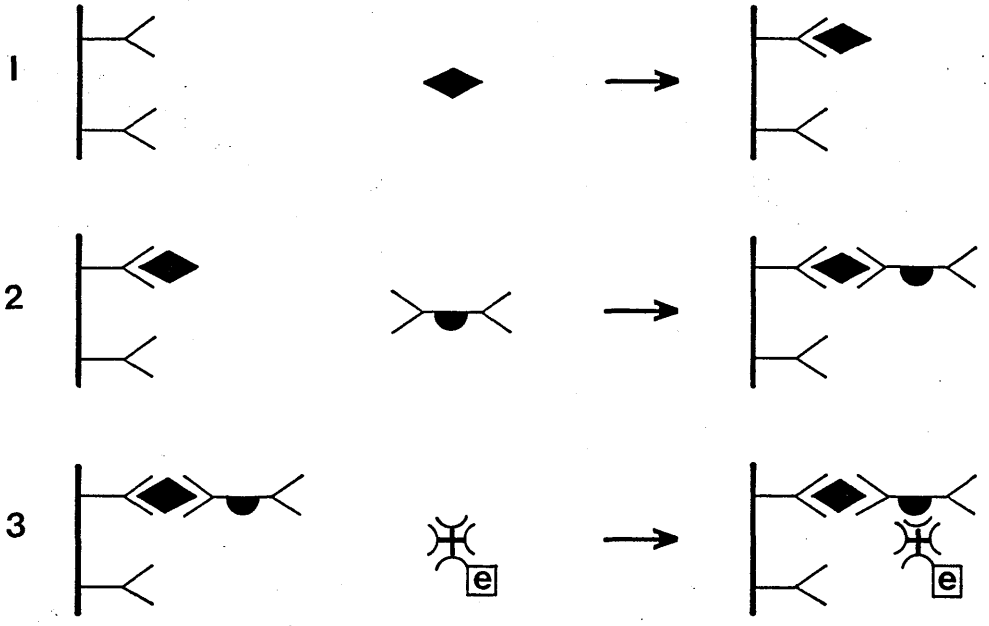
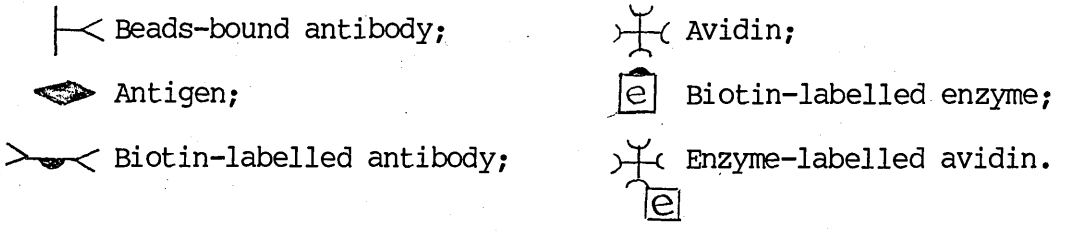


Fig.46 and 47: Principles of the BRAB-LAB techniques for the quantitation of an antigen.

Fig.45: the BRAB technique; 1) fixation of the antigen on the immobilised antibody; 2) fixation of the biotin-labelled antibody on the solid body; 4) binding of the biotin-labelled enzyme to the avidin followed by the determination of the enzymatic activity.

Fig.46: the LAB technique; 1) fixation of the antigen on the immobilised antibody; 2) fixation of the biotin-labelled antibody on the solid phase; 3) binding of the enzyme labelled avidin to the biotin-labelled antibody, followed by the determination of the enzymatic activity (from Guesdon et al, 1979).



4.3. The 3' Terminal Region of PVX RNA (Strain-Group 3): Structural Studies.

With few exceptions, studies to determine the 3' terminal structure of PVX RNA have been limited. Sonenberg (1978) reported the presence of of a methylated terminal at the 5' terminus and the absence of a poly (A) region at the 3' terminus, was determined by affinity chromatography on oligo d(T) sepharose. However, Morozov et al. (1981) and Morozov et al. (1983) reported the presence of 3' poly (A) sequences in PVX RNA of 50-200 nucleotides in length. My results support the results of Morozov et al. (1981)and clearly demonstrate that the 3' terminus possesses poly (A)-rich tracts which are of sufficient size to prime the synthesis of of complementary DNA from the RNA template. Initial experiments compared the synthesis of cDNA in the presence and absence of primers (oligo d(T) and calf thymus DNA). Results from these experiments led to affinity chromatography studies using oligo d(T) cellulose, capable of binding oligo- or poly (A)-rich regions in RNA. These experiments confirmed the results of the primed cDNA synthesis experiments.

However, these results from the cDNA synthesis experiments only indicated the presence of poly (A)-rich regions but gave no indications of the size(s) of these tracts and their exact location in the genome, while the oligo d(T) results suggested tracts of poly (A) of at least 20 nucleotides in length (i.e. the minimum number of nucleotides needed to bind to oligo d(T)). More direct studies were, therefore, undertaken using specific ribonucleases. Ribonuclease H (RNase H), cleaves the RNA endonucleotically of RNA-DNA hybrids to produce a 3' hydroxyl and a 5' phosphate at the point of cleavage (Donis-Keller 1979; Berkower et al., 1973; Henry et al., 1973; Stavrianopoulos and Chargaff, 1973; Haberkern and Cantoni, 1973; Stavrianopoulos and Chargaff, 1978; Stein and Hausen, 1969; Hausen and

Stein, 1970). The resulting fragments were then labelled, purified and sized by electrophoresis. Any poly (A) segments would be hybridised into duplex form. The single stranded RNA would then be cleaved into two or more fragments. This would allow an internal poly (A) tract along the genome to be detected. No such cleavage was detected after RNase digestion, suggesting a terminal location for the poly (A).

The size of the poly (A) sequence was also determined experimentally with a double-digest of RNase A and RNase T1. The RNase A specifically hydrolysed pyrimidine nucleotides leaving pyrimidine 3' phosphates while the RNase T1 hydrolysed the RNA at every guanosine, leaving a guanosine 3' phosphate. Therefore, digestion with both enzymes left poly (A)-rich regions which were resistant to the action of both ribonucleases. These regions could then be sized on polyacrylamide gels and visualised by autoradiography. Contrary to the published literature of Sonenberg (1978) but in agreement with the results of Morozov et al. (1981), the presence of poly (A) sequences at the 3' terminus of PVX RNA was, therefore, conclusively demonstrated. The 3'terminus of the RNA genome was also directly sequenced using a relatively new method which utilised a range of base-specific ribonucleases. Taken together, this range of experiments produced data which directly contradicted Sonenberg's published data (1978) on the absence of poly (A) sequences at the 3' terminus of PVX RNA, while supporting the published data by Morozov et al. (1981).

4.3.1. Synthesis of First-Strand cDNA from PVX RNA.

In the first experiments, cDNA synthesis using in vitro polyadenylated PVX RNA primed with oligo d(T) was compared with:

1) native PVX RNA without primer;

2) BMV RNA with oligo d(T) primer;

3) *in vitro* polyadenylated PVX RNA with calf thymus primer; and

4) *in vitro* polyadenylated PVX RNA without primer.

Results (Table 4) showed that the greatest amount of cDNA synthesised was from the *in vitro* polyadenylated PVX RNA sample followed by native PVX RNA. Depending on the conditions, however, the amount of cDNA synthesised from the RNA template varied a great deal. The percentage copied from the RNA template varied from 2.2 % to 53.0 % for native RNA and from 3.5 % to 21.9 % for the *in vitro* polyadenylated RNA suggesting oligo or poly (A) sequences of sufficient length to bind with the oligo d(T) primer and initiate the synthesis of a complementary strand.

Negruk *et al.* (1979) demonstrated a tRNA-like structure in barley stripe mosaic virus which could self-prime a complementary strand to its RNA. In order to show that this was not the case with PVX, a duplicate reaction mix was set up without the primer. No incorporation of radiolabelled nucleotides was observed (Table 4) suggesting the presence of a poly (A) sequence requiring complementary primers such as oligo d(T) to initiate cDNA synthesis rather than a self-priming tRNA-like structure.

4.3.2. Passage of PVX RNA through Oligo d(T) Cellulose.

Details of the passage of PVX RNA through oligo d(T) cellulose have been given in Section 3. The amounts of bound and unbound RNA were determined spectrophotometrically.

The results showed that, after its first passage through the oligo d(T) cellulose, 56.6 % PVX RNA bound to the oligo d(T) cellulose while 43.4 % passed through the column.

The oligo d(T) cellulose, used for the separation of the poly (A)+ and poly (A)- fractions, contained polymers of about 20 deoxythymidylic acid (dT) which were covalently coupled to washed and defined cellulose by modification of the procedure of Gilham (1964) (P.L.Biochemicals specification sheet). Under appropriate conditions, the 3' poly or oligo (A) segment of the RNA formed hydrogen bonds with the cellulose-bound oligo d(T). Other RNA species lacking a poly (A) segment (such as ribosomal and transfer RNA) do not bind to the oligo d(T) and pass through the column. A buffer of low ionic strength is then used to disrupt hydrogen bonds and to elute the poly A+ RNA (Aviv and Leder, 1972; Nakazato and Edmonds, 1972). The affinity chromatography techniques using oligo d(T) cellulose indicated the presence of poly (A) sequences in the PVX N RNA but did not determine the sizes of these sequences.

Lindberg and Persson (1972) demonstrated that mRNA from polysomes of KB cells (a human cell line containing poly (A) sequences of approximately 180 nucleotides) was isolated by affinity chromatography on columns of polyuridylic acid covalently linked to sepharose. Lindberg et al. (1972) also reported the retention of at least 90 % poly (A)-containing polysomal mRNA by poly (U) sepharose. Nakazato and Edmonds (1972) obtained similar results using affinity chromatography techniques with polythymidylate (poly (T)) cellulose to separate poly (A)-containing and non-poly (A)-containing RNAs. The poly (A) sequences present in the RNAs were sequences of about 150 nucleotides in length. Agranovsky et al. (1982) indicated that a sequence of 25 adenine nucleotides of a similar type to that used in the experiment was needed for binding to the oligo d(T) cellulose. The apparently rather weak binding of PVX RNA to oligo d(T) cellulose suggests a

short oligo (A) tract of less than 25 nucleotides.

4.3.3. Size Determination of the Poly (A) Sequence in PVX RNA: I.

Many plant viruses have been found to lack polyadenylic acid segments at their 3' termini. Sonenberg (1978) similarly reported the absence of a poly (A) region for PVX RNA, based upon studies carried out with affinity chromatography using poly (U) sepharose (contrary to the results of Morozov et al. (1981). However, results from oligo $d(T)_{10}$ cellulose experiments, RNase H, and the synthesis of cDNA using native RNA primers, the presence of a poly (A) segment in the genome of PVX RNA was indicated which was of sufficient length to initiate cDNA synthesis and bind to oligo d(T) cellulose. Sizing experiments were, therefore, undertaken using enzymatic digestion according to the methods of Negruk et al. (1979) and Agranovsky et al. (1983).

Poly (A) fragments were obtained from total PVX RNA by treatment with the RNases A and T1. The isolation of these fragments followed the method of Negruk et al. (1979) and was found to contain tracts of poly (A) of 8-16 nucleotides. These results can be compared with the results of Negruk et al. (1979) who prepared poly (A) fragments from barley stripe mosaic virus (BSMV) using 3 "drastic" conditions in which the concentrations of the RNase solutions were varied. Their results showed that the last method, with the greatest amount of RNase activity, contained over 99.9 % adenosine nucleosides. After polyacrylamide gel electrophoresis, a set of fragments 9 to 28 nucleotides long were produced with most of the fragments being 10-15 residues long. This can be compared with the results obtained with PVX RNA after digestions with RNases A and T1 with the range of fragments between 8-16 nucleotides long. AbouHaidar (1983) reported the presence

of poly (A) sequences of variable size (between 75-100 residues) in another member of the potexvirus family, clover yellow mosaic virus. These sequences were isolated by RNase A and T1 digestion, as well as affinity chromatography with poly (U) sepharose, and subsequent polyacrylamide gel electrophoresis. Results indicated that the poly (A) sequences were located at the 3' terminus of the RNA.

Later work by Agranovsky et al. (1981) and Agranovsky et al. (1982) showed that BSMV, containing an internal polyadenylated sequence, would be resolved by oligo d(T) cellulose into bound (poly (A)+) and unbound (poly (A)-) fractions. Agranovsky et al. (1983) compared the poly (A) length in the total, poly (A)+ and poly (A)- BSMV RNA fractions and found that the length of the poly (A) in total BSMV RNA was 8-40 residues. Fragments of 19-40 residues were predominant in the poly (A)+ RNA fraction while the bulk of the poly (A)- fraction contained tracts having 12 or fewer residues.

4.3.4. Position of the Poly (A) Sequence in PVX RNA: I.RNase H.

The presence of an internal poly (A) sequence in a genome has been demonstrated using RNAse H. The enzyme, RNase H (purified from E.coli) attacks RNA-DNA hybrids, breaking the RNA endonucleotically to produce a 3' hydroxyl and a 5' phosphate at the point of cleavage. The enzyme is specific for RNA-DNA hybrids and does not hydrolyse RNA-RNA hybrids, single-stranded RNA or DNA, or DNA-DNA hybrids (Donis-Keller, 1979; Keller and Crouch, 1972; and Stein and Hausen, 1969). Agranovsky et al. (1982) cleaved BSMV into two fragments with RNase H at the position of the poly (A) or oligo (A) sequence in the presence of oligo $d(T)_{10}$. The shorter fragment (Sh) retained the ability of intact viral RNA to be aminoacylated, i.e. it represented the 3'-terminal

part of BSMV RNA. Electrophoretic analysis of the Sh-RNA revealed three closely positioned subspecies with an average length of about 210 nucleotides. The remaining long 5'-terminal RNA fragment (L) produced by RNase H treatment had an electrophoretic mobility similar to that of intact BSMV RNA. In the method, a synthetic DNA oligomer (e.g. a tetramer of known sequence) will hybridise to complementary single-stranded regions of an RNA molecule and will direct cleavage by RNase H to those sites. DNA oligomers of different sequences will direct cleavage by RNase H to other regions of the RNA molecule. In the case of PVX RNA, the 3' terminal region of the RNA was labelled (see methods 2.9 ii), then hybridised with oligo $d(T)_{12-18}$ nucleotides to produce the RNA-DNA hybrids, and hydrolysed with RNase H. The resulting fragments were then electrophoresed on a 5 % polyacrylamide gel.

Originally, on the basis of the reported lack of a 3' terminal poly (A) by Sonenberg et al., (1978) it was thought that poly (A) regions of PVX N RNA would be of a similar nature to BSMV, having both the shorter fragment representing the 3' terminal region and the much longer fragment representing the rest of the genome when digested with RNase H. However, the results of RNase H-digested PVX RNA produced the long fragment of the genome as expected but without the labelled shorter fragment. This indicated that the poly (A) sequences were sited very close to or at the 3'-terminal region of the RNA as reported by Morozov et al. (1981) rather than some way into the 3' terminus as in BSMV RNA.

The result of the gel after staining (Fig.36) showed only a slight reduction in the size of the RNA (indicated by a faster migration rate of the major, larger fragment) when compared to the control RNA. The

terminally labelled nucleotides could not be detected by autoradiography indicating a 3' terminal or close to the 3' terminal location of the poly (A) sequences in the RNA. These smaller, labelled fragments had then spread out and migrated off the gel.

Therefore, PVX N RNA, though possessing a poly (A) sequence like BSMV, differed in the positioning of this sequence. In BSMV, the poly (A) tract is situated about 210 nucleotides into the 3' terminus producing two distinctive bands after RNAse H digestion. With PVX, the poly (A) sequence is at or very near to the 3' terminus, and after digestion, leaves only the major fragment.

4.3.5. Determination of the 3' Sequence of PVX RNA.

Following the information gained from the digestion of PX RNA with RNases A and T1, the 3' terminal region of PVX RNA was directly sequenced using modifications of the methods by Donis-Keller et al., (1977); Gupta and Randerath (1977); Lockard et al., (1978); Simoncsits et al., (1977); and Levy and Karpetsky (1980). Unlike the DNA sequencing methods of Maxam and Gilbert (1977) and Sanger and Coulson (1975) where double-stranded cDNA copies have to be synthesised and cloned into a suitable plasmid or cloning vehicle prior to amplification and excision with restriction endonucleases then sequenced on polyacrylamide gels, the method of sequence analysis of RNA strands is dependent upon a number-specific enzymes. These RNases, when used together, provide through selective hydrolysis at specific bases, the means by which the sequential arrangement of nucleotides within a polymer chain can be determined. Five enzymes were used for the sequencing: RNase T1 specific for G residues; RNase U_2 specific for A residues; RNase Phy M specific for A and U residues; RNase

B.cereus specific for C and U residues; and RNase CL_3 specific for C residues.

The procedure of sequence analysis was developed by Donis-Keller et al., (1977) who described a procedure for sequence analysis of 5'[^{32}P] labelled RNA by partial chemical and enzymatic digestion of the 5'[^{32}P] RNA followed by separation of the partial digestion products by polyacrylamide gel electrophoresis.

A pyrimidine-specific extracellular RNase from a strain of B.cereus (Tabor et al., 1976) was also used in the sequencing. The enzyme cleaves almost all of the pyrimidine in an RNA providing more information for the location of pyrimidine residues at the 3' end of the RNA.

The RNase U_2 was originally isolated by Arima et al. (1986) from Ustilago sphaerogena and was reported to have a strict specificity for purines. The enzyme releases Ap and Gp but no Cp and Up from RNA, and the isolated oligonucleotide products terminated exclusively in Ap and Gp.

Labelled, purified PVX RNA was partially digested by all five enzymes and the resulting fragments analysed by thin-layer polyacrylamide gel electrophoresis (Sanger and Coulson, 1978). The resulting autoradiograph (Fig.38) showed a series of adjacent ladders resulting from the partial enzyme digestion, together with a size marker lane of RNA fragments caused by patial hydrolysis by alkali. The RNases U_2 (A-specific) and Phy M (A and U-specific) produced the most marked ladders, confirming the presence of poly (A) residues at the 3' terminal of PVX RNA. The fragments ranged in size from 2 nucleotides to 10 nucleotides which corresponded with the heterogeneous size distribution observed with the RNases A and T1

digestion. Very strong bands in the U_2 lane were observed at 2, 8, and 10 nucleotide lengths. The heterogeneous series of bands was expected, rather than a well-defined sequence, since the partial digestion of the RNA by the endoribonucleases produced a series of nested fragments resulting from poly (A) sequences of heterodispersed length at the 3' terminus. Such heterodispersity is already indicated by the varied size of fragments produced by RNase A and T1 digestion.

It was also shown that, as reported by Levy and Karpetsky (1980), enzyme activity of RNase CL3 was inhibited by free poly (A) or tracts of the polypurine present at the 3' terminus of the RNA. Restoration of enzyme activity was achieved by the addition of low concentrations (1 mM) spermidine to the reaction mixtures.

Morozov et al. (1981), Morozov et al. (1983), and Abou Haidar (1981) reported the existence of a poly (A) sequence of a length of 50-200 nucleotides at the 3' terminus of PVX RNA. In affinity chromatography experiments with oligo d(T) cellulose, Morozov et al. (1981) found that up to 90 % of the native PVX RNA was bound to the column although this value dropped sharply with even slightly degraded PVX RNA. They suggest that the absence of binding of RNA with poly (U) sepharose by Sonenberg et al. (1978) was caused by the degradation of the uniformly labelled virion PVX RNA preparation. Morozov et al. (1981) also synthesised a cDNA strand of the 3' terminus of PVX RNA using oligo d(T) as a primer suggesting that the poly (A)-rich region was of sufficient length to prime with oligo d(T). They also noted that PVX is the first plant virus with both a "cap" at the 5' terminus and a poly (A sequence at the 3' terminus to occur in the genomic RNA.

The sequence data presented by Morozov et al., (1983) are not necessarily inconsistent with the sequences derived from the direct

sequencing of the 3' terminus of PVX RNA. Heterogeneity of sequence would not be apparent since the cloning procedure would result in the isolation of one particular sequence. Similarly, the occasional non-adenine nucleotide would be difficult to detect by direct sequencing within a heterogenous A-rich tail. The direct sequencing results and those of the RNAse A and T1 digestion do indicate that, in contrast to the data of Morozov et al. (1983), the longest runs of adenine may be as long as 16 nucleotides. These differences may result from differences in viral strains.

4.3.6. The Presence and Importance of 3' Polyadenylate Sequences in Viruses.

An important feature of the heterogeneous nuclear RNA and messenger RNA (mRNA) of most mammalian cells is the presence of a segment of polyadenylic acid poly (A) (Brawerman, 1974). The poly (A) segment is covalently linked to the 3' terminus of the RNA. Such regions are also found in many positive-stranded RNA viruses. In the picornavirus group, the genomes of poliovirus (Armstrong et al., 1972), encephalomyocarditis virus (Gillespie et al., 1973), Columbia S-K virus (Johnston and Bose, 1972) and Rhinovirus (Nair and Owens, 1974). The size of the poly (A) on these viruses ranges from 50-100 nucleotides in length. Another member, Mengovirus, possessess a poly (A) sequence of an average of 15-17 nucleotides in Length (Miller and Plagemann, 1972).

Poly (A) has also been found in the mRNA of many plant viruses (Manahan et al., 1973; Verma et al., 1974) such as bean pod mottle virus (BPMV) and cowpea mosaic virus (CPMV)(Semanik, 1974). Some plant virus groups which contain the poly (A) sequence include the Comovirus

and Nepovirus. Alternatively, many plant viruses, e.g. TMV and BMV, have a tRNA-like 3' terminus which can bind a specific amino acid.

The biological significance of the poly (A) segment or the aminoacylating (amino acid binding) moiety of the RNA is not known although it has been postulated that the poly (A) segment may play some role either in the processing and transport of mRNA fom nucleus to cytoplasm in mammalian cells or in the translation of mRNA in general (Darnell et al., 1973; Armstrong et al., 1972). Spector and Baltimore (1974) have shown that reduction in the size of the poly (A) segment markedly decreases the specific infectivity of poliovirus RNA and indicates that poly (A) is necessary to the infectivity of the RNA.

The earliest report of a terminal poly (A) region in a plant virus RNA was reported by El Manna and Bruening (1973) for cowpea mosaic virus with an average length of 200 nucleotides. El Manna and Bruening found no evidence for internal polyadenylate sequences or for terminal sequences of 16 or fewer residues. Atabekov and Morozov (1979) suggest that the poly (A) sequences play a stabilising role in the maintenance of the mRNA structural integrity in animal cells. They suggest that proteins, bound to the poly (A) sequences, may protect the message from RNase action and secondly, that the poly (A) tracts may inhibit endonuclease mRNA cleavage. It is, therefore, possible that the presence of the poly (A) segment in plant viruses such as PVX may play the same roles as those present in mammalian viruses and aid in the ability of the virus to infect susceptible hosts.

CONCLUSION

The screening of plants for viruses has been important with a variety of crops. Methods which are reliable, fast, and able to cope with large numbers of samples are, therefore, advantageous. Newer detection methods were tested in comparison with the more traditional methods such as the use of indicator plants (which requires large amounts of space as well as the continual maintenance of a large number of plants) and the use of the electron microscope (which is expensive and requires skilled operators).

A variety of methods were studied and compared for the screening of plant viruses. The methods were all capable of screening mass numbers of plant samples and were based on either viral particle detection (ELISA) or viral RNA detection (dot blot).

It was found that the sensitivity of each method varied, depending on whether viral particles/viral RNA were detected in crude sap, viral particles, or (in the case of the dot-blot) purified RNA.

The use of an immunoassay for the detection of viral antigen, using polyclonal antibodies, was tested against cDNA probes hybridised to samples bound to nitrocellulose.

With the ELISA, the assay was initially more sensitive in the detection of virus in crude sap (by up to 3 logs) than the dot-blot assay with either a biotinylated or radiolabelled probe. However, this difference in sensitivty was greatly reduced with extended exposure times for the radiolabelled probes. The ELISA was also found to increase in sensitivity with incubation time where colour production in the samples were intensified (up to 10^{-6} x dilution). The assay also detected both strain 2 and 3 (better with strain-Group 2 purified viral particles in some cases than the strain-Group 3 isolates). The method was found to be fast, taking 24 h, and also capable of handling large numbers of samples. With the purified viral particles, not much

difference was observed in the sensitivity of detection between the strains although the absorbance values varied greatly between 20 ng and 200 pg, showing that the ELISA (with polyclonal antibodies) will detect both strains with equal ease.

However, as previously mentioned, the ELISA does not detect infectious RNA in the samples but rather, only the viral antigen and therefore, the alternatives are complementary probes. Radiolabelled probes have been previously used for the detection of mammalian viral DNA (such as Epstein-Barr virus (Brandsma and Miller, 1980)) and for the detection of plant viral RNA (such as potato spindle tuber viroid (Owens and Diener, 1981)). The sensitivity of the radiolabelled probes were compared with a non-radiolabelled probe in the detection of PVX viral RNA in crude sap extracts, purified viral particles and purified RNA. The results showed that, in comparison with the ELISA, the oligo d(T)-primed probes were less sensitive by up to 3 logs in the detection of viral RNA in the crude sap extracts with a 4 h exposure, but by extending the exposure time, the sensitivity of the ^{32}P-labelled probe could be increased by up to 2 logs. The calf thymus-primed radiolabelled probes were comparable to the ELISA results, detecting PVX N RNA in crude sap samples to 10^{-4} with a 4 h exposure. From previous results of extended exposure times after hybridisation with the oligo d(T)-primed probe, this sensitivity of detection was expected to increase making detection of the samples at least equal to those of the ELISA. This increase in sensitivity was not observed with the biotinylated probe. Therefore, the ^{32}P-labelled probe was comparable to the ELISA in sensitivity for crude sap extracts when the exposure time was extended.

The hybridisation techniques using the probes were relatively easy to handle with the advantage that the method could handle large

numbers of samples at the one time (up to 100 samples per filter). In general, radiolabelled probes gave clearer signals with less background, although both probes were comparable in their sensitivity. The probes also detected PVB (strain-Group 2) less than the strain-Group 3 isolates which may present problems when more than one strain is detected.

The use of streptavidin/biotin was chosen for the high affinity of the molecules for each other, their stability in storage, as well as being non-hazardous to health. The probe was economic since it could be stored without decay. However, attempts to re-use the probes two or three times gave disappointing results. It was found that the sensitivity of the method in a second and third hybridisation for crude sap samples, was reduced especially for the PVX (strain-Group 3, PBI) isolate. This was thought to be due to the removal of sequences of high specificity from the probe after hybridisation with the isolate on the filter. The signals from the second and third hybridisations were also reduced for the sap samples. With PVX N RNA, re-using the probe in the second and third hybridisations, produced a marked reduction in the strength of signals for the second hybridisation and almost fading totally in the third hybridisation.

A third nick-translated probe, pPVX N 21, was far less sensitive for PVX N RNA, detecting purified PVX N RNA to only 20 ng with high background signals with a 7 day exposure. The probe did not hybridise with PVB (strain-Group 2) or PVX (strain-Group 3, PBI) RNA. The only crude sap isolate detected with the nick-translated probe was the PVX (strain-Group 3, PBI) isolate, and this thought to be due to the high levels of virus present in the sap spot.

Therefore, for rapid results in the detection of virus, the use of the ELISA would be as rapid as the dot-blot assay. For the detection

of of RNA, however, it was concluded that, although the biotinylated probes have the potential advantages of being non-hazardous to health, being re-useable, and are reported to be equally sensitive as radiolabelled probes, in practice, sensitivities were slightly lower, considerably so if exposure times for the autoradiography were increased. Re-using the biotinylated probes caused difficulties and with crude sap samples, there were potential problems with false positives.

For the structural studies of the 3' terminal region of PVX N RNA (strain-Group 3), it was conclusively proved that, contrary to some reported data (Sonenberg, 1978) but supporting others (Morozov et al., 1983), the 3' end of the genome possessed a sequence of poly (A) residues of sufficient length to i) initiate cDNA synthesis in the presence of the primer (oligo $d(T)_{12-18}$); ii) bind to oligo d(T) cellulose in affinity chromatography experiments; iii) appears as a tract of 7-12 poly (A) nucleotides on polyacrylamide gels after digestion with RNases A and T1, and iv) as a heterogenous series of poly (A) sequences when the RNA was directly sequenced. This poly (A) region was shown to be terminally positioned by RNase experiments. The precise role of the poly (A) sequence is not known although its presence may help in the infectivity of the RNA.

AbouHaidar, M. (1981). Polar Assembly of Clover Yellow Mosaic Virus. Journal of General Virology, 57, 199-203.

AbouHaidar, M. (1983). The Structure of the 5' and 3' ends of Clover Yellow Mosaic virus RNA. Canadian Journal of Microbiology, 29, 151-156.

Abu Salih, H.S., Murant, A.F. and Daft, M.J. (1968). The use of Antibody-Sensitised latex Particles to Detect Plant Viruses. Journal of General Virology, 3, 299-302.

Adams, J.M., Jeppesen, P.G.N., Sanger, F. and Barrel, B.G. (1969). Nucleotide Sequence from the Coat Protein Cistron of R17 Bacteriophage RNA. Nature (London), 223, 1009.

Agranovsky, A.A., Dolja, V.V., Gorbulev, V.G., Kozlov, Y.V., and Atabekov, J.G. (1981). Aminoacylation of Barley Stripe Mosaic Virus RNA: Polyadenylate-Containing RNA has a 3' Terminal Tyrosine-Accepting Structure. Virology, 113, 174-187.

Agranovsky, A.A., Dolja, V.V. and Atabekov, J.G. (1982). Structure of the 3' Extremity of Barley Stripe Mosaic Virus RNA: Evidence for Internal Poly (A) and a 3' Terminal tRNA-like Structure. Virology, 119, 51-58.

Agranovsky, A.A., Dolja, V.V. and Atabekov, J.G. (1983). Differences in Polyadenylate Length between Individual Barley Stripe Mosaic Virus RNA Species. Virology, 129, 344-349.

Allard, H.A. (1914). The Mosaic Disease of Tobacco. Bulletin of the United States Department of Agriculture, No.40, 33.

Allison, A.V. and Shalla, T.A. (1974). The Ultrastructure of Local Lesions Induced by Potato Virus X: A Sequence of Cytological Events in the Course of Infection. Phytopathology, 64, 784-793.

Alwine, J.C., Kamp, D.J. and Stark, G.R. (1977). Method for Detection of Specific RNAs in Agarose gels by Transfer to Diazobenzyloxymethyl-paper and Hybridisation with DNA Probes. Proceedings of the National Academy of Sciences, 74, 5350-5354.

Alwine, J.C., Kamp, D.J., Parker, B.A., Reiser, J., Stark, G.R. and Wahl, G.M. (1980). Detection of Specific RNAs or Specific Fragments of DNA by Fractionation in Gels and Transfer to Diazobenzyloxymethyl Paper. Methods in Enzymology, 68, 220-242.

Arima, T., Uchida, T. and Egami, F. (1968). Studies on Extracellular Ribonucleases of Ustilago sphaerogena: Characterisation of Substrate Specificity with Special Reference to Purine-Specific Ribonucleases. Biochemical Journal, 106, 601.

Armstrong, J.A., Edmonds, M., Nakazato, H., Philips, B.A. and Vaughan, M. (1972). Polyadenylic Acid Sequences in the Viorion RNA of Poliovirus and Eastern Equine Encephalitis Virus. Science, 176, 526-528.

Atabekov, J.G. (1975). Host Specificity of Plant Viruses. Annual Review of Phytopathology,13, 127-145.

Atabekov, J.G. and Morozov, S.Y. (1979). Translation of Plant Virus Messenger RNAs. Advances in Virus Research, 25, 1.

Attathom, S., Weathers, L.G. and Gumpf, D.J. (1978). Identification and Characterisation of a Potexvirus from California Barrel Cactus. Phytopathology, 68, 1401-1406.

Aviv, H. and Leder, P. (1972). Purification of Biologically Active Globine Messenger RNA by Chromatography on Oligothymidylic acid-cellulose. Proceedings of the National Academy of Sciences, U.S.A., 69, 1408-1412.

Ball, E.M. (1974). Serological Tests for the Identification of Plant Viruses. American Phytopathological Society. St Paul, Minn., 31.

Ball, E.M. and Brakke, M.K. (1968). Leaf-Dip Serology for Electron Microscopic Identification of Plant Viruses, Virology, 36, 152-155.

Barnard, D.L., Johnson, F.B., Richards, D.F. (1985). Comparison of an Avidin-Biotin Immunoassay with Three Commercially Available Immnunofluorescence Kits for Typing of Herpes Simplex Virus. 38, 1158-1162.

Baulcombe, D., Flavell, R.B., Boulton, R.E. and Jellis, G.J. (1984). The Sensitivity and Specificity of a Rapid Nucleic Acid Hybridization Method for the Detection of Potato Virus X in Crude Sap Samples. Plant Pathology, 33, 361-370.

Bawden, F.C. and Kleczkowskia, A. (1948). Variations in the Properties of Potato virus X and their effects on its Interactions with Ribonuclease and Proteolytic Enzymes. Journal of General Microbiology, 2, 173-185.

Bawden, F.C. and Crook, E.M. (1947). Some Properties of Potato Virus X in Leaf Extracts Made in Different Ways. British Journal of Experimental Pathology, 28, 403-415.

Bawden, F.C. and Roberts, F.M. (1947). The Influence of Light Intensity on the Susceptibility of Plants to Certain Viruses. Annals of Applied Biology, 34, 286-296.

Bawden, F.C. and Sheffield, F.M.L. (1944). The Relationships of Some Viruses causing Necrotic Diseases of the Potato. Annals of Applied Biology, 31, 33-40.

Beemster, A.B.R. and Rozendaal, A. (1972). In Viruses of Potatoes and Seed Potato Production (de Bokx, JA. Ed.), 115-143. Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands.

Bercks, R. (1966). in Viruses of Plants (Beemster, A.B.R. and Dikjstra, J.,eds). 205-212. North Holland, Amsterdam.

Bercks, R. (1970). CMI/AAB. Descriptions of Plant Viruses. No.4.

Bercks, R. (1971). CMI/AAB. Descriptions of PLant Viruses. No.41.

Bercks, R. and Brandes, J. (1963). Electronenmilkroskopishe und serologische Untersuchungen zur klassifizierung des clover yellow mosaic virus. Phytopathologische Zeitschrift, 47, 381-390.

Bercks, R. and Querfurth, G. (1971). The Use of the Latex Test for the Detection of Distant Serological Relationships among Plant Viruses. Journal of General Virology, 12, 25-32.

Berkower, I., Leis, J. and Hurwitz, J. (1973). Isolation and Characterisation of an Endonuclease from Escherichia coli specific for Ribonucleic Acid in Ribonucleic Acid Deoxyribonucleic Acid Hybrid Structures. Journal of Biological Chemistry, 248, 5914-5921.

Birnboim, H.C., and Doly, J. (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Nucleic Acids Research, 7, No.6, 1513-1523.

Brandes, J. (1966). in Viruses of Plants (Beemster, A.B.R. and Dikjstra, J. eds), 218-229. North Holland, Amsterdam.

Brandes, J. and Bercks, R. (1965). Gross morphology and Serology as a Basis for Classification of Elongated Plant Viruses. Advances in Virus Research, 11, 1-24.

Brandes, J. and Wetter, C. (1959). Classification of Elongated Plant Viruses on the basis of particle Morphology. Virology, 8, 99-115.

Brandsma, J. and Miller, G. (1980). Nucleic Acid Spot Hybridisation: Rapid Quantitative Screening of Lymphoid Cell Lines for Epstein-Barr Viral DNA. Proceedings of the National Academy of Sciences, 77, 6851-6855.

Broker, T.R., Angerer, L.M., Yen, P.H., Hershey, N.D. and Davidson, N. (1978). Electron Microscopic Visualisation of tRNA Genes with Ferritin-Avidin:Biotin Labels. Nucleic Acids Research, 5, No.2, 363-384.

Brunt, A.A. (1966). Narcissus Mosaic Virus. Annals of Applied Biology, 58, 13-23.

Carpenter, J.M. (1971). Rothamsted Annual Report, 129-130.

Chester, K.S. (1936). Separation and Analysis of Virus Strains by Means of Precipitin Tests. Phytopathology, 26, 778-785.

Chiko, A.W. and Guthrie, J.W. (1969). Changes in Potato Virus X Particle Length following Purification by Differential Centrifugation. Phytopathology, 59, 1021.

Chou, S. and Merigan, T.C. (1983). Rapid Detection and Quantitation of Human Cytomegalovirus in Urine through DNA Hybridisation. New England Journal of Medicine, 308, 921-925.

Christie and Edwardson, (1977). Florida Agricultural Experimental Station Monogram Service, No 9, 155.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the Microplate method of Enzyme-linked Immunosorbent Assay for the Detection of Plant Viruses. Journal of General Virology, 34, 475-483.

Cockerham, G. (1954). Strains of Potato Virus X. Proceeding of Second Conference on Potato Virus Diseases. Lisse-Wageningen, 89-92.

Cockerham, G. and Davidson,, T.M.W. (1963). Note on an Unusaul strain of Potato Virus X. Scottish plant Breeding Station Report for 1963.

Corbett, M.K. (1961). Purification of Potato Virus X Without Aggregation. Virology, 15, 8-15.

Costa, A.S. and Kitajama, E.W. (1972). CMI/AAB Descriptions of Plant Viruses, No.90.

Coyier, D.L., Stace-Smith, R., Allen, T.C. and Leung, E. (1977). Viruslike Particles Associated with a Rhododendron Necrotic Ringspot Disease. Phytopathology 67, 1090-1095.

Dagert,M. and Ehrlich, S.D. (1979). Prolonged Incubation in Calcium Chloride Improves the Competence of Escherichia coli cells. Gene, 6, 23-28.

Dasgupta, R., Harada, F., and Kaesberg, P. (1976). Blocked 5' termini in brome mosaic virus RNA. Journal of virology, 18, 260-267.

Darnell, J.E., Jeliner, W.R., and Mollov, G.R. (1973). Biogenesis of mRNA: Genetic Regulation in Mammalian Cells. Science, 181, 1215-1221.

de Bokx, J.A. (1965). Hosts and Electron Microscopy of Two Papaya Viruses. Plant Disease Reporter, 49, 742-746.

de Bokx, J.A. (1972). Viruses of Potatoes and Seed Potato Production. Centre for Agricultural Publications and Documentation, Wageningen, The Netherlands, 232.

Derrick, K.S. (1972). Quantitative Assay for plant Viruses Using Serologically Specific Electron Microscopy. Virology, 56, 652-653.

Derrick, K.S. and Brlansky, R.H. (1976). Assay for Viruses and Mycoplasmas using Serologically Specific Electron Microscopy. Phytopathology, 66, 815-820.

Dimitrov, D.H., Graham, D.Y., and Estes, M.K. (1985). Detection of Rotaviruses by Nucleic Acid Hybridisation with Cloned DNA of Simian Rotavirus SAll Genes. The Journal of Infectious Diseases, 153, No.2, 293-300.

Donis-Keller, H. (1979). Site Specific Enzymatic Cleavage of RNA. Nucleic Acids Research, 7, no.1, 179-192.

Donis-Keller, H., Maxam, A.M., Gilbert, W. (1977). Mapping Adenines, Guanines and Pyrimidines in RNA. Nucleic Acids Research, 4, No.8, 2527-2538.

Edgall, M.A., Hutchinson, C.A., and Sinsheimer, R.L. (1969). Large Pore Disc Electrophoresis. Modern Separation Methods of Macromolecules and Particles (Progress in Separation and Purification, Volume 2). New York: Wiley.

El Manna, M.M. and Bruening, G. (1973). Polyadenylate Sequences in the Ribonucleic Acids of Cowpea Mosaic Virus. Virology, 56, 198-206.

Emtage, J.S., Catlin, G.H. and Carey, N.H. (1979). Polyadenylation and Reverse Transcription of Influenza Viral RNA. Nucleic Acids Research, 6, No.4, 1221-1239.

Fenner, F. (1976). Classification and Nomenclature of Viruses. Second Report of the International Commitee on Taxonomy of Viruses. Intervirology, 7, 4-115.

Francki, R.I.B. (1970). CMI/AAB Descriptions of Plant Viruses. No.27.

Francki, R.I.B. and McClean, G.D. (1968). Purification of Potato Virus X and Preparation of Infectious Ribonucleic Acid by Degradation with Lithium Chloride. Australian Journal of Biological Science, 21, 1311-1318.

Fribourg, C.E. (1975). Studies on Potato Virus X Isolated from Peruvian Potatoes. Potato Research, 18, 216-226.

Gilham, P.T. (1964). Synthesis of Polynucleotide Celluloses and their uses in the Fractionation of Polynucleotides. Journal of the American Chemical Society, 86, 4982.

Gillespie, D. and Spiegelman, S. (1965). A Quantitative Assay for DNA-RNA Hybrids with DNA Immobilised on a membrane. Journal of Molecular Biology, 12, 829-842.

Gillespie, D., Takemoto, K., Robert, M. and Gallo, R.C. (1973). Polyadenylic Acid in Visna Virus RNA. Science, 179, 1328-1330.

Goelet, P. and Karn, J. (1982). Methods for cDNA Cloning and Sequencing TMV RNA. Gene, 29, 331-342.

Goodman, R.M. (1975). Reconstitution of Potato Virus X in vitro. I. Properties of the Dissociated Protein Structural Subunits. Virology, 68, 287-298.

Goodman, R.M., Horne, R.W. and Hobart, J.M. (1975). Reconstitution of Potato Virus X in vitro. II. Characterisation of the Reconstituted Product. Virology, 68, 299-308.

Goodman, R.M. and Ross, A.F. (1974a). Enhancement of potato Virus X Synthesis in Doubly Infected Tobacco Occurs in Doubly Infected Cells. Virology, 58, 16-24.

Goodman, R.M. and Ross, A.F. (1974b). Enhancement of Potato Virus X Synthesis in Doublly Infected Tobacco Depends on the Timing of Invasion by the Viruses. Virology, 58, 263-271.

Goodman, R.M. and Ross, A.F. (1974c). Independent Assembly of Virions in Tobacco Doubly Infected by Potato Virus X and Potato Virus Y or Tobacco Mosaic Virus. Virology, 59, 314-318.

Grunstein, M. and Hogness, D. (1975). Colony Hybridisation: A Method for the Isolation of Cloned DNAs that Contain a Specific Gene. Proceedings of the National Academy of Sciences, USA, 72,3961-3965.

Guesdon, J-L., Ternynck, T. and Avrameas, S. (1979). The Use of Avidin-Biotin Interaction in Immunoenzymatic Techniques. The Journal of Histochemistry and Cytochemistry, 27, No.8, 1131-1139.

Gupta, R.C. and Randerath, K. (1977). Use of Specific Endonuclease Cleavage in RNA Sequencing - An Enzymic Method for Distinguishing between Cytidine and Uridine Residues. Nucleic Acids Research, 4, No.10, 3441-3454.

Gustafson, G.D., Milner, J.J., McFarland, J.E., Pedersen, K., Larkins, B.A. and Jackson, A.G. (1982). Investigation of the Complexity of Barley Stripe Mosaic Virus RNAs with Recombinant DNA Clones. Virology, 120, 192-193.

Haberkern, R.C. and Cantoni, G.L. (1973). Studies on a Calf Thymus Ribonuclease Specific for Ribonucleic Acid-Deoxyribonucleic Acid Hybrids. Biochemistry, 12, 2389-2395.

Hampton, R., Beczner, L., Hagedorn, D., Bos, L.Inouye,T., Barnett, O., Musil, M. and Meiners, J. (1978). Host Reactions of Mechanically Transmissable Legume Viruses of the Northern Temperate Zone. Phytopathology, 68, 989-997.

Harrison, B.D. (1970). CMI/AAB Descriptions of Plant Viruses. No.12.

Harrison, B.D., Finch, J.T., Gibbs, A.J., Hollings, M., Shepard, R.J., Valenta, V. and Wetter, C. (1971). Sixteen Groups of Plant Viruses. Virology, 45, 356-363.

Hausen, P. and Stein, H. (1970). Ribonuclease H: An Enzyme Degrading the RNA Moiety of DNA-RNA Hybrids. European Journal of Biochemistry, 14, 278-283.

Henry, C.M., Ferdinand, F.J. and Knippers, R. (1973). A Hybridase from Escherichia coli. Biochemical and Biophysical Research Communications, 50, 603-611.

Hibi, T. and Saito, Y. (1985). A Dot Immunobinding Assay for the Detection of Tobacco Mosaic Virus in Infected Tissues. Journal of General Virology, 66, 1191-1194.

Hiebert, E. (1970). Some Properties of Papaya Mosaic Virus and its Isolated Constituents. Phtopathology, 60, 1295.

Hill, S.A. (1981). Laboratory Methods for Diagnosing Virus Diseases of Plants. Review of Plant Pathology, 60, No.8, 377-382.

Hitchborn, J.H. and Hills, G.J. (1965). The Use of Negative Staining in the Electron Microscopic Examination of Plant Viruses in Crude Extracts. Virology, 27, 528-540.

Hollings, M. (1958). Hydrangea Ringspot in Britain. Journal of Horticultural Science, 33, 275-281.

Hollings, M., and Stone, O.M. (1974). Some Properties of Chrysnthemum Stunt, A Virus with the Characteristics of an Uncoated Ribonucleic Acid. Annals of Applied Biology, 74, 333-348.

Hollings, M. and Stone, O.M. (1970). The Long-Term Survival of Some Plant Viruses Preserved by Lyophilization. Annals of Applied Biology, 65, 411-418.

Holmes, D.S. and Quigley, M. (1981). A Rapid Boiling Method for the Preparation of Bacterial Plasmids. Analytical Biochemistry, 114, 193.

Honda, Y., Kajita, S., Matsui, C., Otsuki, Y. and Takebe, I. (1975). An Ultrastructure Study of the Infection of Tobacco Mesophyll Protoplasts by Potato Virus X. Phytopathologische Zeitschrift. 84, 66-74.

Horne, R.W. (1978). Structure and Function of Viruses. The Camelot Press Ltd., Southhampton.

Horvarth, J. (1978). New Artificial Hosts and Non-Hosts of Plant Viruses and their role in the Identification and Separation of Viruses: II. General Remarks and Proposals on the Experimental Work. Acta Phytopathologica Academiae Scientiarum Hungaricae, 13, 269-280.

Hsu, S-M., Raine, L., and Fanger, H. (1981).Use of Avidin-Biotin-Peroxidase Complex (ABC) in Immunoperoxidase Techniques. The Journal of Histochemistry and Cytochemistry, 29, no.4, 577-580.

Ireland, R.J. and Pierpoint, W.S. (1980). Reaction of Strains of Potato Virus X with Chlorogenoquinone in vitro and the Detection of a Naturally Modified Form of the Virus. Physiological Plant Pathology, 16, 81-92.

Johnston, R. and Bose, H.R. (1972). Correlation of Messenger RNA Function with Adenylate Rich Sequences in the Genome of Single Stranded RNA Viruses. Proceedings of the National Academy of Sciences, 69, 1514-1516.

Jones, A.T. (1977). Partial Purification and Some Properties of Wineberry Latent, a virus obtained from Rubus phoenicolanius. Annals of Applied Biology, 86, 199-208.

Kassanis, B. and Govier, D.A. (1972). CMI/AAB Descriptions of Plant Viruses. No.98.

Keller and Crouch (1972). Degradation of DNA-RNA Hybrids by Ribonuclease H and DNA Polymerases of Cellular and Viral Origin. Proceedings of the National Academy of Sciences, 69, 3360-3364.

Kelley, R.G., Cozzarelli, N., Deutscher, M.P., Lehman, I.R., and Kornberg, A. (1970). Enzymatic Symthesis of Deoxyribonucleic Acid. Journal of Biological Chemistry, 254, 39.

Kingsbury, N. and Masters, C.J. (1970). On the Determination of Molecular Weights in Complex Protein Mixtures by Means of Disc Electrophoresis. Analytical Biochemistry, 36, 144.

Koenig, R. (1971). Nucleic Acids in the Potato Virus X Group and in some other plant Viruses: Comparison of the Molecular Weights by Electrophoresis in Acrylamide-Agarose Composite Gels. Journal of General Virology, 10, 111-114.

Koenig, R. (1973). CMI/AAB Descriptions of Plant Viruses. No.114.

Koenig, R. (1978b). ELISA in the Study of Homologous and Heterologous Reactions of Plant Viruses. Journal of General Virology, 40, 309-318.

Koenig, R. and Bercks, R. (1968). Changes in the Heterologous Reactions of Antisera against Members of the Potato Virus X Group during the Immune Response. Phytopathologische Zeitschrift, 61, 382-398.

Koenig, R and Lesemann, D.E. (1974). A Potyvirus from Gloriosa rothschildiana. Phytopathologische Zeitschrift, 80, 136-142.

Koenig, R and Lesemann, D.E. (1978). CMI/AAB Descriptions of Plant Viruses. No.200.

Koenig, R., Tremaine, J.H. and Shepard, J.F. (1978). In situ Degradation of the Protein Chain of Potato Virus X at the N- and C-termini. Journal of General Virology, 38, 329-337.

Kozar, F.E. and Shedludko, K.M. (1969). Ultrastructure of Potato and Datura stramomium Plant Cells Infected with Potato Virus X. Virology, 38, 220-229.

Ladeburg, R.C., Larson, R.H. and Walker, J.C. (1950). Wisconsin Agricultural Experimental Station Research Bulletin, No.165, 1-47.

Lange, L. (1978). Synchytium endobioticum and Potato Virus X. Phytopathologische Zeitschrift, 92, 132-142.

Langer, P.R., Waldrop, A.A. and Ward, D.C. (1981). Enzymatic synthesis of Biotin-labelled polynucleotides: Novel Nucleic Acid Affinity Probes. Proceedings of the National Academy of Sciences, U.S.A., 78, No.11, 6630-6637.

Leary, J.J., Brigati, D.J. and Ward, D.C. (1983). Rapid and Sensitive Colorimetric Method for Visualising Biotin-labelled DNA Probes Hybridised to DNA or RNA Immobilised on Nitrocellulose: Bioblots.Proceedings of the Academy of Sciences, U.S.A., 80, 4045-4049.

Lesemann, D.E. and Koenig, R. (1977) in Insect and Plant Viruses: An Atlas (Maramorosch, K. ed). 331-345. Academic Press, N.Y.

Lesnaw, J.A. and Reichmann, M.E. (1970). Determination of Molecular Weights of Plant Viral Protein Subunits by Polyacrylamide Gel Electrophoresis. Virology, 42, 724-731.

Levy, C.C. and Karpetsky, T.P. (1980). The Purification and Properties of Chicken Liver RNase. The Journal of Biological Chemistry, 255, No.5, 2153-2159.

Lin, M.T., Kitajima, E.W., Cupertino, F.P. and Costa, C.L. (1977). Partial Purification and Some Properties of Bamboo Mosaic Virus. Phytopathology, 67, 1439-1443.

Lindberg, U. and Persson, T. (1972). Isolation of mRNA from KB-cells by Affinity Chromatography on Polyuridylic Acid Covalently linked to Sepharose. European Journal of Biochemistry, 31, 246-254.

Lisa, V. and Delavalle, G. (1977). Viola Mottle Virus, a New Member of the Potexvirus Group. Phytopathologische Zeitschrift, 89, 82-89.

Lockard, R.E., Alzner-Deweerd, B., Hecjman, J.E., MacGee, J., Tabor, M.W. and RajBhandary, U.L. (1978). Sequence Analysis of 5' [^{32}P] Labelled mRNA and tRNA using Polyacrylamide Gel Electrophoresis. Nucleic Acids Research, 5, No.1, 37-56.

Loughlane, J.B. and Murphy, P.A. (1938). Dissemination of Potato Viruses X and F by Leaf Contact. The Scientific Proceedings of the Royal Dublin Society, 22, 1-15.

Luunsgaard, T. (1976). Routine Seed Health Testing for Barley Stripe Mosaic Virus in Barley Seeds using the Latex Test, Zeitschrift fur pflan zenkrankheiten und pflanzenschutz, 83, 278-283.

Maat, D.Z., and de Bokx, J.A. (1978). Enzyme-linked Immunosorbent Assay (ELISA) for the Detection of Potato Viruses A and Y in Potato Leaves and Sprouts. Netherlands Journal of Plant Pathology, 84, 167-173.

Mackey, J.K., Barckman, K.H., and Green, M. (1977). Preparation and Characterization of Highly Radioactive in vitro Labelled Adenovirus DNA and Restriction Fragments. Biochemistry, 16, 4478-4483.

Manahan, C.O., App, A.A., and Still, C.C. (1973). The Presence of Polyadenylate Sequences in the Ribonucleic Acid of a Higher Plant. Biochemical Biophysical Research Communications, 53, 588-595.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Matthews, R.E.F. (1948a). Studies on Potato Virus X: I. Types of Change in Potato Virus X Infections. Annals of Applied Biology, 36, 448-459.

Matthews, R.E.F. (1948b). Studies on Potato Virus X: II. Criteria of Relationships Between Strains. Annals of Applied Biology, 36, 460-474.

Matthews, R.E.F. (1949). Criteria of Relationships betwen Strains of PVX. Annals of Applied Biology, 36, 460-474.

Matthews, R.E.F. (1979). Classification and Nomenclature of Viruses. Third Report of the Internatinal Commitee on Taxonomy of Viruses. Intervirology, 12, no.3-5, 132.

Matthews, R.E.F. (1981). in Plant Virology (Second Edition). Academic Press.

MacKinnon, J.P. and Bagnall, R.H. (1972). Potato Research, 15, 81-85.

Maxam, A. and Gilbert, W. (1977). A New Method for Sequencing DNA. Proceedings of the National Academy of Sciences, U.S.A., 74, 560.

McKinney, H.H. and Silber, G. (1968). in Methods in Virology, 491-501, (Maramorosch, K. and Koprowski, H. eds. Academic Press, N.Y)

McLauglin, M.R. and Barnett, O.W. (1978). Enzyme-linked Immunoassay for Detection and Identification of Forage legume Viruses. Proceedings of the South Pasture Forage Crop Improvement Conference (35), 138-145.

Mellor, F.C. and Stace-Smith, R. (1970). Virus Strain Differences in Eradication of Potato Virus X and S. Phytopathology, 60, 1587-1590.

Miki, T. and Knight, C.A. (1967). Some Chemical Studies on a Strain of White Clover Mosaic virus. Virology, 31, 55-63.

Miki, T. and Knight, C.A. (1968). The Protein Subunit of Potato Virus X. Virology, 36, 168-173.

Miki, T. and Oshima, N. (1972). Chemical Studies on the Structural Protein of Potato Acuba Mosaic Virus. Virology, 48, 386-393.

Milbrath, G.M., Nelson, M.R. and Wheeler, R.E. (1973). The Distribution and Electron Microscopy of Viruses of Cacti in Southern Arizona. Phytopathology, 63,1133-1139.

Miller, R.L. and Plagemann, P.G.W. (1972). Purification of Mengovirus and Identification of an A-Rich Segment in its RNA. Journal of General Virology, 17, 349-353.

Milne, R.G. and Luisoni, E. (1977). in Methods in Virology, 6, 265-281, Maramorosch, K. and Kopowski, H, eds.). Academic Press, N.Y.

Milner, J.J. and Jackson, A.O. (1979). Sequence Complementarity of Sonchus Yellow Net Viral RNA with RNA from Polysomes of Infected Tobacco. Virology, 97, 90-99.

Moore, L.W. and Guthrie, J.W. (1965). Effect of Incubation Temperature on Length and Infectivity of Potato Virus X Particles in Four Host Species. Phytopathology, 55, 607-611.

Morozov, S.Y., Gorbulev, V.G., Novikov, V.K., Agranovskii, A.A., Kozlov, Yu.V., Atabekov, I.G., and Baev, A.A. (1981). Primary Structure of 5' and 3'-Terminal regions of Potato Virus X Genomic RNA. Doklady Akademii Nauk, 259, No.3, 723-725.

Morozov, S.Y., Zakharov, V.M., Chernov, B.K., Prasolov, V.S.,Koslov, Y.V., Atabekov, I.G. and Skriabin, K.G. (1983). Analysis of the Original Structure and Localisation of a Gene of Shell Protein in Genome Potato Virus X RNA. Reports of the Academy of Sciences of the USSR, 271, No.1, 211-215.

Nair, C.N. and Owens, M.J. (1974). Preliminary Observations Pertaining to Polyadenylation of Rhinovirus RNA. Journal of Virology, 13, 535-537.

Nakane, P.K. and Pierce, G.B. (1966). Enzyme-labelled antibodies: Preparation and Application for the Localisation of Antigens. The Journal of Histochemistry and Cytochemistry, 14, 929-931.

Nakazato, H., and Edmonds, M. (1972). The Isolation and Purification of Rapidly Labelled Polysome-Bound Ribonucleic Acid on Polythymidylate Cellulose. Journal of Biological Chemistry, 247, No.10, 3365-3367.

Negruk, V.I., Agranovsky, A.A.,Skryabin, K.G., and Atabekov, J.G. (1979). Size Determination of Poly (A) Sequences in Barley Stripe Mosaic Virus RNA. Analytical Biochemistry, 99, 450-453.

Nienhaus, F. and Stille, B. (1965). Ubertragung des Kortoffel X-Virus durch Zoosporen von Synchytrium endobioticum. Phytopathologische Zeitschrift, 54, 335-337.

Nygaard, A.P. and Hall, B.D. (1963). A Method for the Detection of RNA-DNA Complexes. Biochem.Biophys.Chem.Res.Commun, 12, 98-104.

Nygaard, A.P. and Hall, B.D. (1964). Formation and Properties of RNA-DNA Complexes. Journal of Molecular Biology, 9, 125-142.

Orlob, G.B. (1968). Relationships between Tetranychus urticae Koch and some Plant Viruses. Virology, 35, 121-133.

Otsuki, Y., Takebe,I., Honda, Y., Kajita, S. and Matsui, C. (1974). Infection of Tobacco Mesophyll Protoplasts by Potato Virus X. Journal of General Virology, 22, 375-385.

Ouchterlony, O. (1953). Diffusion-In-Gel Methods for Immunological Analysis II. Progress in Allergy, 6, 30-154.

Owens, R.A. and Diener, T.O. (1981). Sensitive and Rapid Diagnosis of Potato Spindle Tuber Viroid Disease by Nucleic Acid Hybridisation. Science, 213, 670-671.

Pierpoint, W.S. and Carpenter J.M. (1978). An Infective Peroxidyl-derivative of Potato virus X: PVX-PLP. Journal of General Virology, 38, 505-518.

Pirone, T.P. and Kassanis, B. (1975). Polyamino Acid Induced Aphid Transmission of Plant Viruses. Journal of General Virology, 29, 257-266.

Pratt, M.J. (1961). Studies on Clover Yellow Mosaic and White Clover Mosaic Viruses. Canadian Journal of Botany, 39, 655-665.

Pratt, M.J. and Reichmann, M.E. (1961). Characterization of Clover Yellow Mosaic Virus and its Distinction from White Clover Mosaic Virus. Proceedings of the Canadian Phytopathological Society, 28, 13.

Pugh, S.F., Slack, R.C., Caul, E.O., Paul, I.D., Appleton, P.N. and Gatley, S. (1985). Enzyme Amplified Immunoassay: A Novel Technique Applied to Direct Detection of Chlamydia trachomatis in Clinical Specimens. Journal of Clinical Pathology, 38, 1139-1141.

Purcifull, D.E., Edwardson, J.R. and Christie, R.G. (1966). Electron Microscopy of Intracellular Aggregates in Pea (Pisum sativum). Virology, 29, 276-284.

Purcifull, D.E. and Shepard, J.F. (1964). Preparation of the Protein Fragments of Several Rod-shaped Plant Viruses and their use in Agar-Gel Diffusion Tests. Phytopathology, 54, 1102-1108.

Querfurth, G. and Paul, H.L. (1979). Protein-A CoatedLinked Antisera (PALLAS), New Reagents for a Sensitive Test Permitting the use of Antisera unsuitable for the Latex Test. Phytopathologische Zeitschrift, 94, 282-285.

Radloff, R., Bauer, W. and Vinogard, J. (1967). A Due-Buoyant-Density Method for the Detection and Isolation of Cloned, Circular Duplex DNA: the Closed Circular DNA in HeLa Cells. Proceedings of the National Academy of Science, U.S.A., 57, 1514-1521.

Rave, N., Crkvenjakov, R. and Boedtker, H. (1979). Identification of Procollagen mRNAs Transferred to Diazobenzyloxymethyl Paper from Formaldehyde Agarose Gels. Nucleic Acids Research, 6, 3559-3567.

Reunova, G.D., Reunov, A.V. and Reitman V.G. (1973). Effect of Actinomycin on Potato Virus X Multiplication in Datura stramomium leaves. Virology, 52, 502-506.

Reichmann, M.E. (1959). Potato X Virus. II. Preparation and Propperties of Purified, Nonaggregated virus from Tobacco. Canadian Journal of Chemistry, 37, 4-10.

Reichmann, M.E. (1960). Degradation of Potato Virus X. Journal of Biological Chemistry, 235, 2959-2963.

Ricciardi, R.F., Goodman, R.M. and Gottlieb, D. (1978). Translation of PVX RNA in vitro by Wheat Germ. I. Characterisation of the Reaction and Product Size. Virology, 85, 310-314.

Rich, (1978). Transfer RNA: Three-Dimensional structure and Biologic Function. Trends in Biochemical Science, 3, 34-37.

Roberts, F.M. (1948). Experiments on the Spread of Potato Virus X between Plants in Contact. Annals of Applied Biology, 35, 266-278.

Rochow, W.F. and Ross, A.F. (1955). Virus Multiplication in Plants Doubly Infected by Potato Viruses X and Y. Virology, 1, 10-27.

Salaman, R.N. (1939). PVX: Its Strains and Reactions. Philosophical Transactions of the Royal Society. Series B, 229, 137-217.

Sampson, P.J. and Taylor, R.H. (1968). A Comparison of the Electron Microscope , Microprecipitin Tests, and Indicator Plants for the Detection of Potato Viruses s, X, and Y. Phytopathology, 58, 489-493.

Sanger, F. and Coulson, A.R. (1975). A Rapid Method for Determining Sequences in DNA by Primed Synthesis with DNA Polymerase. Journal of Molecular Biology, 94, 441.

Sanger, F. and Coulson, A.R. (1978). The Use of Thin Acylamide Gels for DNA Sequencing. FEBS Letters, 87, No.1, 107-110.

Schlegel, D.E. and Delisle, D.E. (1971). Viral Protein in Early Stages of Clover Yellow Mosiac Virus Infection of Vicia faba. Virology, 45, 747-754.

Seeburg, P.H., Shine, J., Marshall, J.A., Baxter, J.D., and Goodman, D.H. (1977). Nucleotide Sequence and Amplication in Bacteria of Structural Gene for rat Growth Hormone. Nature, 220, 486.

Semanik, J.S. (1974). Detection of Polyadenylic Acid Sequences in Plant Pathogenic RNAs. Virology, 62, 288-291.

Shalla, T.A. and Peterson, L.J. (1973). Infection of Isolated Plant Protoplasts with Potato Virus X. Phtopathology, 63, 1125-1130.

Shalla, T.A. and Shepard, J.F. (1970a) An Antigenic Analysis of Potato Virus X and of its Degraded Protein. II: Evidence for a Conformational Change Associated with the Depolymerisation of Structural Proteins. Virology, 42, 835-847.

Shalla, T.A. and Shepard, J.F. (1970b). A Virus Induced Soluble Antigen Associated with Potato Virus X Infection. Virology, 42, 1130-1132.

Shalla, T.A. and Shepard, J.F. (1972). The Structure and Abtigenic Analysis of Amorphous Inclusion Bodies Induced by Potato Virus X. Virology, 49, 654-667.

Sharp, P.A., Sugden, B., and Sambrook, J. (1973). Detection of Two Endonuclese Activities in Haemophilus parainfluenzae using Analytical Agarose. Biochemistry, 12, 3055.

Shaw,, J.G., Reichmann, M.E. and Hatt, D.L. (1962). Amino Acid Analysis of the Proteins of two Strains of Potato Virus X. Virology, 18, 79-88.

Shepard, J.F. (1975). Regeneration of Plants from Protoplasts of Potato Virus X-Infected Tobacco Leaves. Virology, 66, 492-501.

Shepard, J.F. and Claflin, L.E. (1975). Critical Analyses of the Principles of Seed Potato Certification. Annual Review of Phytopathology. 13, 271-293.

Shepard, J.F. and Secor, G.A. (1972). The Effects of Enzymatic Digestion on the Molecular Weight and Antigenic Specificity of Potato Virus X Protein. Phytopathology, 62, 1154-1160.

Shepard, J.F. and Shalla, T.A. (1970). An Antigenic Analysis of Potato Virus X and of its Degraded Protein. I. Evidence for and Degree of Antigenic Disparity. Virology, 42, 825-834.

Shepard, J.F. and Shalla, T.A. (1972). Relative Antigenic Specificities of Two PVX Strains and their D-Protein Oligomers. Virology, 47, 54-60.

Simoncsits, A., Brownlee, G.G., Brown, G.S., Rubin, J.R. and Guilley, H. (1977). New Rapid Gel Sequencing Method for RNA. Nature, 269, 833-836.

Sippel, A.E. (1973). Purification and Characterization of Adenosine Triphosphate: Ribonucleic Acid Adenyltransferase from Escherichia coli. European Journal of Biochemistry, 37, 31-40.

Skofenko, A., Kushnirenko, O. and Kolomiyetz, L. (1975). Third International Congress of Virology (Abstract), 84.

Sonenberg, N., Shatkin, A.J., Ricciardi, R.P., Rubin, M. and Goodman. (1978). Analysis of Terminal Structures of RNA from Potato Virus X. Nucleic Acids Research, 5, No.7, 2501-2511.

Southern, E.M. (1975). Detection of Specific Sequences among DNA Fragments Separated by Gel Electrophoresis. Journal of Molecular Biology, 98, 503-517.

Spector, D. and Baltimore, D. (1974). Requirement of 3' Terminal Polyadenylic Acid for the Infectivity of Poiovirus RNA. Proceedings of the National Academy of Sciences, U.S.A., 71, 2983-2987.

Spector, S.A., Rua, J.A., Spector, D.H. and McMillan, R. (1984). Detection of Human Cytomegalovirus in Clinical Specimens by DNA-DNA Hybridisation. Journal of Infectious Diseases, 150, 121-126.

Stalhandskkke, P. and Pettersson, U. (1982). Identification of DNA Viruses by Membrane Filter Hybridisation. Journal of Clinical Microbiology, 15, 744-747.

Stanly, C.J.; Paris, F. and Plumb, A. (1985). Enzyme Amplification: A New Technique for Enhancing the Speed and Sensitivity of Enzyme in Enzyme Immunoassays. Int. Biotech. Lab. 3, No.3, 46-51.

Stavrianopoulos, J.G. and Chargaff, E. (1973). Purification and Properties of Ribonuclease H of Calf Thymus. Proceedings of the National Academy of Sciences, USA, 70, 1959-1963.

Stavrianopoulos, J.G. and Chargaff, E. (1978). Simplified Method for Purification of Ribonuclease h from Calf Thymus. Proceedings of the National Academy of Sciences, USA, 75, 4140-4144.

Stein, H. and Hausen, P. (1969). Calf Thymus Degrading the RNA Moiety of DNA-RNA Hybrids: Effect on DNA-dependent RNA Polymerase. Science, 166, 393-395.

Stols, A.L.H., Hill-van de Meulen, G.W. and Toen, M.K.I. (1970). Electron Microscopy of Nicotiana glutinosa Leaf Cells Infected with Potato Virus X. Virology, 40, 168-170.

Stryer, L. (1981).in Biochemistry. W.H. Freeman and Company, San Francisco.

Tabor, M.W.; Leuke, B.H., and MacGee, J. (1976). Federation Proceedings Abstracts, 35, 298.

Thomas, P.S.(1980).Hybridisation of Denatured RNA and Small DNA Fragments Transferred to Nitrocellulose Proceedings of the National Academy of Sciences, U.S.A., 77, No.9, 5201-5205.

Thomson, A.D. (1961a). Effect of Tobacco Mosaic Virus and Potato Virus Y on Infection by Potato Virus X. Virology, 13, 262-264.

Thomson, A.D. (1961b). Interactions between Plant Viruses. I. Appearance of New Strains after Mixed Infection with Potato Virus X Strains. Virology, 13, 507-514.

Todd, J.M. (1957). Proceedings of the Third Conference of Potato Virus Diseases, 132, Lisse-Wageningen, The Netherlands.

Tomlinson, J.A. and Walkey, D.G.A. (1967). Effects of Ultrasonic Treatment on Turnip Mosaic Virus and Potato Virus X. Virology, 32, 267-278.

Torrance, L. and Ross, R.A.C. (1981). Recent Developments in Serological Methods Suited for use in Routine Testing for Plant Viruses. Plant Pathology, 30, 1-24.

Tremaine, J.H. and Agrawal, H.O. (1972). Limited Proteolysis of Potato Virus X by Trypsin and Plant Proteases. Virology, 49, 735-744.

Tung, J-S. and Knight, C.A. (1972). The Coat Protein Subunits of Potato Virus X and White Clover Mosaic Virus. A Comparison of Methods for Determining their Molecular Weights and some *in situ* Degradation Products of Potato Virus X Protein. Virology, 49, 214-223.

van Regenmortel, M.H.V. (1963). Serologically Related Plant Contaminants in Preparations of Partially Purified Plant Viruses. Virology, 21, 657-658.

van Regenmortel, M.H.V. (1966). in Viruses of Plants (Beemster, A.B.R. and Dikjstra, J. eds.). 213-217. North Holland, Amsterdam.

van Regenmortel, M.H.V. (1978). Applications of Plant virus Serology. Annual Review of Phytopathology, 16, 57-81.

van Slogteren, D.H.M. (1955). Proceedings of the Second Conference of Potato Virus Diseases, 45-50. Lisse-Wageningen, The Netherlands.

Varma, A., Gibbs, A.J. and Woods, R.D. (1968). Some Observations of the Structure of the Filamentous Particles of Several Plant Viruses. Journal of General Virology, 2, 107-114.

Varma, A., Gibbs, A.J., and Woods, R.D. (1970). A Comparative Study of Red Clover Vein Mosaic Virus and Some Other Plant Viruses. Journal of General Virology, 8, 21-32.

Verma, D.S., Nash, D.T. and Schulman, H.M. (1974). Translation and In Vitro Translation of Soy bean legheamoglobin, Nature (London), 251, 74-77.

Veira, J. and Messing, J. (1982). The pUC Plasmids, an M13mp7-derived System for Insertion Mutagenesis and Sequencing with Synthetic Universal Primers. Gene, 19, 259-268.

Virtanen, M.,Syvanen, A.C., Oram, J.,Soderlund, H. and Ranki, M. (1984). Journal of Clinical Microbiology, 20, 1083-1088.

Voller, A., Draper, C., Bidwell, D., and Bartlett, A. (1974). Microplate Enzyme-linked Immunosorbent Assay for Chagas Disease. Lancet I(7904), 426-428.

Voller, A., Bartlett, A., Bidwell, D.E., Clark, MF., and Adams, AN. (1976). The Detection of Viruses by Enzyme-linked Immunosorbent Assay (ELISA). Journal of General Virology, 33, 165-167.

Voller, A., Bidwell, D.E. and Bartlett, A. (1976b). Enzyme Immunoassays in Diagnostic Medicine: Theory and Practice. Bulletin of the World Health Organisation, 53, 55-65.

Wahl, G.M., Stern, M., Stark, G.H. (1979). Efficient Transfer of large DNA Fragments from Agarose Gels to Diazobenzyloxymethyl-Paper and Rapid Hybridisation by using Dextran Sulphate. Proceedings of the National Academy of Sciences, U.S.A., 76, No.8, 3683-3687.

Walters, H.J. (1952). Some Relationships of Three Plant Viruses to the Differential Grasshopper, Melanoplus differentialis, Phytopathology, 42, 355-362.

Waterhouse, P.M., Gerlach, W.L., and Miller, W.A. (1986). Serotype-Specific and General Luteovirus Probes from Cloned cDNA Sequences of Barley Yellow Dwarf Virus. Journal of General Virology, 67, 1273-1281.

Welsh, M.F., Stace-Smith, R. and Brennan, E. (1973). Clover Mosaic Virus from Apple Trees with Leaf Pucker Disease. Phytopathology, 63, 50-57.

Wetmur, J.G. (1975). Acceleration of DNA Renaturation Rates. Biopolymers, 14, 2517-2524.

Wicker, R. and Avrameas, S. (1969). Localisation of Virus Antigens by Enzyme-labelled Antibodies. Journal of General Virology, 4, 465-471.

Wodnar-Filipowicz, A., Szczeana, E., Zan-Kowalczewska, M., Muthukrishnan, S, Szybiak, U., Legocki, A.B. and Filipowicz, W. (1978). 5'-Terminal 7-Methylguanosine and mRNA Function. The Effect of Enzymatic Decapping and of Cap Analogs on Translation of Tobacco mosaic Virus RNA and Globin mRNA in vitro. European Journal of Biochemistry, 92, 69-80.

Wodnar-Filipowicz, A., Skrzeckowski, L.J. and Filipowicz, W. (1980). Translation of Potato Virus X RNA into High Molecular Weight Proteins. FEBS Letters, 109, 151-155.

Yolken, R.H. (1979). Enzyme-Linked Immunosorbent Assay (ELISA): A Practical Tool for Rapid Diagnosis of Viruses and Other Infectious Agents. Journal of Clinical Microbiology, 10, 85-92.