Molecular Characterisation of Barley Yellow Mosaic Virus

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By

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Abstract

Barley yellow mosaic virus (BaYMV) causes a disease in winter barley resulting in up to 40% loss in yield. It was first reported in England in 1980 and due to increased winter barley production and the soil-borne nature of the virus the disease will continue to be economically important until resistant barley cultivars are available. Little is known about the molecular biology of the virus which does not apparently fit into any of the major plant virus groupings.

Methods have been developed for the transmission, propagation and purification of the virus and extraction of viral RNA. Particle structure and cytopathological effects have been studied using electron microscopy. Total single- and double-stranded and polysomal RNA has been extracted from BaYMV-infected plants and analysed by gel electrophoresis, Northern blotting and hybridisation to BaYMV-specific cDNA. Virion and polysomal RNA have been used to direct *in vitro* translation systems. Soluble proteins of healthy and BaYMV-infected plants have been analysed for the presence of 'pathogenesis-related' (PR) proteins and cultivar- or temperature-dependant gene expression. Root tissue of infected plants has been investigated for evidence of virus replication. Recombinant M13 phage clones have been obtained using cDNA synthesised from template RNA enriched for BaYMV RNA.

BaYMV encapsidates two RNA species ($\simeq$1.35 & 2.7 $\times 10^6$) which do not have extensive sequence homology and were not found to be polyadenylated or infectious. Northern blot analysis revealed four RNA species in polysomal RNA extracts and at least seven dsRNA species in infected plants that hybridised to BaYMV-specific cDNA. *In vitro* translation of virion RNA produced a number of polypeptides none of which comigrated with capsid protein. BaYMV infection of barley did not induce high levels of PR-like proteins.

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Abbreviations of Virus Names

AlMV	Alfalfa Mosaic Virus
AuMV	Araujia Mosaic Virus
BaYMV	Barley Yellow Mosaic Virus
BMV	Brome Mosaic Virus
BNYVV	Beet Necrotic Yellow Vein Virus
BSMV	Barley Stripe Mosaic Virus
CARNA-5	Cucumber Mosaic Virus Associated RNA-5
CMV	Cucumber Mosaic Virus
CPMV	Cowpea Mosaic Virus
CLSV	Chlorotic Leaf Spot Virus
CTV	Citrus Tristeza Virus
MRDV	Maize Rough Dwarf Virus
NVMV	Nicotiana Velutina Mosaic Virus
OMV	Oat Mosaic Virus
PeMV	Pepper Mottle Virus
PMTV	Potato Mop-Top Virus
PPV	Plum Pox (Sharka) Virus
PVA	Potato Virus A
PVMV	Pepper Veinal Mottle Virus
PVX	Potato Virus X
PVY	Potato Virus Y
RNMV	Rice Necrosis Mosaic Virus
SBWMV	Soil-Borne Wheat Mosaic Virus
TBSV	Tomato Bushy Stunt Virus
TBV	Tulip Breaking Virus
TEV	Tobacco Etch Virus
TMV	Tobacco Mosaic Virus
TNV	Tobacco Necrosis Virus
TYMV	Turnip Yellow Mosaic Virus
WMV-1	Watermelon Mosaic Virus 1
WMV-2	Watermelon Mosaic Virus 2
WSSMV	Wheat Spindle Streak Mosaic Virus
WMV	Wheat Mosaic Virus
WYMV	Wheat Yellow Mosaic Virus

1 INTRODUCTION

Barley yellow mosaic virus (BaYMV) causes stunting of winter barley and up to 40% loss of yield (INOUYE & SAITO, 1975). The virus has been known in Japan since 1940 (IKATA & KAWAI) but has only recently been recorded in Germany (HUTH & LESEMANN, 1978), France (LAPIERRE, 1980), Belgium (MAROQUIN *et al.*, 1982) and the UK (HILL & EVANS, 1980) where its increasing incidence is a cause for concern. Recent trends in winter barley production have made BaYMV an economically important virus and, since it is soil-borne, difficult to control. These factors, along with a lack of knowledge about the virus have prompted an increasing interest in BaYMV.

BaYMV bears a superficial resemblance to the potyviruses with regard to its particle morphology and its ability to induce cytoplasmic 'pinwheel'-type inclusion bodies. However, as noted by HOLLINGS & BRUNT (1981) there seems no cogent reason to include BaYMV in such a homogeneous grouping until differences in mode of transmission, particle-size distribution and symptomatology are shown to be insignificant. Hence studies on the molecular characterisation of a virus which may merit a new classification are of considerable interest.

1.1 Discovery and international spread of BaYMV

BaYMV was recorded for the first time in Japan where by 1958 it had become one of the most important diseases of barley (MIYAMOTO, 1958). The disease appeared to be restricted to the Far East until 1974 when it was recorded in Bulgaria. Outbreaks were later reported in the Federal Republic of Germany (HUTH & LESEMANN, 1978) where by 1979, in a large area of northern Germany bounded by the cities of Braunschweig and Göttingen to the east and Köln and Bonn

to the west, the virus was found to completely infect more or less limited areas as large as 1000 ha. (HUTH, 1979). The rapid increase in number of reports of the virus, in West Germany as well as in other parts of Europe, can be explained at least in part by a greater awareness of and the ability to recognise the virus. In Germany yellow patches had been reported in autumn-sown barley since about 1960 and these patches were subsequently found to be infected with BaYMV. By 1984 more than one third of all arable land in northern Germany had become affected (HUTH, 1985).

In 1980 BaYMV was reported in France (LAPIERRE, 1980), where it appeared to be confined to the eastern half of northern France and an isolated area south-west of Paris, and in England (HILL & EVANS, 1980). Most recently in 1982 BaYMV was reported in Belgium (MAROQUIN *et al.*) where it is likely to have been present in isolated patches along the Sambre and Meuse valleys since 1977.

After the first report in England at a site near Abingdon, Oxfordshire, more than 40 outbreaks were documented in 1980, mainly in East Anglia. Further outbreaks were recorded in 1981 including the first in the south-west. By 1982 more than 200 reports had been confirmed and by 1983 the virus was found to be prevalent in many parts of the country including the West Country, the Home Counties, East Anglia, the Midlands, North Yorkshire, Humberside and the Grampians (CUTTING, 1983).

Even allowing for the fact that the virus probably went unnoticed for many years these records reflect a rapid and widespread dissemination which was undoubtedly expedited by the soil-transmission, and hence persistence, of the virus and recent increases in winter barley production.

1.2 Agronomic Trends

A combination of underlying factors has led to an increase in barley production of 74% over the period 1974-82 during which time the proportion of autumn-sown barley has risen from 10 to 40% (GOVERNMENT STATISTICAL SERVICE, 1983). Similarly winter barley production has more than doubled in the same period in West Germany (FRIEDT & FOROUGHI-WEHR, 1985). Autumn sowing spreads the timing of the cereal harvest, relieving pressure on resources and allowing early entry for following crops such as oilseed rape and stubble turnips or, alternatively, greater time for soil-conditioning. Autumn-sown barley predominates over autumn-sown wheat because of its greater yield potential. Increasingly, malting varieties of barley (traditionally spring-sown) have been autumn-sown in preference to more cold-tolerant winter varieties. The higher yield of the latter is compensated for by high malting premium and early selling time with associated reductions in storage problems and improvements in cash-flow though this has led to increased disease-susceptibility and winter losses.

In 1982 in England and Wales, 829 kilohectares of land were cultivated with winter barley and were consequently at risk from BaYMV.

1.3 Transmission

It is now widely accepted that BaYMV has at least one fungal vector, the plasmodiophoraceous root parasite *Polymyxa graminis* (Led.). Evidence put forward by KUSABA *et al.* (1971), to support this contention includes: the acquisition of infectivity by sterilised soil on addition of resting spores of the fungus collected from the roots of naturally infected barley; the stability of infectivity in resting spores to treatment with 10% Teepol, 5% trisodium

orthophosphate, or at pH 2.3; acquisition of infectivity by virus-free fungus when grown on plants infected with BaYMV by mechanical inoculation; and the close correlation between infectivity of washings from naturally infected roots and the concentration of fungal spores therein. Additionally, infectivity was found to be retained in air-dried soil for over 5 years (YASU & YOSHINO, 1964), though an alternative explanation for this was put forward by MIYAMOTO (1959a,b) who reported infectivity associated with soil particles of the clay fraction which includes organic and inorganic colloidal material. Mixtures of sap from infected plants and sterilised soil, kaolin or bentonite remained infectious for at least 8 months at 10-15°C. While the possibility exists of contamination by viruliferous resting spores from the infected plants (though none were found) a demonstration of the ability of soil particles to stabilise infectivity does not rule out a vector role for *Polymyxa graminis in vivo*. Indeed there is a growing body of evidence linking the transmission of many soil-borne viruses with zoosporic fungi (for review see BRUNT & SHIKATA, 1986). It is not unlikely that BaYMV is transmitted by other zoosporic root parasites such as *Pythium* spp.

An unambiguous demonstration of the ability of *P.graminis* to transmit BaYMV has recently been given (K.LAING, pers. comm.). Infectivity was found to be associated with fungal cystosori isolated from BaYMV-infected plants and freed from contamination by differential centrifugation and micromanipulation.

Soil-transmission is the only known method of spread "in the field" as there have been no reports of transmission through seed, by dodder or by any insect (INOUYE & SAITO, 1975; HUTH, 1985). Spread within and between fields is facilitated by farm machinery leading to elongation of virus-infected patches along lines of cultivation (HILL & EVANS, 1980; HUTH, 1984). HUTH (1985) has also proposed

that high winds can carry soil dust contaminated with viruliferous spores over long distances.

Barley (*Hordeum vulgare*) is the only susceptible species known in the field.

BaYMV is transmissible to barley by mechanical inoculation. The virus is not readily transmissible (INOUYE & SAITO, 1975) but improvements in inoculation procedure (FRIEDT, 1982) have resulted in success rates of up to 100%. Plants were inoculated at the 3-4 leaf stage by rubbing with inoculum consisting of plant sap and various additives, left in the shade for 24h then incubated at temperatures of 10/8°C, day/night, with 12h daylength. Under this regime addition of potassium phosphate (0.04M, pH7) and/or sodium sulphite (0.1M, pH9) to the inoculum and repetition of inoculation after 7 days resulted in the first appearance of symptoms in plants after 3 weeks and maximum infection rates of between 80 and 100% after 5-6 weeks.

Mechanical transmission to any but *Hordeum* spp. has not been reported.

Results of recent work by HUTH *et al.* (1984) and EHLERS & PAUL (1986) suggest that more than one strain of BaYMV exists in Germany at least one of which is not sap-transmissible. This work is discussed below (see 1.6).

Mechanical transmission is unlikely to be significant in the field.

1.4 Symptomatology and Yield Effects

The youngest leaves of BaYMV-infected plants have chlorotic streaks (1-5mm in length) running along their veins (Figs.3-5) and often exhibit upward rolling of leaf margins causing an upright, spiky appearance. In the older leaves of some cultivars (eg. Maris Otter) chlorotic streaks often become necrotic, particularly at the tip, while other

cultivars (eg. Igri) display an orange-yellow discolouration with age. Early in the season during onset of stem elongation at growth stage (GS) 30 (ZADOKS *et al.*, 1974), affected patches have a yellow or greying and spiky appearance and by GS 32 stunting is apparent (HILL & EVANS, 1980). The severity of symptoms depends on the cultivar and environmental conditions, symptoms not usually being produced over 18°C (INOUYE & SAITO, 1975), appearing in early spring and tending to disappear as the weather becomes warmer. Under mild conditions some cultivars (eg. Sonja, Augusta & Dura) suffer less damage but lower temperatures throughout the growth period lead to height reductions of up to 50% (HUTH, 1985). Climatic differences could thus explain the apparent resistance of cv. Sonja in England (HILL, 1985; PLUMB, 1985) as compared with its susceptibility in Germany (HUTH, 1985). Infection of susceptible cultivars, which are generally less cold-tolerant anyway (HILL, 1985), may lead to early senescence and increased vulnerability to frost damage and winter kill. In Germany frost damage due to prolonged cold spells has resulted in barley being ploughed in.

The degree of damage is further influenced by the concentration of viruliferous spores of *P.graminis* and thus the age of the infestation, cropping-history and soil-type, heavy soils being richer in fungal spores (HUTH, 1985; HILL, 1985). It may also be affected by the sowing time, *Polymyxa*-infectivity being greater at the higher temperatures found earlier in the autumn. In a study in England (PLUMB, 1985), early sowing was found to increase the proportion of plants infected with *P.graminis*, the severity of its infection and the proportion of plants displaying BaYMV-symptoms throughout the growing season. The interpretation of these results is complicated by the finding that early sowing increased *P.graminis* infection in the symptomless plants as well, though of course it is not possible to distinguish between viruliferous and virus-free

cystosori. Furthermore HILL, (1985) found that this late-sowing attenuation was short-lived and that by March early- and late-sown plants were equally infected with BaYMV.

The effects of BaYMV on components of yield are analogously influenced. In Germany regional climatic differences are associated with differences in yield reduction, greatest reductions being found in areas of generally lowest temperatures (HUTH, 1985). In a study in England, PLUMB (1985) found all components of yield (height, grain number, grains/ear, 1000 grain weight and yield) to be significantly decreased by BaYMV infection in both Maris Otter and Sonja plants indicating that, when infected, cv. Sonja is damaged as much as the more susceptible cv. Maris Otter. In a study in Germany (HUTH, 1982), yield reductions were found to be due to lower shoot number rather than 1000 grain weight which actually increased relatively in some cultivars. The author acknowledged that these results were from small sample plots and that no account was taken of winter kill thus biasing the figures towards resistant/virus-free plants.

1.5 Varietal Reactions, Resistance and Control

General patterns of varietal reaction to BaYMV that have emerged after years of trials can be summarised as follows:

- UK malting cultivars of winter barley are universally susceptible whilst feed cultivars vary in susceptibility;
- cultivars grown in the UK fall into three broad categories: very susceptible (eg. Maris Otter, Halcyon, Tipper, Triumph), moderately resistant (eg. Pirate, Gerbel, Hexa) and resistant (eg. Igri, Sonja, Birgit, Athene);
- such groups appear to be related to the breeding parentage of the cultivar but varietal reaction is highly variable with respect to the proportions of plants infected and the degree of symptom-expression, either because of

lack of genetic uniformity within one cultivar or erratic distribution of virus/vector populations;

- resistance is associated with cold tolerance though susceptibility of some cultivars is increased in cold temperatures, thus cv. Sonja appears resistant in the UK but susceptible in Germany;
- in the UK but not Germany reactions may differ according to varietal pre-cropping in that cultivars sharing common parentage are more resistant in fields pre-cropped with cultivars of different parentage. Thus pre-cropping with Igri as compared with Maris Otter changed the proportion of infected plants from 34% to 97% for Maris Otter, 26% to 75% for Halcyon (related to Maris Otter), 49% to 28% for Igri and 35% to 1% for Sonja (related to Igri);
- this effect is apparently reversible by repeated cropping with another cultivar since Sonja, grown in fields in which Maris Otter had been grown and infected, showed no symptoms until the fourth year of repeated cultivation. The character of a virus or vector population may thus be changed by selection over a period of only 3 years (HILL, 1985).

Attempts at control of BaYMV including deep ploughing, use of pre-cropping soil-sterilants such as methyl bromide, diazomet, dichloropropene and formaldehyde, and seed-dressings and fungicide sprays such as fosetyl-aluminium (Alliette), have all proved unsuccessful (HILL, 1985). Yield loss can only apparently be avoided by growing resistant cultivars. However, presently recommended resistant cultivars lack other agronomically desirable characteristics (FRIEDT & FOROUGHI-WEHR, 1985), so breeding programmes designed to combine resistance with superior grain quality have been initiated.

In an evaluation of sources of resistance in 480 cultivars and strains in Germany, HUTH (1982) found 6 cultivars with high resistance (Barbo, Birgit, Franka, Ogra, Hexa and Bison) and a further 23 cultivars, 8 of them

2-rowed, with intermediate resistance. A similar survey of 800 lines in Japan (TAKAHASHI *et al.*, 1973) had revealed one resistant cultivar , "Mokusekko 3", a Chinese 6-rowed spring barley carrying a dominant resistance gene Ym 1 on chromosome 4 ; Japanese breeders succeeded in transferring this gene into a 2-rowed cultivar which they named "Resistant-Ym No.1" (MURAMATSU, 1976).

FOROUGHI-WEHR & FRIEDT (1984) crossed resistant 6-rowed cultivars with susceptible but higher malting quality 2-rowed cultivars and used the rapid technique of anther culture to produce doubled haploids of F_1 progeny which they screened for resistance by mechanical inoculation. Their results established that all sources of resistance in German cultivars can be explained by one or two recessive genes and that resistance and row-number segregate independently. A subsequent programme (FRIEDT & FOROUGHI-WEHR, 1985) indicated that this resistance is probably determined by a single recessive gene. After confirmation of the dominance of the Ym 1 gene by crosses between susceptible German 2-row cultivars and the resistant "Mokusekko 3" and "Resistant-Ym No.1" cultivars the Ym 1 was introduced into German cultivars carrying the single recessive gene. The absence of susceptible plants in F_1 or F_2 progeny suggested that the two genes are either allelic or that "Resistant Ym No.1" carries an additional recessive gene allelic to that of the German cultivars. Further work is necessary but it appears that it is possible to introduce a broad basis of resistance into high yielding 2-rowed cultivars. In the UK such a blend has apparently been achieved in "Palomino", from Miln Masters. It is described as a resistant, 2-rowed cultivar with yield between that of Sonja and Igri.

In the German breeding programmes resistance or susceptibility was assessed by reaction to mechanical inoculation with BaYMV. Recent reports of at least one strain of BaYMV that is not sap-transmissible (HUTH *et al.*,

1984, and see 1.6 below) thus have important implications. However, so far all plants found to be resistant to infection by mechanical inoculation have also proved resistant in the field, though HUTH (1985) has expressed a fear that some cultivars may only have strain-specific resistance.

In summary, one might conclude that two separate factors are at work influencing the BaYMV-responsiveness of different barley cultivars: a non-specific hardiness associated with cold-tolerance which can be overcome by prolonged cold periods and a genetically based resistance which, although usually only found in 6-rowed cultivars at present, is determined by gene(s) independent of gene(s) for row-number. Potential thus exists for incorporating these two favourable characteristics into new breeding lines.

1.6 BaYMV Strains and Serological Relationships

In 1970 in Japan, an antiserum was raised to a partially purified preparation of BaYMV which, after adsorption with healthy plant sap, gave a titre of 1/1280 and reacted with wheat yellow mosaic virus (WYMV) and rice necrosis mosaic virus (RNMV) in complementation fixation tests (USUGI & SAITO, 1970). In similar tests no serological relationship was found with wheat yellow virus (USUGI & SAITO, 1970) or soil-borne wheat mosaic virus (USUGI & SAITO, 1976). More recently (USUGI & SAITO, 1979) BaYMV has been shown to be serologically related to wheat spindle streak mosaic virus (WSSMV). BaYMV resembles RNMV (INOUYE & FUJII, 1977), WYMV (SAITO *et al.*, 1966), WSSMV (SLYKHUIS, 1976) and oat mosaic virus (OMV) (HEBERT & PANIZO, 1975) in its soil-transmission, particle morphology and ability to induce cytoplasmic pinwheel-type inclusion bodies and membranous network structures in infected cells. Of the last two named

viruses, which are prevalent in N.America, OMV is not known to be serologically related to any of the other three viruses which until recently have been confined to Japan. There is no evidence for the existence of different strains of BaYMV in Japan but differences in varietal reaction to BaYMV have been found (KUSABA *et al.* 他, TAKAHASHI *et al.*, 1973).

In Germany at least three types of BaYMV exist which are identical in particle morphology and symptomatology and soil-transmission but differ in buoyant density, serology and sap-transmissibility (HUTH *et al.*, 1984). One type (designated BaYMV-M) could be mechanically transmitted and did not react with the antiserum raised to the Japanese isolate (BaYMV-J), whereas the other (BaYMV-NM) could not be so transmitted but did react with BaYMV-J antiserum. BaYMV-J is sap-transmissible. The third type, designated BaYMV-So and described only recently (EHLERS & PAUL, 1986), reacts with BaYMV-J antiserum and is closely related to BaYMV-NM but is mechanically transmissible, though with some difficulty. Of the three types BaYMV-So thus most closely resembles BaYMV-J.

Using Japanese antiserum to WYMV, BaYMV-NM could be trapped on activated grids but decoration was not observed, indicating a serological relationship more distant than that to BaYMV-J. However field samples of barley from western Germany contained a virus which reacted strongly with WYMV antiserum in both tests but only weakly with BaYMV-J antiserum, suggesting that even more types may exist. BaYMV-M did not react with WYMV antiserum and Japanese antiserum to RNMV did not react with either virus.

Using antisera raised to BaYMV-M alone and a mixture of BaYMV-M and -NM and the BaYMV-J antiserum, HUTH *et al.* (1984) showed that the proportion of BaYMV-M tended to increase throughout the growing season though there was great variability in samples even from the same site. Selectivity by barley cultivars for one type of virus was

not found. These authors also observed different degrees of intensity in decoration experiments and proposed mixed encapsidation with both types of capsid protein to explain this result.

These data on serological relationships present a confusing picture: RNMV is related to BaYMV-J but not any of the German isolates; WYMV antiserum cross-reacts with BaYMV-J and, less strongly, with BaYMV-NM and BaYMV-So but not with BaYMV-M; BaYMV-J is related to BaYMV-NM and BaYMV-So but not BaYMV-M although BaYMV-J is reported to be sap-transmissible; additional virus types may exist in German barley that are more closely related to WYMV than BaYMV although WYMV does not infect barley. The properties of BaYMV-M are particularly anomalous in that, despite resembling other BaYMV isolates in symptomatology, soil-transmission, particle-morphology and cytopathology, it bears no serological relationship to any of them. Furthermore, plants resistant to infection with BaYMV-M by mechanical inoculation also seem to be resistant to infection with a mixture of BaYMV-M and BaYMV-NM isolates in the field. Differences in coat protein stability may explain variations in sap-transmission (see 1.8 below) but not the serological anomalies. Further information such as sequence data is required to show the relatedness of these two viruses and whether they can both be designated BaYMV.

The implications of the existence of strains to resistance-breeding programmes and testing have been mentioned (1.5, above). At present results of field trials all concur with those of greenhouse tests using mechanical inoculation to assay BaYMV-susceptibility (in HUTH *et al.*, 1984). However, considering the anomalous serological characteristics of BaYMV-M, it is conceivable that differences in varietal behaviour may emerge. Such behaviour would help make sense of differences in varietal reaction as a consequence of pre-cropping though, equally,

these results could be explained by different vector populations.

HILL (1985) reported that from limited testing it appears that both types of BaYMV found in Germany are also found in the UK. Further evidence (K.LAING, pers. comm.) suggests that other UK serotypes may exist as well. An isolate from Little Clacton, used in this study, and one from Streatly in Bedfordshire react in ISEM and ELISA tests with antiserum to BaYMV-M and a mixture of BaYMV-M and -NM. Both are mechanically transmissible so appear to be similar to BaYMV-M. An isolate from Ipsden in Oxfordshire does not react with BaYMV-M antiserum and reacts only weakly with the mixed antiserum in ELISA tests. However in decoration tests using protein A-gold a positive reaction is seen with the mixed antiserum. It is mechanically-transmissible with great difficulty so seems more closely related to BaYMV-NM or -So.

1.7 Taxonomy

Barley yellow mosaic virus was not included in any internationally recognised virus grouping until recently when it was placed in sub-group 2 (fungal-transmitted) of the potyviruses. It is similar to potyviruses in particle morphology and ability to induce pinwheel-type inclusion bodies, but its soil-transmission and lack of serological-relationship to any known potyviruses argue against inclusion in this group. Other soil-borne viruses such soil-borne wheat mosaic virus and beet necrotic yellow vein virus (see BRUNT & SHIKATA, 1986) have shorter, more rigid particles, do not induce cytoplasmic inclusions and show no serological relationship to BaYMV. BaYMV shares many characteristics with WYMV, RNMV, WSSMV and OMV (see 1.6 above) and common antigenic properties with at least the first three of these viruses which may thus constitute a

new virus grouping. Unambiguous classification will not be possible until more biochemical and molecular data are available.

1.8 Particle Structure, Composition and Properties

Barley Yellow Mosaic Virus particles are slightly flexuous filamentous rods (Fig.1). They are 13nm in diameter and have a bimodal length distribution with maxima reported to be at 275nm and 550nm by INOUYE & SAITO, (1975), and at 270-289nm and 568-600nm by HUTH *et al.* (1984), who also found a minor maximum at 855-873nm, a value near to the sum of the other two maxima. Size distributions were similar from leaf-dip or partially purified preparations and, for the German virus, from BaYMV-M or BaYMV-NM. Negative-staining sometimes revealed a faint central canal and indistinct cross-banding (HUTH *et al.*, 1984).

The uv-absorption spectrum of BaYMV is typical for a nucleoprotein having a maximum at 265-270nm with a shoulder at 290nm and an A260/280 ratio of 1.14 indicating a nucleic acid content of 5% (INOUYE & SAITO, 1975). Buoyant density in caesium chloride is 1.294g/ml (USUGI & SAITO, 1976). HUTH *et al.*, (1984), found two RNA species in partially purified virus preparations of molecular weights 2.7-2.8 and 1.4-1.5 $\times 10^6$ as determined by electrophoresis through 2.8% polyacrylamide gels under non-denaturing conditions. It was not shown whether both species are sufficient or necessary to cause infection. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) of denatured capsid protein from similar preparations revealed a band of size 35 $\times 10^3$ as well as bands of molecular weights between 29.5- and 33 $\times 10^3$ which were ascribed to degradation since their levels could be reduced by the use of protease inhibitors during purification (EHLERS & PAUL, 1986).

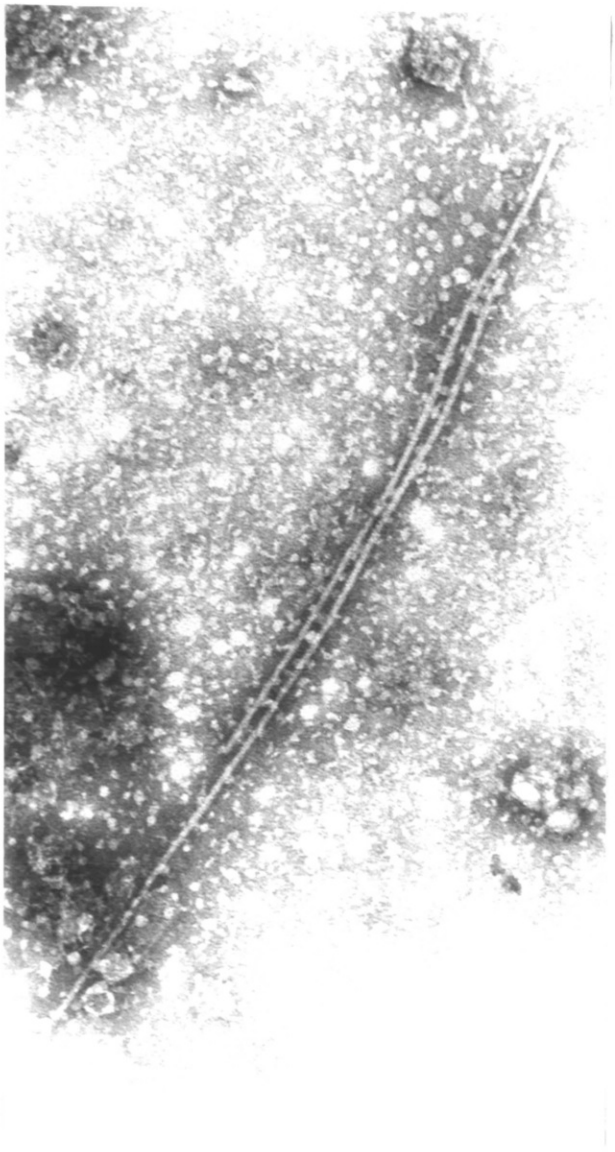


Fig.1 Electron micrograph of BaYMV particles in a leaf-dip preparation (×100,000).

All bands were shown to be BaYMV-specific by Western blotting experiments which also detected proteins of higher molcular weight in BaYMV-M extracts. The significance of these bands is not known. Coat proteins of BaYMV-NM and -So were found to be more labile than that of BaYMV-M so degradation of capsid may explain the poor mechanical transmission and higher bouyant density of the former.

Virus concentration in plants is low, the dilution end point being less than 10^{-3} (KUSABA *et al.*, 1971). Infectivity of dried leaves is retained for two years at 5-10°C (MIYAMOTO, 1958). Freezing at -22°C results in about 20% loss of infectivity (FRIEDT, 1982). Thermal inactivation point and longevity *in vitro* are not known but particles purified by equilibrium centrifugation lose infectivity within 24h if left in CsCl at 4°C (USUGI & SAITO, 1975).

1.9 Relations With Cells and Tissues

Cells of infected plants are characterised by voluminous cytoplasm containing hypertrophied endoplasmic reticulum (ER), numerous small vesicles, a high ribosome content and occasionally lipid droplets and a high electron density associated with necrosis (HUTH *et al.*, 1984). Electron microscopy of thin sections of infected leaves reveals prominent membranous network structures, cytoplasmic pinwheel-type inclusions and filamentous aggregates often several µm in length which may represent bundles of virus particles. These cytoplasmic alterations are found in mesophyll, xylem parenchyma and epidermal cells of infected tissue (SAITO *et al.*, 1966, MAROQUIN *et al.*, 1982, HUTH *et al.*, 1984).

Pinwheel inclusions resemble those of potyviruses consisting in cross-section of an axial tubule and more or less curved arms which, in longitudinal section, are often

seen associated at one end with ER. Scroll formation has not been observed.

In some cells highly ordered, sometimes paracrystalline, three-dimensional membranous arrays of up to 5μm in diameter are observed, peripherally continuous with rough ER. Relatively loose "coat of mail" arrays are found in cells of plants with mild infections but in severe infections arrays appear more condensed. Differences in cytology may reflect environmental influence.

Similar cytological effects have been found in plants infected with all BaYMV isolates.

1.10a Purification of BaYMV

There are two published protocols for the purification of BaYMV, one by Japanese workers (USUGI & SAITO, 1970, 1975, 1976) and the other by German workers (HUTH *et al.*, 1984). In the former the virus was purified by clarification with carbon tetrachloride followed by differential, sucrose density gradient and isopycnic gradient centrifugation, the last in caesium chloride. Optimal yields were obtained using citrate buffer (0.1M, pH7.0) as opposed to phosphate (0.1M, pH7,0), borate (0.005M, pH9.0), acetate (0.1M, pH5.0) or citrate (0,01M, pH7.0) buffers. The virus was said to be stable in carbon tetrachloride but not in chloroform or butanol. Sucrose density gradient centrifugation resulted in two virus containing zones, the top consisting mainly of short particles and the bottom of a mixture of both long and short particles. The bottom zone and mixtures of the two were made highly infectious by addition of of healthy plant sap. Equilibrium density centrifugation in caesium chloride for 20h at 36,000rpm further purified the virus which was found to have a buoyant density of 1.2943 and was highly infectious though storage in caesium chloride for even one

day entirely destroyed this infectivity. Electron microscopy revealed a highly pure product with occasionally some degree of aggregation of the rods.

HUTH *et al.* (1984), however, found this protocol to be unsatisfactory in terms of quality and virus yield. They introduced modifications to reduce the number and shorten the length of steps during purification as they found BaYMV had a tendency to aggregate and become insoluble in elongated protocols. The major modifications were the use of only one round of differential centrifugation, through a 30% (w/v) sucrose cushion, and omission of the sucrose gradient step. The final isopycnic gradient centrifugation step was retained and yielded a partially purified preparation suitable for production of antisera and the study of some particle properties. The authors found the choice of starting material to be critical; green leaves displaying clear symptoms gave highest yields while older chlorotic or necrotic leaves gave poor results despite containing more particles, as revealed by ISEM. This phenomenon is probably due to association and aggregation of the virus with some host component(s) found at higher levels in necrotic tissue leading to loss at one of the low speed centrifugation steps and anomalous properties in caesium chloride gradients. This sort of behaviour was found in preparations containing high ferritin levels. Sucrose density centrifugation was unsuccessful since breakdown and aggregation led to a wide range of sedimentation characteristics.

The caesium chloride centrifugation step gave the first indications of the existence of strains of BaYMV in Germany. Material from the field produced two closely spaced opalescent bands whereas mechanically inoculated material only produced one, the upper band. This discovery led to the further characterisation which has been described earlier (1.8). EHLERS & PAUL (1986) proposed that degradation of capsid, leading to a lower protein:RNA

ratio, could account for the observed increase in buoyant density.

The main problems encountered by the German authors during purification were the low levels of virus found in plants, instability and a tendency for particles to aggregate with each other and host components leading to insolubility. Aggregation and fragmentation lead to heterogeneity in sedimentation properties so, from the information available, it is difficult to understand how the Japanese authors had any success with sucrose density gradient centrifugation at all.

1.10b Purification of Rod-Shaped Plant Viruses

It is not unreasonable to expect that virus particles sharing biophysical and morphological characteristics require similar considerations with regard to purification. Amongst rod-shaped plant viruses the problems of irreversible aggregation and/or particle fragility are frequently encountered during the purification of Hordeiviruses (JACKSON & LANE, 1981), Potexviruses (PURCIFULL & EDWARDSON, 1981), Carlaviruses (WETTER & MILNE, 1981), Potyviruses (HOLLINGS & BRUNT, 1981), Closteroviruses (LISTER & BAR-JOSEPH, 1981) and "furoviruses" (BRUNT & SHIKATA, 1986). Tobraviruses and Tobamoviruses are relatively stable and easy to purify (HARRISON & ROBINSON, 1981, VAN REGENMORTEL, 1981).

Aggregation of Hordeiviruses can be caused by interaction between polycations and the negatively charged particles, particularly at low ionic strengths, or with plant lectins on account of the carbohydrate moieties of the virus. High ionic strengths (>0.2M) alone also lead to aggregation so a compromise level must be used. Detergents help to solubilise particles but at too high concentrations (>1%) lead to loss of infectivity. This behaviour is not

particularly important for Hordeivirus purification because of the large numbers of particles found in plants but is illustrative of the type of physico-chemical properties rod-shaped plant viruses can exhibit.

Potexviruses and Carlaviruses both have slightly flexuous filamentous particles which are prone to fragmentation and aggregation during purification but because of their moderate (Carla-) and high (Potex-) concentrations in plants these problems have not been addressed to any great extent. Interestingly, in contrast to Hordeiviruses, aggregation of Carlaviruses is minimised by high molarity (WETTER & MILNE, 1981).

Some, but not all, potyviruses have proved difficult to purify. As with other flexuous rods the major problem is aggregation leading to insolubility. A number of different approaches to this problem have been used. Concentration of virus in the host plant is another important aspect and can sometimes be manipulated by judicious choice of propagation host, though not for viruses such as those of the *Gramineae* which have restricted host ranges.

Many different buffers for purification have been used and it appears each virus may have its own specific requirements (eg low ionic strengths for plum pox virus (PPV), (VAN OOSTEN, 1972) and avoidance of borate for tulip breaking virus (TBV), (DERKS *et al.*, 1982)). High ratios of buffer to plant material are universally useful, probably by dispersing virus and host material, but practical considerations limit this to 3:1 or 4:1 (ml:g). Additives such as chelating agents, reducing agents and protease inhibitors are often helpful. The addition of diethyl dithiocarbamate (DIECA), a copper-chelator and hence polyphenol oxidase inhibitor, at a concentration of 10mM doubled the infectivity of PPV preparations (VAN OOSTEN, 1972), minimised degradation of potato virus A (PVA) (FRIBOURG & DE ZOETEN, 1970), and in general is probably useful where necrotic tissue is involved. EDTA reduces

aggregation for some viruses (eg. PPV (VAN OOSTEN, 1972), PVY (DERRICK & BRLANSKY, 1975) and watermelon mosaic virus 2 (WMV2) (in HIEBERT & CHARUDATTAN, 1984)) but not others such as araujia mosaic virus (AuMV) and watermlon mosaic virus 1 (WMV1) which require divalent cations for particle stability and solubility (HIEBERT & CHARUDATTAN, 1984). The most commonly used reducing agent is 2-mercaptoethanol (0.1-1%) but ascorbate, thioglycollate and sodium sulphite have also been used. Phenylmethyl sulphonyl fluoride (PMSF) is sometimes added to inhibit the action of serine proteases (HIEBERT & CHARUDATTAN, 1984).

Several different clarifying agents have been used, one of the most common of which is n-butanol though HOLLINGS & BRUNT, (1981), found this solvent disruptive for many potyviruses. Other solvents which have been used are chloroform and carbon tetrachloride, alone or in combination. Clarification with the non-ionic detergent Triton X-100 proved the most successful method for PPV (VAN OOSTEN, 1972) and TBV (DERKS *et al.*, 1982), viruses for which clarification with organic solvents, especially carbon tetrachloride, led to fragmentation and loss of activity.

Ultracentrifugation and polyethylene glycol (PEG) precipitation are the most widely used concentration methods although the former can sometimes cause fragmentation and the latter irreversible aggregation of potyviruses. However, many potyviruses are stable to one round of ultracentrifugation, especially through sucrose cushions, or they can be resuspended from PEG-pellets. Resuspension of virus pellets has been aided by urea (TOLIN *et al.*, 1975, SUN *et al.*, 1974, DAMIRDAGH & SHEPHERD, 1970, THOUVENEL *et al.*, 1976, VAN OOSTEN, 1972), EDTA (TOLIN *et al.*, 1975, BOND & PIRONE, 1971), 2-mercaptoethanol (THOUVENEL *et al.*, 1976, VAN OOSTEN, 1972, LANGENBERG, 1973) and polyvinyl pyrrolidone (LANGENBERG, 1973). Further purification is usually achieved by sucrose density

centrifugation but chromatography on controlled-pore glass (CPG) columns was successful for pepper veinal mottle virus (BARTON, 1977) and centrifugation onto a caesium sulphate cushion gradient was used for AuMV (HIEBERT & CHARUDATTAN, 1984).

Closterovirus particles only reach low levels in plants and being even longer and more flexuous than potyviruses are prone to sheering during homogenisation and compaction during centrifugation (LISTER & BAR-JOSEPH, 1981). "Mild" clarifying agents such as bentonite (DUNN & HITCHBORN, 1965; BAR-JOSEPH & HULL, 1974) and Triton X-100 (KASSANIS *et al.* 1977) have often been used. Concentration is usually by PEG-precipitation and further purification has been achieved by isopycnic density gradient centrifugation (BAR-JOSEPH & HULL, 1974) or CPG-column chromatography (BRUNT, 1978). Caesium sulphate is preferable to caesium chloride as a gradient material especially for citrus tristeza virus (CTV) which is stable in the former (GONSALVES *et al.*, 1978) but not the latter (BAR-JOSEPH *et al.*, 1972). Recently BAR-JOSEPH *et al.*, (1985), have used sucrose/caesium sulphate cushion gradients to improve yields of CTV. Divalent cations are required for particle integrity of apple chlorotic leaf spot virus (CLSV) (LISTER & HADIDI, 1971) though at high levels (0.1M) are deleterious. Interestingly particles of this virus are sensitive to RNase A.

Sucrose/caesium sulphate cushion step gradients have also been used to purify WSSMV (HAUFLER & FULBRIGHT, 1983), a virus sharing many characteristics with BaYMV (see 1.7 above), but little further information is available.

The main difficulties encountered with purification of "furoviruses" (fungal-borne rod-shaped viruses) are low concentration in plants , instability *in vitro*, fragmentation and aggregation (BRUNT & SHIKATA, 1986). The extent of aggregation between virus and host components is so great for two of these viruses (potato mop top virus

(PMTV) and nicotiana velutina mosaic virus (NVMV)) that they are both sedimented on centrifugation of plant sap at centrifugal forces as low as 9-12,000×g. This property has been used in the purification of both viruses. Other members of the furovirus group have been purified by methods outlined above, or variations thereof, with varying degrees of success but little comparative work has been done.

Rod-shaped virus particles thus exhibit a wide range of those physico-chemical properties relevant to their purification. Even within a homogeneous grouping such as the potyviruses there is variation in buffer preference, cation requirements, solvent tolerance, resistance to ultracentrifugation forces and solubility. It is also clear that, of the purification methods most commonly used (sucrose, caesium chloride or caesium sulphate gradient centrifugation and column chromatography), the technique of preference is not predictable *a priori*.

In the case of viruses that are difficult to purify some methods may be suitable for the purification of virus capsid, free from host proteins, whereas others may yield more intact particles suitable for nucleic acid analysis. Practical considerations also influence choice of method especially if large amounts of material must be processed. In such cases a balance must be achieved between simplicity of method and optimisation of yield.

1.11 Plant Viral Double-Stranded RNA

Investigations of double-stranded (ds) RNA forms found in plants are based on the premise that plants not infected with RNA viruses or virus-like agents do not contain detectable amounts of homogeneous segments of high molecular weight ($>0.1\times10^6$) dsRNA. In general this assumption holds true as most dsRNA found in healthy plants

is of heterogeneous low molecular weight (FRAENKEL-CONRAT, 1983; IKEGAMI & FRAENKEL-CONRAT, 1979; GOULD & FRANCKI, 1981) but there have been two reports of high molecular weight dsRNAs in apparently uninfected plants. One (GRILL & GARGER, 1981) is of a factor associated with male sterility in *Vicia faba* and the other (WAKARCHUK & HAMILTON, 1985) concerns two species of dsRNA, of sizes estimated to be 14.9 and 13.0 kilobase pairs (kbp), found consistently in *Phaseolus vulgaris* cv. Black Turtle Soup (BTS). The latter have homology with the genome of the French Bean but their functional significance, if any, is not known. Other dsRNAs found in some seed lots are probably due to undetected virus infection though it is not unlikely that further host-encoded dsRNA species will be found. Therefore in studies of plant viral infections proper analysis of healthy controls is important. However, the absence of dsRNAs in healthy controls grown from the same seed stocks as infected plants is a strong indication that dsRNAs are infection- if not virus-specific.

The value of dsRNA analysis in detection and diagnosis of plant virus infection has been the subject of a recent review (DODDS *et al.*, 1984). Methodologies based on cellulose chromatography (FRANKLIN, 1966) have been developed so that dsRNA can be extracted and analysed quickly and sometimes from small amounts of tissue (as little as 0.5g). Techniques are simple, cheap and do not require special equipment. No knowledge of the identity of the infectious agent is needed for detection which is possible even in the absence of virions. Diagnosis is based on the recognition of a gel-electrophoretic pattern of dsRNA species characteristic of a particular virus or group. In most cases studied so far the patterns produced are complex but consistent and illustrate differences between viruses of different groups (MORRIS, 1983), similarities between viruses of the same group (MORRIS, 1983) and small differences between different strains of

the same virus (GILDOW *et al.*, 1983). A comprehensive catalogue of expected dsRNAs would therefore allow diagnosis of mixed infections as well.

If sufficiently purified from host nucleic acids dsRNAs are useful as comparatively stable and accessible sources of viral RNA sequences. End-labelling with ^{32}P as in JORDAN & DODDS, (1983) provides a source of both (+) and (-) sense probes without the necessity of virus purification, often a more complex and difficult procedure than dsRNA purification. Strategies for the specific molecular cloning of dsRNAs have been reported though two of them (BOTH *et al.*, 1982; IMAI *et al.*, 1983) have the inherent problem of keeping the strands apart long enough for complementary DNA (cDNA) synthesis by the enzyme reverse transcriptase. The third (SHOTNIKI *et al.*, 1985) employs direct cloning of dsRNAs in plasmid/bacteriophage vectors by addition of DNA linkers though there is little information available on this procedure. *In vitro* translation of melted dsRNAs again has the problem of reannealling during the incubation but has been reported for the yeast killer-associated dsRNA (BOSTIAN *et al.*, 1985) and tobacco necrosis virus (TNV) subgenomic dsRNAs (CONDIT & FRAENKEL-CONRAT, 1979) although evidence for the latter was said (in 1979) to be preliminary. A more recent attempt to translate melted tobacco mosaic virus (TMV) dsRNAs was unsuccessful (PALUKAITIS *et al.*, 1983). Unlike animal picornavirus dsRNAs plant viral dsRNAs do not seem to be infectious unless first denatured (JACKSON *et al.*, 1971; SHANKS *et al.*, 1985) but as such could be useful for infectivity studies when virion single-stranded (ss) RNA is not available.

Double-stranded RNA species are often referred to as replicative form (RF) though there is no evidence that they have this function *in vivo*. Indeed, partially double-stranded RNAs with single-stranded tails (so-called replicative intermediates, RIs) in a complex with membranes

and proteins are more likely to be the true replicative structures within the cell, as has been found for turnip yellow mosaic virus (TYMV) (GARNIER *et al.*, 1980). RF may be a by-product or end-product or even an artifact of extraction methods. However, the existence of dsRNAs of the same length as genomic and subgenomic ssRNAs of many viruses implies that analysis of ds forms gives valid information on the genome strategies of the viruses involved. For instance, on extraction of dsRNA from plants infected with cowpea mosaic virus (CPMV), a virus whose gene-products arise by polyprotein processing rather than translation of subgenomic RNAs (PELHAM, 1979, GOLDBACH & REZELMAN, 1983), no ds forms shorter than genomic length are found (SHANKS *et al.*, 1985). Tripartite genome viruses (eg Cucomoviruses) produce dsRNAs for each genomic RNA in addition to a species for subgenomic RNA-4 and for satellite RNA when present (KAPER & DIAZ-RUIZ, 1977). For TMV, a single component virus, four dsRNAs are found, three of which appear to be double-stranded forms of polysome-associated ssRNAs (PALUKAITIS *et al.*, 1983). Other workers found an additional three (BAR-JOSEPH *et al.*, 1983) or eight (DAWSON & DODDS, 1982) dsRNA species in TMV-infected plants and the latter authors found nine "subgenomic" ssRNAs some of which corresponded in size to some of their dsRNAs but these ssRNAs were not found on polysomes and all but the original four ssRNAs (one genomic and three subgenomic) were shown by PALUKAITIS *et al.* (1983) to be electrophoretic artifacts. Similar artifactual bands produced on electrophoresis of mixtures of plant RNA and viral RNA from tobacco etch virus (TEV) (DOUGHERTY, 1983) and tomato bushy stunt virus (TBSV) (HAYES *et al.*, 1984) have been reported. It has become evident that interpretation of such results is impossible without the appropriate controls. These sorts of effects cannot, however, explain differences in the number of TMV dsRNAs found by the three groups since purified preparations of dsRNA

should not contain any plant RNA. The disparity may be due to the different resolving powers of the gel systems employed to visualise the dsRNAs or to anomalous behaviour of some dsRNAs in one or more of the systems.

The structures of RF molecules from two plant viruses have recently been investigated with a view to elucidating replication strategy. The terminal sequences found in both RF-M and RF-B of CPMV were found to be identical (LOMONOSSOFF *et al.*, 1985), suggesting a role for these structures in the replication process. The (-) strands form perfect duplexes with (+) strands even to the extent of a poly(U) sequence at 5' ends complementary to poly(A) tracts on virion 3' ends. This implies that poly(A) tails are copied from the (-) sense template rather than being added on later by a host poly(A)-polymerase, a situation that would explain the lack of eukaryotic polyadenylation signals in either viral RNA. The 5' ends of both strands were found to be linked to a VPg, as with polioviruses, though some RF molecules lacked the VPg on their (+) strands. The proposed role of the VPg in initiation of RNA synthesis (WIMMER, 1982) is in agreement with the suggestion that such molecules had the VPg removed after RNA synthesis and were destined for messenger RNA activity instead of encapsidation.

The double-stranded forms of cucumber mosaic virus (CMV) RNA-3 and the satellite RNA (CARNA-5) and some RNA-4 dsRNAs contain an unpaired (G) at the 3' end of the (-) strand (COLLMER & KAPER, 1985) as do many alphaviruses (WENGLER *et al.*, 1982), thus begging comparison of their replication strategies. The presence of the unpaired (G) on CARNA-5 RF suggests that the satellite shares at least part of the replicative machinery of its helper virus. The finding that some ds forms of RNA-4 (a subgenomic of RNA-3, coding for coat protein) contain an unpaired (G) on their (-) strand 3' ends implies that not all (+) strand RNA-4 molecules are synthesised on (-) strand RNA-3 templates

since in this case double-strandéd RNA-4 would arise by degradation of the non-homologous single-stranded region of the (-) strand. Thus RNA-4 may replicate independently with its own RF. Alternatively the (-) strand synthesis on an RNA-4 (+) strand , implied by the terminal (G), may be an aberration induced by the presence of the satellite. Analysis of the (+) strands revealed that the 3' terminal (A) of RNAs-3 and -4 and the 5' cap of CARNA-5 are lacking so these must both be added post-transcriptionally.

There have been no reports of BaYMV-associated ss or dsRNAs apart from the finding of two encapsidated ssRNAs in virus preparations (HUTH *et al.*, 1984). An investigation of dsRNAs found in BaYMV-infected tissue might yield a method of detection and diagnosis, with the advantages outlined above, a potential footprint of the virus to aid in its classification and in strain comparisons, and a source of RNA for molecular characterisation without the need for virus purification.

1.12 RNA Plant Virus Genome Analysis

A viral genome must contain all the information required for infectivity, replication, encapsidation and pathogenicity within the host plant. The organisation of this information, i.e. 'genome strategy', can be investigated by a number of methods, the most important of which are probably infectivity studies (in plants and protoplasts), *in vitro* translation of viral RNA, identification of viral RNAs by blotting and hybridisation, and sequence analysis of viral RNAs or cloned cDNAs. The use of dsRNA analysis in studies of genome organisation has been discussed above. Most recently the technique of 'reverse genetics' has become available where the effects of *in vitro* mutation of cloned full-length cDNAs are studied after RNA transcription. From such studies it has emerged that in

(+)-strand RNA viruses genetic information is organised and expressed in different ways and viral RNAs have characteristic structures that are probably involved in the expression of the genetic information and/or its regulation, though not all have known function.

The variety of RNA structures and genome strategies employed by plant viruses has been reviewed extensively (ZAITLIN, 1979; LANE, 1979; DAVIES, 1979; HIRTH, 1982; VAN VLOTEN-DOTING & NEEDLEMAN, 1982 and DAVIES & HULL, 1982). Viral genes can be contained on a single RNA species or divided between two or more components. Monocistronic RNAs can be translated directly but internal cistrons of polycistronic RNAs must be made available for translation by the use of subgenomic mRNAs (encapsidated or not) or readthrough of 'leaky' termination codons producing two or more proteins from the same RNA region. In some viruses functional proteins are not synthesised by direct translation of mRNA but by processing of polyproteins which are the primary products. More than one of these strategies is sometimes employed by a single virus.

At 5' ends of viral RNAs different terminal structures have been found including $m^7G^{5'}ppp^{5'}$ caps, di- or triphosphates and VPgs. Most small, efficient mRNAs have relatively short leader sequences compared to those of larger genomic RNAs although the lengths do not correlate with translational efficiency *in vitro*. Cap structures are probably involved in translation initiation and VPgs in initiation of RNA synthesis but functions for none of the 5' structures are known for certain and structure type does not correlate with translational strategy. At the 3' ends terminal structures include tRNA-like sequences that can be aminoacylated *in vitro*, polyadenylate tracts, neither structure or both. The tRNA-like structure is almost certainly involved in replication since it contains signals directing interaction with replicase, nucleotidyl transferase and aminoacyl tRNA synthetase. Polyadenylate

tracts may afford protection against 3' exonuclease digestion but probably have other roles. Viral RNAs with 3' poly(A) sequences usually but not always have 5' VPgs and use post-translational protein processing. Those with 3' tRNA-like sequences show 3' sequence homology within virus groups and conservation of amino acid charging. In general, however there is little or no correlation between terminal structures and translational strategy or particle morphology.

The way in which different experimental approaches can give information on viral genome organisation is best illustrated with examples. These will be taken from two virus groups that share certain features with BaYMV: Furoviruses are fungal-transmitted and have rod-shaped particles of more than one modal length; Potyviruses are flexuous rods and induce cytoplasmic inclusions.

Beet necrotic yellow vein virus (BNYVV) and soil-borne wheat mosaic virus (SBWMV) are possible members of the newly proposed Furovirus group (BRUNT & SHIKATA, 1986). They have particles of three (SBWMV) or four (BNYVV) modal lengths and contain an RNA species for each length. Infectivity studies with purified virions of SBWMV showed the two smaller sizes (0.5 & 0.35L) were not infectious. Infectivity of 1.0L virions may have been due to contamination with smaller virions (TSUCHIZAKI *et al.*, 1975). Using sucrose-gradient purified RNAs SHIRAKO & BRAKKE (1984a) showed that the SBWMV genome is bipartite since 1.0L and one or other of 0.5L and 0.35L RNAs were required for infectivity. Of the two smaller RNAs the 0.35L was proposed to be a deletion mutant of the 0.5L RNA. Spontaneous deletion was subsequently demonstrated by the detection of 0.35L virions and RNA in plants originally inoculated with purified 1.0L and 0.5L RNAs (SHIRAKO & BRAKKE, 1984b). Infectivity studies with BNYVV RNAs have not been reported so the significance of the four RNAs is not known. The two smallest RNAs display length

heterogeneity in different isolates as does SBWMV RNA-2 (RICHARDS *et al.*, 1985).

The 3' and 5' ends of both viruses have been examined. The presence of poly(A) tails on all BNYVV RNAs was demonstrated by sequencing (PUTZ *et al.*, 1983) using wandering spot analysis of RNAs, 3'-labelled with [5'-^{32}P]pCp and T4 ligase, after electrophoresis and homochromatography. The absence of such structures in SBWMV RNAs was suggested by their inability to bind to oligo(dT)-cellulose columns or to serve as substrates for reverse transcriptase with oligo(dT) as a primer (HSU & BRAKKE, 1985a). Determination of the precise 3' ends of these RNAs awaits sequence analysis. The presence of a cap structure (m^7GpppN) at the 5' ends of BNYVV RNAs was indicated by the failure to detect a VPg by iodination, the failure to label 5' ends with [γ-^{32}P]ATP and polynucleotide kinase without prior treatment with tobacco acid pyrophosphatase and by DEAE-Sephadex chromatography of total RNase P_1 or T_2 digests of RNAs labelled at the 5'-end using NaB[^{3}H]$_4$ (PUTZ *et al.*, 1983). The absence of either VPg or cap structure in SBWMV RNAs was proposed following the failure of iodination labelling and of m^7Gp to inhibit *in vitro* translation, and the success of labelling experiments using [γ-^{32}P]ATP and polynucleotide kinase after alkaline phosphatase treatment of RNAs (HSU & BRAKKE, 1985). The differences between ends of these two viruses tends to argue against their inclusion in the same group.

Hybridisation experiments using cDNA copies of SBWMV and BNYVV (cloned in the latter case) revealed sequence relationships. In Northern blots cDNA from SBWMV RNA-1 hybridised to RNA-1 from other isolates but not to smaller RNAs. cDNA from 0.5L RNA hybridised to RNAs from 0.4L and 0.35L virions thus supporting the proposal of their derivation through deletion (HSU & BRAKKE, 1985a). Cloned cDNAs from BNYVV RNAs 3 and 4 did not hybridise to RNAs 1 or 2 ruling out the possibility that they are subgenomic

messengers derived from the larger RNAs (RICHARDS *et al.*, 1985). BNYVV RNAs 3 and 4 showed some cross hybridisation and sequence analysis revealed 90% homology for their terminal 200 nucleotides (BOUZOUBAA *et al.*, 1985). Sequence analysis also confirmed that internal deletions accounted for length heterogeneity between isolates. Deletions were within open reading frames which are thus unlikely to encode essential functions.

In *in vitro* translation experiments SBWMV RNA-1 directed synthesis of five major polypeptides of 180K, 152K, 135K, 80K and 45K. The total coding capacity of RNA-1 is 228K so some products must overlap. The situation is similar to that found for TMV where large products arise *in vitro* through readthrough of leaky termination codons. Translation of RNA-2 from all isolates produced a polypeptide of 19,700 which comigrated with coat protein, a protein of 28K and another protein whose size varied with RNA length (55K, 66K or 90K for 0.35L, 0.4L & 0.5L respectively) (HSU & BRAKKE, 1985b). All polypeptides were immunoprecipitated with antiserum to virus and peptide mapping suggested that the two larger products arose from readthrough. Products appeared in ascending order of magnitude so the coat protein gene seems to be located at the 5' end of RNA-2 and deletions occur in 3' sequences. All three proteins were immunoprecipitated from extracts of SBWMV-infected wheat so the readthrough strategy appears to operate *in vivo* also. Translation of gel purified BNYVV RNAs showed that RNA-2 encodes a 22K polypeptide that reacts with antiserum to virus and RNAs 3 and 4 of the F2 isolate direct synthesis of 27K and 33K proteins respectively (RICHARDS *et al.*, 1985). RNA-1 gave no detectable translation products. Sequence analysis confirmed that F2 RNA-3 contains an ORF encoding a 25K polypeptide and F2 RNA-4 contains an ORF encoding a 31K protein though it is not known if these proteins are synthesised *in vivo*.

These two viruses thus employ a split genome strategy with at least coat protein being directly translated from genomic RNA. There is no evidence for the generation of subgenomic RNAs or polyprotein processing and products of SBWMV RNAs arise by readthrough, *in vitro* at least. SBWMV has a bipartite genome as may BNYVV if RNAs 3 and 4 prove to be satellites but terminal structures are different and the viruses are not serologically related. These data along with further sequence analysis and data on other possible members of the proposed Furovirus group (BRUNT & SHIKATA, 1986) will help in taxonomic assignment.

Studies on the genome strategies of potyviruses have led to a division of opinion as to whether expression of genes in the single RNA species (≃10kb) is achieved by polyprotein processing or subgenomic RNAs. Most work has been done on tobacco etch virus (TEV).

Potyviruses have a single RNA component as shown by infectivity of wheat streak mosaic virus (WSMV) RNA (BRAKKE & VAN PELT, 1970) for example. Tobacco etch virus RNA exists in two populations differentiated by ability to bind to poly(U)-agarose both of which are infectious (HARI *et al.*, 1979). The presence of a short poly(A) tail is not eliminated by the inability to bind to these columns. TEV RNA has a VPg at the 5'-end (HARI, 1981).

Potyviruses induce large amounts of inclusion proteins in infected plants and the characterisation of these greatly helped genome analysis. Two nuclear inclusion proteins (49K & 55K for TEV) and the cylindrical inclusion protein (70K for TEV) were purified and antisera raised against them.

Translation of TEV RNA in rabbit reticulocyte lysate (DOUGHERTY & HIEBERT, 1980) yielded a major product of 87K and five other discrete products. Immunoprecipitation using antisera to capsid and the inclusion proteins tentatively identified immunoreactive polypeptides comigrating with respective antigens in addition to a number of other

products which were presumed to be due to premature termination or readthrough. Precipitation of these products with more than one antiserum allowed a gene map to be proposed which was 5'-end - 87K protein - 49K nuclear inclusion protein - 50K protein - 70K cylindrical inclusion protein - 54K nuclear inclusion protein - 30K coat protein - 3'-end. The order of appearance of products gave no indications of polyprotein processing.

KOZIEL *et al.*, (1980) translated TEV RNA in a wheat-embryo-derived cell-free system and found only a single polypeptide of 40K. They accounted for this by proposing that the wheat embryo system was more stringent and did not allow readthrough.

RNA extracted from TEV virions and from infected plants was analysed by Northern blot hybridisation to TEV cDNAs in two separate studies. OTAL & HARI (1983) found ten subgenomic sized RNAs four of which were found in both virion RNA and infected tissue. Three each of the others were found in only one of these. All RNAs were polyadenylated and products of cell-free translation of these RNAs comigrated with known viral polypeptides so a role for subgenomic mRNAs in TEV replication was proposed. In the other study (DOUGHERTY, 1983) similar experiments again revealed less than full-length RNAs in infected tissue but reconstitution experiments using healthy plants and TEV virions or RNA suggested these were electrophoretic artefacts. Only one polyadenylated viral RNA (of genomic length) was found. Translation of polyadenylated RNA produced a slightly different set of polypeptides from the previous study by DOUGHERTY & HIEBERT (1980) but the 87K and 49K proteins were present along with a 120K protein that reacted with cylindrical inclusion antiserum and an 85K protein that reacted with antisera to both capsid protein (30K) and the 55K nuclear inclusion.

Northern blot hybridisation of polysomal RNA from soybean mosaic virus (SMV) revealed a single species of

genomic length (VANCE & BEACHY, 1984a). These workers also found evidence for protein processing *in vitro* (VANCE & BEACHY, 1984b) and the presence of the single genomic length RNA on polysomes suggested the same mechanism of gene expression *in vivo*. Many other RNA viruses that have 5' VPgs and 3' poly(A) tails employ this strategy and further evidence for processing in potyviral gene expression was provided by sequence analysis of the 3' terminal 2324 nucleotides of TEV cDNA (ALLISON *et al.*, 1985). The sequence begins with an open reading frame of 2135 nucleotides and contains an untranslated region of 189 nucleotides adjacent to a polyadenylate tract. Thus the initiation codon is upstream of the sequence. Sequencing of the N-terminal of capsid protein allowed location of the start of its gene at amino acid -263 (nucleotide -974) thus indicating that maturation of capsid requires cleavage of a larger precursor. This could be either the 85K protein seen among translation products or a single protein consisting of the entire coding capacity of the TEV genome. Sequence analysis of the rest of TEV RNA will help to decide this question.

These studies illustrate the difficulties in interpretation of data from some experiments and show that certain *in vitro* techniques can realise different results in different laboratories. In the analysis of viral genome strategy it is therefore important to utilise as many techniques as are available.

1.13 Pathogenesis-Related Proteins

Infection of plants with viruses, viroids, fungi and bacteria is often accompanied by the expression of novel soluble proteins. First found in tobacco, similar proteins have now been detected in 16 dicotyledonous species (VAN LOON, 1985). They are not pathogen-specific and are often

inducible by chemical agents, indicating they constitute a host response, and have come to be known as pathogenesis-related (PR)-proteins. PR-proteins are characteristically of low molecular weight but can be separated in native polyacrylamide gels because of charge differences. In tobacco pIs vary from 4.0-7.5 (PIERPOINT, 1983) and in tomato P14 has a pI of 10.7 (CAMACHO-HENRIQUEZ *et al.*, 1983). They are selectively extractable at low pH (VAN LOON, 1975), occur mainly in the intercellular fluids (PARENT & ASSELIN, 1984) though are not peroxidases, ribonucleases, proteases or phosphatases (VAN LOON, 1985) and are resistant to a variety of proteases (PIERPOINT, 1983) though are not protease inhibitors (PIERPOINT *et al.*, 1981).

PR-proteins are also found in healthy tobacco plants at the onset of flowering (FRASER, 1981), in callus (ANTONIW *et al.*, 1981) and crown gall tissue (ANTONIW *et al.*, 1983) and can be induced in cucumber and cowpea by plasmolysis (WAGIH & COUTTS, 1981). A number of chemical agents are also capable of inducing PR-proteins including polyacrylic acid (GIANINAZZI & KASSANIS, 1974), benzoic acid derivatives (WHITE, 1974), plant hormones (VAN LOON, 1977) and culture filtrates of pathogenic fungi (MAISS & POEHLING, 1983) and bacteria (LEACH *et al.*, 1983). Their induction by ethylene suggests a participation in a stress response since ethylene production is itself a stress response acting to stimulate aromatic biosynthesis which may be mimicked by benzoic acid derivatives.

PR-protein induction is inhibited by protein-synthesis inhibitors and pulse-chase experiments suggest $^{14}CO_2$ incorporation is due to *de novo* synthesis rather than turnover (PIERPOINT, 1983). In *N. tabacum cv Xanthi-nc* induction is not inhibited by RNA synthesis inhibitors and *in vitro* translation of mRNA from healthy plants leads to the synthesis of polypeptides precipitable by PR-protein antisera implying translational control of expression (CARR

et al., 1982). Conversely, in cv. Samsun NN *in vitro* translation and Northern blotting experiments using cloned probes showed a >100-fold induction of PR-protein mRNA on TMV infection (HOOFT VAN HUIJSDUIJNEN *et al.*, 1985), implying a transcriptional level of control.

Tobacco PR-proteins 1a, b & c have been purified and shown to be polypeptides of ≈15K. They show serological cross-reactivity with each other and with cowpea and tomato PR-proteins (NASSUTH & SANGER, 1986). Cloning and sequencing have shown 90% homology between PR1 proteins and 60% amino acid homology between PR1b and tomato P14 (CORNELISSEN *et al.*, 1986a). Tobacco PR1 protein mRNAs have leader sequences consistent with their extracellular location and sequence analysis revealed abundant trypsin and chymotrypsin sites which are presumably inaccessible in native proteins. TMV infection induces at least five other mRNA families three of which code for polypeptides precipitable by PR-protein antisera (HOOFT VAN HUIJSDUIJNEN *et al.*, 1986). One of these is highly homologous to thaumatin, the sweet-tasting protein from *Thaumatococcus daniellii* Benth (CORNELISSEN *et al.*, 1986b).

The functions of PR-proteins are not clear. Accumulation to high levels at lesion margins and lower levels in uninoculated leaves in which fewer lesion develop on challenge inoculation has been taken as evidence for their participation in virus localisation and a role in systemic acquired resistance (VAN LOON, 1975). Chemical induction of PR-proteins also leads to acquired resistance and both PR-proteins and resistance are confined to the treated leaf. Ethylene treatment leads to PR-protein production and systemic acquired resistance which are not limited to the treated leaf. In the interspecific hybrids *N. glutinosa* × *N. debneyi*, amphidiploids express a PR-protein and exhibit resistance constitutively (AHL & GIANINAZZI, 1982). When such hybrids are used as root stock, scions express their own PR-protein constitutively suggesting that

a mobile compound, constitutive in the hybrids, is inducing PRs in the scions (GIANINAZZI & AHL, 1983). One interpretation is thus that infection causes ethylene production which stimulates aromatic biosynthesis and one such compound, which is mobile and mimicked by benzoic acid derivatives, induces PR-proteins and hence resistance.

However PR-protein induction and systemic acquired resistance may be separate effects. Resistance in tobacco is manifested before PRs can be detected and PRs are not sufficient for virus localisation (FRASER & CLAY, 1983). Similar discrepancies have been found in cowpea and cucumber (COUTTS & WAGIH, 1983). Furthermore in tobacco plants containing the *N* gene, which is temperature sensitive, when PRs are induced by TMV infection at 20°C their presence is not sufficient to prevent systemic spread of the virus upon a temperature shift to 30°C. Thus additional factors seem to be necessary for virus localisation.

In an investigation of the proteins found in the intercellular fluid of stem rust-affected wheat and barley (HOLDEN & ROHRINGER, 1985) it was concluded that the novel proteins found were probably of fungal origin. There have been few other such studies and no reports of PR-proteins in monocots. Such considerations prompted a study on the presence of PR-like proteins in BaYMV-infected plants.

2. MATERIALS & METHODS

2.1 Abbreviations

The following abbreviations are used:

dH_2O	distilled water
BSA	bovine serum albumin
SDS	sodium dodecyl sulphate
SDOC	sodium deoxycholate
EDTA	ethylenediamine tetracetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether) N,N'-tetracetic acid
tris	tris(hydroxymethyl)aminomethane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IPTG	isopropylthio-β-D-galactopyranoside
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
DTT	dithiothreitol
PEG	polyethylene glycol
TCA	trichloroacetic acid
TEMED	N,N'-tetramethyl ethylenediamine
AMPS	ammonium persulphate
PMSF	phenylmethyl sulphonyl fluoride
MeHgOH	methylmercuric hydroxide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
TTP	thymidine triphosphate
s	second(s)
min	minute(s)
h	hour(s)
vol	volume(s)
µl	microlitre(s)
ml	millilitre(s)
l	litre(s)
ng	nanogram(s)

µg	microgram(s)
mg	milligram(s)
g	gram(s)
u	unit(s)
M	molar
mM	millimolar
mol wt	relative molecular mass
d	dalton(s)
C	centrigrade
mm	millimetre(s)
cm	centimetre(s)
mA	milliamp(s)
V	volt(s)
cv	cultivar
rpm	revolutions per minute
psi	pounds per square inch
OD	optical density
A	absorbance
uv	ultraviolet
ss	single-stranded
ds	double-stranded
RF	replicative form
RI	replicative intermediate

2.2 Safety Precautions

A protective laboratory coat was worn at all times and gloves were worn when handling hazardous chemicals. Gloves and glasses were worn when using radioisotopes and work was carried out behind a Perspex screen. All radioactive waste was disposed of in accordance with Imperial College and John Innes Institute Safety Regulations. All manipulations involving toxic chemicals were carried out in a fume hood.

2.3 Companies and Suppliers of Reagents and Materials

Chemicals and equipment were supplied by the following companies:

Alpha Laboratories, 40 Parham Drive, Eastleigh, Hants. SO5 4NU

American Can Co., Greenwich, Conn. 06830, USA.

Amersham International plc, White Lion Road, Amersham, Bucks. HP7 GLL.

Anglian Biotechnology Ltd., Unit 8, Hawkins Road, Colchester, Essex CO2 8JX.

BCL: Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex BN17 1LG.

BDH Chemicals Ltd., Freshwater Road, Dagenham, Essex RM8 1R2.

Beckman Instruments Inc., 2500 Harbor Blvd., Fullerton, CA, USA.

Bio-Rad Laboratories (UK) Ltd., Caxton Way, Watford Business Park, Watford, Herts. WD1 8RP.

BRL: Bethesda Research Laboratory (UK) Ltd., PO Box 145, Science Park, Cambridge CB4 4BE.

Carborundum Company Ltd., Trafford Park, Manchester M17 1HP.

Difco Laboratories, PO Box 14B, Central Avenue, East Molesey, Surrey KT8 08E.

EMscope, Kings North Industrial Estate, Wotton Rd., Ashford, Kent.

Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leics. LE11 0R6.

Fluka: UK agents, Fluorochem Ltd., Peakdale Rd., Glossop, Derby SK13 9XE.

Gilson Company Inc., PO Box 677, Worthington, Ohio 43085, USA.

Koch-Light Laboratories Ltd., 37 Holland Rd., Haverhill,Suffolk CB9 8PU.

Kodak Ltd., Distribution Southern Region, PO Box 33, Hemel Hempstead, Herts. HP2 7EU.

Lancaster Synthesis, Eastgate, White Lund, Morecambe, Lancs.

LKB Instruments Ltd., 232 Addington Road, Selsdon, South Croydon, Surrey CR2 8YD.

Marine Colloids Division, FMC Corporation, Rockland, ME 04841, USA.

Medicell International, 239 Liverpool Rd., London N1 1LX.

Microflow Ltd., South Way, Walworth Estate, Andover, Hants. SP110 5AG.

Millipore Corp., 80 Ashby Road, Bedford, MA, USA.

MSE Scientific Instruments, Manor Royal, Crawley, West Sussex RH10 2QQ.

New England Biolabs Inc., UK suppliers CP Laboratories Ltd., PO Box 22, Bishops Stortford, Herts. CM22 7RQ.

New England Nuclear, Du Pont (UK) Ltd., Wedgewood Way, Stevenage, Herts. WD1 8RP.

P&S Biochemicals Ltd., 38 Queensland Street, Liverpool L7 3JG.

Packard Instruments Ltd., 13-17 Church Rd., Caversham, Reading, Berks. RG4 7AA.

Pharmacia (Great Britain) Ltd., Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Bucks. MK9 3HP.

P-L Biochemicals Ltd., PO Box 98, Northampton NN3 1AW.

Rose Chemicals Ltd., 83 Darenth Road, London N16 6EB.

Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset BH17 7NH.

Sterilin Ltd., 43-45 Broad Street, Teddington, Middx. TW11 8QZ.

Universal Scientific Ltd., The Broadway, Woodford Green, Essex IG8 0HL.

Whatman Labsales, Springfield Mill, Maidstone, Kent ME14 2LE.

Worthington Diagnostics Systems Inc., UK suppliers Flow Laboratories Ltd., PO Box 17, Second Avenue, Industrial Estate, Irvine, Scotland KA12 8NB.

Unless otherwise stated all chemicals were supplied by BDH Chemicals Ltd and were of analytical grade or equivalent.

2.4 Buffers and Media

The following buffers and media were used commonly and usually made up in concentrated form, e.g. 10×. Other buffers which were used for specific purposes will be detailed with reference to the method being described. Concentrations given in the form of percentages are weight per volume (w/v) unless otherwise stated.

BE : 40mM boric acid (pH8.2), 1mM EDTA.

SSC : 150mM NaCl, 15mM trisodium citrate (pH7.0).

STE : 50mM tris-HCl (pH7.5), 100mM NaCl, 1mM EDTA.

TAE : 40mM tris-HCl (pH7.8), 20mM sodium acetate, 1mM EDTA.

TE : 10mM tris-HCl (pH8.0), 1mM EDTA.

2TY : 1.6% Bactotryptone (Difco), 1% Yeast Extract (Difco), 0.5% NaCl, 1.5% Bacto Agar (Difco) (omitted for 2TY broth).

H Top Agar: 1% Bactotryptone (Difco), 0.8% NaCl, 0.8% Bacto Agar (Difco).

2.5 General Methods

2.5.1 Miscellaneous

All Eppendorf tubes and micropipette tips were autoclaved before use for 20min at 120°C, 15psi. All solutions were also autoclaved when possible and if not were filter-sterilised (Millipore) before use. Graduated and Pasteur pipettes were sterilised by baking at 200°C overnight. Glassware for RNA work was siliconised by rinsing with 5% (v/v) dichlorodimethyl silane (Sigma) in chloroform followed by at least ten washes in dH_2O prior to baking overnight at 200°C.

All dH_2O was glass distilled.

Dialysis tubing (Medicell) was boiled in two changes of 10mM EDTA, 10mM sodium bicarbonate for 15min each, rinsed in several changes of dH_2O, autoclaved in dH_2O for 20min at 15 psi and stored at 4°C.

Formamide (Rose) and glyoxal were deionised by stirring 100ml with 5g "Amberlite" monobed resin (MB-3) for 4h. The slurries were filtered through Whatman No.1 filter paper to remove the resin and the filtrates were stored in 10ml aliquots at -20°C.

Stocks of buffered phenol contained 0.1% (w/w) 8-hydroxyquinoline as an anti-oxidant unless stated otherwise. Stocks of chloroform used in nucleic acid extractions contained 4% (v/v) isoamyl alcohol. Stocks of buffered phenol:chloroform (1:1) were made up using the stocks described above. The presence of 8-hydroxyquinoline and isoamyl alcohol will be taken as implied by the terms phenol and chloroform in the text.

Ethanol precipitation of nucleic acids was achieved with 2vol of ethanol for DNA or 2.5vol of ethanol for RNA in the presence of 100mM sodium acetate (pH6.5) at -20°C overnight or at -70°C for at least 2h unless otherwise stated.

Sorvall RC-5B or MSE Hi-spin 21 centrifuges were used for high speed centrifugation and a Beckman L8-55 was used for ultracentrifugation. Centrifugation of Eppendorfs (microcentrifugation) was carried out in a Hettich Mikroliter or a MSE Microcentaur.

Radioactivity detection by mini-monitor was carried using a Mini-Instruments g-m meter type 5.10.

2.5.2 Preparation of Bentonite

Bentonite suspensions were prepared essentially as described in FRAENKEL-CONRAT *et al.* (1961). Bentonite (Technical grade) was suspended in dH_2O at 1g/20ml and centrifuged at 2,500rpm for 15min. The supernatant was decanted and centrifuged at 8000 rpm for 20min. Bentonite pellets were resuspended in 50mM tris-HCl (pH7.0) for 48h at 25°C then subjected to differential centrifugation as above. The final pellet was resuspended in a small volume of dH_2O and its concentration determined by dry weight.

2.5.3 Proteinase K-Treatment of DNase I

DNase I was rendered RNase-free by treatment with proteinase K as described by TULLIS & RUBIN (1980). DNase I (Worthington) was dissolved to 1mg/ml in 20mM tris-HCl (pH7.5), containing 10mM $CaCl_2$ and equilibrated at 37°C for 20min. Proteinase K (Sigma) was added to 1mg/ml and the solution was incubated at 37°C for 1h then stored at -20°C. Solutions were tested for DNase and RNase activity as follows: 1µg each of RNA and DNA were incubated together at 37°C for 30min with RNase A (50µg/ml) (Sigma) or a mixture of RNase A (50µg/ml) and proteinase K-treated DNase I (50µg/ml each) then nucleic acid integrity was inspected

using agarose gel electrophoresis (2.6.1) and ethidium bromide staining (2.6.4) (see Fig.2).

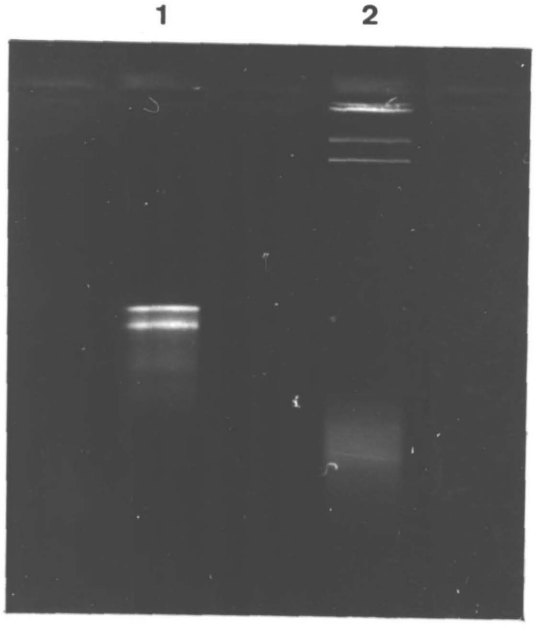


Fig.2 Efficacy of proteinase K treatment of DNase I.
1µg each BSMV RNA and plasmid DNA were incubated together with a mixture of RNase A and proteinase K-treated DNase I (Lane 1) and RNase alone (Lane 2). Incubations were carried out as in the text.

2.5.4 Spectrophotometry

Uv absorption spectra of nucleic acid preparations were determined using a Beckman Model 35 spectrophotometer with quartz cells of path length 1cm. Concentrations of nucleic acids were calculated from the relationship 1 $A_{260} \equiv 40\mu g/ml$ and purities estimated from the $A_{260}:A_{230}$ and $A_{260}:A_{280}$ ratios which should both be ca. 2.

Crude estimates of virus concentration were made by assuming an absorbance coefficient at 260nm of 2.5 and estimates of purity using the published $A_{260}:A_{280}$ ratio of 1.14.

2.6 Gel Electrophoresis

2.6.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis of nucleic acids was carried out using horizontal "submarine" slab gels, mainly "half-gels" (15.5×11.5×0.4cm), "quarter-gels" (10×11.5×0.4cm) or "mini-gels" (6.5×10×0.2cm) (Bio-Rad, "mini-sub") and (10×8×0.2cm) (Cambridge Scientific). Direct current was provided by LKB Biochrom 2103 and Pharmacia EPS 500/400 power-supplies.

a Non-denaturing gels

1% agarose gels were prepared by briefly boiling the required amount of agarose (Sigma No.A-6013) in 1×TAE buffer and cooling to 60°C prior to pouring into the relevant plates. Sometimes ethidium bromide (Sigma) was added to the molten gel (at 60°C) to a final concentration of 0.5μg/ml from a 10mg/ml aqueous stock (stored at 4°C in the dark) thus enabling gels to be visualised directly after electrophoresis. Samples (2-10μl for mini-gels, usually 20μl for larger gels) contained 1×TAE, 8% (v/v)

glycerol and 0.05% bromophenol blue and were electrophoresed at 100mA, constant current, in 1×TAE for 1-4h.

b Methyl mercury gels

RNA samples denatured with methyl mercuric hydroxide (MeHgOH, Lancaster Synthesis) (BAILEY & DAVIDSON, 1976) were electrophoresed in gels in the presence or absence of MeHgOH. All manipulations involving MeHgOH were carried out in a fume hood. Gels (1%) were prepared by briefly boiling the required amount of agarose in dH_2O, cooling to 60°C and adding 10×BE (pH8.2) and 1M MeHgOH to final concentrations of 1×BE and 0, 5 or 15mM MeHgOH, the last concentration being required to fully denature dsRNA. Samples (5-15µl) contained 1×BE, 5 or 15mM MeHgOH, 10%(v/v) glycerol and 0.05% bromophenol blue and were electrophoresed at 80-100V in 1×BE for 1-4h. Following electrophoresis gels were treated with 10mM 2-mercaptoethanol (Sigma) for 30min and washed extensively to remove solubilised mercury.

c Glyoxalated RNA gels

RNA samples were denatured with formamide in the presence of glyoxal by the method of McMASTER & CARMICHAEL (1977) modified as below. Gels were prepared as in 2.6.1a in TPE buffer (0.08M tris-phosphate (pH7.0),0.008M EDTA). Samples were 1vol nucleic acid solution: 9vol GFP (1.11M glyoxal, 77.8% formamide, 11.1mM sodium phosphate (pH7.0)) and were denatured by heating to 65°C for 15min prior to addition of glycerol and bromophenol blue to 10%(v/v) and 0.05% respectively then electrophoresed in 1×TPE as above. GFP was a gift from D.Turner, John Innes Institute.

d Alkaline gels

DNA samples were electrophoresed in alkaline gels as in McDONNELL *et al.* (1977). Gels were prepared as above in 50mM NaCl, 1mM EDTA, allowed to set then soaked in 30mM

NaOH, 1mM EDTA for at least 30min prior to electrophoresis. The presence of alkali during melting of the gel results in hydrolysis of the agarose. Samples contained 30mM NaOH, 1mM EDTA, 10% (v/v) glycerol and 0.05% bromophenol blue and were electrophoresed in 30mM NaOH, 1mM EDTA at 20mA for 16h.

e Low Melting Point (LMP) agarose gels

Preparative non-denaturing or methyl mercury low melting point gels were prepared and run as in 2.6.1a and b except that LMP-agarose (Marine Colloids) was used and gels were allowed to set at 4°C. In the latter case MeHg was used to denature RNA samples but was omitted from the gel itself.

2.6.2 Polyacrylamide Gel Electrophoresis (PAGE) of ds RNA

dsRNAs were analysed on 4% polyacrylamide gels. Gels were made by adding to a Buchner flask:

5ml stock aqueous acrylamide (40% acrylamide, 0.4% N,N'-methylene bisacrylamide)
2ml 25×TAE buffer
0.5ml 5% (v/v) TEMED
40ml dH_2O

After mixing the solution was degassed using a water pump for at least 30min, 2.5ml 1.5% AMPS was added then the gel was mixed, poured and allowed to set for at least 2.5h. Gels were poured into glass plates (20×16.5cm) separated by spacers (0.2cm). A 13.2×2.5cm slot was cut out of the top of one plate to allow contact between gel and electrophoresis buffer. Sample wells were 0.85cm wide giving a maximum loading volume of 60µl. Samples contained 1×TAE, 8% (v/v) glycerol and 0.05% bromophenol blue and were electrophoresed at 70V (constant voltage) for 16-18h with TAE as the electrophoresis buffer.

2.6.3 PAGE of Proteins

Proteins were analysed by gradient or discontinuous PAGE. Protein slab gels were cast using a Pharmacia GSC-2 Gel Slab Casting Apparatus and electrophoresis was performed using a Pharmacia Gel Electrophoresis Apparatus GE-2/4 and a Pharmacia EPS 500/400 power pack. Gel sizes were 180 or 90×140×2mm. Tube gels were cast in siliconised glass tubes 7.5×0.25cm (length×radius) sealed at one end with Parafilm (American Can Co.) and electrophoresis was performed in a Shandon Kit using the power pack described above.

a 5-15% Gradient Gels

5-15% polyacrylamide gradient gels containing 0.4M tris-HCl (pH8.5), 0.1% SDS, 0.03% (v/v) TEMED and 0.06% AMPS were cast using a LKB gradient mixer. The stock acrylamide solution used to produce the gradient contained 30% acrylamide (Sigma) and 0.8% N,N'-methylene bisacrylamide (Sigma). Prior to addition of AMPS the gel solutions were degassed using a water pump to prevent the formation of air-bubbles and ensure even setting of the gel. A well former was put in place before pouring the gels.

Electrophoresis buffer contained 0.025M tris, 0.192M glycine and 0.1% SDS (pH8.3). Gels were pre-electrophoresed at 70V for 20min and samples were electrophoresed at 150V for 16h.

Samples (20-100µl) were denatured, prior to loading, in 0.01M tris-HCl (pH8.0), 0.001M EDTA, 1% SDS, 5% (v/v) 2-mercaptoethanol (Sigma), 10% (v/v) glycerol and 0.05% bromophenol by heating at 100°C for 5min. Any insoluble material was removed by centrifugation in a microfuge for 10min.

b Discontinuous SDS-PAGE

A discontinuous SDS-PAGE system was employed as described by LAEMMLI (1970). The stock acrylamide solution used was as described above. A stacking gel containing 4% acrylamide, 0.125M tris-HCl (pH6.8), 0.1% SDS, 0.1% (v/v) TEMED and 0.1% AMPS was layered onto a resolving gel containing either 10, 12.5 or 15 % acrylamide, 0.375M tris-HCl (pH8.8), 0.1% SDS, 0.1% TEMED and 0.1% AMPS. Gel solutions were degassed as described prior to addition of AMPS. The resolving gel was poured first to within 3cm of the top of the gel plates and overlayed with dH_2O to ensure a level surface. After the gels had set (1-2h) they were set stored for later use wrapped in 'clingfilm' at 4°C or a stacking gel was poured immediately and a well former put into place.

Preparation and denaturation of samples was as described for gradient gels. The electrophoresis buffer contained 0.05M tris, 0.384M glycine (pH8.3) and 0.1% SDS. Samples were electrophoresed at 70V until the tracking dye had migrated to the top of the resolving gel and then electrophoresis was continued at 200-300V for 3-4h.

c Tube Gels

Denaturing and non-denaturing PAGE both employed a discontinuous system, in the presence or absence respectively of SDS. The stock acrylamide solution used was as described (2.6.3a). A stacking gel containing 3% acrylamide, 0.0625M tris-HCl (pH6.7), 0.1% SDS (if used), 0.05% (v/v) TEMED and 0.1% AMPS was layered onto a resolving gel containing 10% acrylamide, 0.375M tris-HCl (pH8.9), 0.1% SDS (if used), 0.05% (v/v) TEMED and 0.05% AMPS. The gel solutions were degassed as described prior to addition of AMPS.

The resolving gel was poured to within 2cm of the top of the tubes and overlayed with dH_2O. When gels had set (1-2h) they were either stored wrapped in 'clingfilm' at 4°C or a stacking gel (1-1.5cm) was poured directly. Samples (50-250µl) were either denatured as above (2.6.3b) or were adjusted to 5% sucrose and 0.05% bromophenol blue then electrophoresed at 2mA/gel (constant current) for 20min followed by 4mA/gel until the gel front was a few mm from the end of the gel (1-2h). Electrophoresis buffer contained 0.05M tris, 0.384M glycine (pH8.3) and 0.1% SDS (if used). After electrophoresis gels were removed from tubes by inserting a syringe needle between gel and tube and squirting dH_2O while rotating the tube.

2.6.4 Gel Staining and Photography

Nucleic acids were visualised in agarose and polyacrylamide gels on a Chromato-Vue Transilluminator C-63 ultraviolet light box after staining with 0.5µg/ml ethidium bromide for 15-30min followed by destaining in dH_2O. Gels containing methylmercuric hydroxide were stained as above concurrently with 2-mercaptoethanol treatment (2.6.1b). Gels run with glyoxalated nucleic acids were treated with 50mM NaOH for 15min to dissociate the glyoxal then neutralised with 0.5M ammonium acetate (pH6.5) while staining as above. Alkaline gels were neutralised with three 10min washes of 1M tris-HCl (pH7.5), 1.5M NaCl prior to staining as above.

Protein gels were stained with 0.25% Coomassie Brilliant Blue R250 (Bio-Rad) in 45% (v/v) methanol and 9% (v/v) glacial acetic acid for 2h at room temperature with shaking. Gels were destained in 45% (v/v) methanol, 9% (v/v) acetic acid for 24h with frequent changes. Protein bands were visualised on a light box. Tube gels were stained and destained individually in test-tubes.

Gels were photographed using (1) a Polaroid or (2) an Exa.1 camera fitted with two Y(K2) yellow filters and one UV(O) filter (all Hoya) on 35mm Ilford HP5 or Pan F film which was subsequently developed in Promicrol ultrafine grain developer.

2.6.5 Gel Drying

Agarose and polyacrylamide gels were placed on wet Whatman 3MM paper, covered with 'Saran Wrap' and dried at 60-80°C under vacuum using a Bio-Rad gel drier. If unlabelled molecular weight markers had been used in agarose gels marker bands were excised after staining and spots of bromophenol blue dye were placed on the Whatman paper to determine their positions. After drying spots of radioactive ink ([α^{32}P]dCTP suspended in ink) of equivalent activity to bands in the gel were placed on top of the dye spots. In protein gels marker bands were excised after gel drying and their positions determined analogously using [^{3}H]-leucine or [^{35}S]-methionine suspended in ink as appropriate.

2.7 Virus Propagation

2.7.1 Plants and Plant Cultivation

Barley (*Hordeum vulgare* cvs.: Maris Otter, Tipper, Halcyon, Triumph, Gerbel, Igri, Sonja, Birgit and Franka) plants were grown from seed kindly provided by S.Hill (MAFF, Harpenden). *Eragrostis siciliensis*, *Atriplex hortensis* and *Tourenia fournieri compacta nymphe* seeds were kindly provided from seed stocks at Kew Gardens. *Chenopodium* spp. (*foliosum*, *murale*, *ficifolium*, *opifolium*,

hybridum, *amaranticolor* and *quinoa*), *Commelina communis*, *Vicia faba*, *Vigna unguiculata* and *Brassica rapa* plants were grown from seeds stocked at the John Innes Institute, Norwich. *Gomphrena globosa* seeds were a gift from Dr.S.E.Adams (Imperial College, London). *Nicotiana benthamiana* and *tabacum* cvs. White Burley and Xanthi seeds were a gift from Dr.R.H.A.Coutts (Imperial College, London).

All seeds were sown in 4-inch pots, using Levingtons or John Innes No.2 compost and grown in an insect-free greenhouse at a temperature of 16-25°C with 70-80% relative humidity and illumination of 7-8,000 lux at pot level for 16h per day from mercury vapour lamps. Some plants were transferred to glass cloches and others to a growth cabinet at temperatures 15/10°C (day/night) with a 12h day length (100μeinsteins light intensity)

2.7.2 Origin of Virus

BaYMV-infected plants were collected from an ADAS trial site at Woodlands Farm, Little Clacton, Essex and maintained at Imperial College in the greenhouse, cloches or growth cabinet as above (2.8.1a). Infected leaves were also harvested from infected plants at Little Clacton and stored at -20°C. Tissue from this site was the initial source of inoculum for transfer of BaYMV to and maintenance in *Hordeum vulgare* cvs. Tipper and Maris Otter as in 2.7.3.

2.7.3 Inoculation of Plants and Virus Propagation

a Inoculation with sap

Barley seedlings were inoculated with infected plant sap by a method adapted from that of FRIEDT (1982). Usually cvs. Tipper and Maris Otter were used for propagation of BaYMV. Plants (10-15/pot) were germinated and grown in the greenhouse to the 2-3 leaf stage as described in 2.7.1 and then placed in the dark for 24-48h prior to inoculation. Inoculum was freshly prepared by grinding 1-2g leaves showing clear symptoms in a chilled mortar with 2-5ml/g cold inoculation buffer (0.04M K_2HPO_4, 1% Na_2SO_3 (pH7.0)). Seedlings were dusted with 600-mesh carborundum (Carborundum Co.), rubbed with fresh inoculum using a small sterile sponge, put in the shade for 24h without post-washing then incubated in growth cabinets as above (2.7.1a,b). Fresh inoculum was prepared for every batch of 6 pots inoculated.

Plants of other species were inoculated in a similar fashion and then incubated in growth cabinets or in the greenhouse.

After harvesting of infected leaves, provided that meristems had not been removed, plants could be returned to the growth cabinet to allow secondary growth. Such growth was not prolific but all new leaves showed strong symptoms and were useful sources of virus.

Plants to be used as healthy controls (eg in dsRNA extraction) were "mock"-inoculated as above but using buffer alone.

b Inoculation with RNA

Barley seedlings at the 2-3 leaf stage were inoculated with RNA by the method described in AHLQUIST *et al.*, (1984). Plants were dusted with baked carborundum and inoculated with RNA in 50mM tris-PO_4 (pH8.0), 250mM NaCl, 5mM EDTA, 5mg/ml bentonite using a gloved finger which was wiped with methanol and dried between inoculations. 20-40 plants were treated with the same inoculum, using 10-30µl per plant, at BaYMV RNA concentrations of 0.3, 3 & 30 ng/µl. Pure viral RNA (2.11.2), crude BaYMV RNA (2.11.1) and total RNA from infected plants (2.11.3) were used as inocula and BaYMV RNA concentrations in the latter two were measured by comparison with known quantities of RNA after denaturing gel electrophoresis (2.6.1b). RNA used for inoculation was extracted with or without the use of proteinase K. For each set of RNA inoculations a batch of plants was inoculated with infected sap as above for comparisons of inoculation efficiency and speed of symptom expression. After RNA inoculation plants were treated as described for sap-inoculation.

2.8 Virus Purification

Many different purification protocols were used during this study with varying degrees of success. The principal considerations were the low levels of virus found in plants, the relative fragility of the particles and the tendency of particles to aggregate and become insoluble. These difficulties were addressed in a number of different ways with reference to the purification of other rod-shaped plant viruses (see 1.10.2). The success or failure of techniques was determined by several methods and usually involved comparisons of two or more techniques differing in only one aspect. A working protocol is detailed below (2.8.8).

2.8.1 Assays

The simplest and quickest method for monitoring purifications was electron microscopy (EM) (2.9). When antiserum became available this was complemented by ISEM (2.10.1). Samples from different stages and different protocols were viewed and monitored in terms of purity, particle concentration and degrees of aggregation and fragmentation. The effects of buffers, pH, salt and cation concentration and other treatments were determined by EM after incubating the same crude preparation in each of the test solutions.

Purity of virus was further monitored by SDS-PAGE (2.6.3) and comparison with extracts from healthy plants at the same purification stage. SDS-PAGE also allowed estimation of yield in terms of capsid protein.

Yield and purity of virus preparations were measured spectrophotometrically assuming an absorbance coefficient at 260nm of 2.5 and an A_{260}/A_{280} ratio of 1.14 (2.5.4).

Once the techniques had been established RNA extraction and denaturing agarose electrophoresis were used to assay yields of full-length RNA (see 2.11.1). This method, with reference to other assays, gave information on the degree of fragmentation or degradation of particles. Estimates of total yield were not possible since degraded viral RNA was indistinguishable from degraded plant RNA.

Virus zones in caesium gradients were located by determination of refractive index. Assuming an RNA content of 5% BaYMV banding densities are 1.32g/cm^3 in CsCl and 1.27g/cm^3 in Cs_2SO_4. Refractive indices are thus 1.3644 and 1.3561 respectively.

Infectivity tests were not usually used because of the long delay in obtaining results and their irreproducibility.

2.8.2 Plant Material and Homogenisation

Fresh and frozen barley leaves and roots were broken up by cutting with scissors, freezing to -70°C and crushing manually or grinding in a mortar with liquid nitrogen. Material was further homogenised to a fine breis in cold extraction buffer (2-4ml/g) in a Waring blendor either once or twice. In the latter case the first homogenisation was at low speed and the second at high speed. Homogenates were then filtered through two layers of sterile muslin.

2.8.3 Extraction buffers

The following extraction buffers were used:

	Reference
0.067M sodium phosphate (pH7.3)	
0.1M tris-thioglycollic acid (TGA) (pH9.0)	HUTTINGA (1973)
0.1M sodium citrate/0.1% TGA (pH6.5)	A.A.BRUNT (pers. comm.)

0.1M sodium citrate (pH7.0)	USUGI & SAITO (1976)
20mM HEPES-NaOH (pH7.4)	DAMIRDAGH & SHEPHERD (1980)

The following additives were included in extraction buffers:

1-5mM EDTA

0.1-1% (v/v) 2-mercaptoethanol

1% Na_2SO_4

10mM sodium diethyl dithiocarbamate (DIECA)

1mM phenylmethyl sulphonyl fluoride (PMSF) (from 100mM stock in isopropyl alcohol)

2.8.4 Clarification

Clarifying agents were added during homogenisation, after filtration through muslin or after a preliminary centrifugation step. The following clarifying agents were used:

1vol $CHCl_3/CCl_4$ (1:1)

0.2vol CCl_4

0.3vol ether/CCl_4 (1:1)

8% (v/v) butanol

0.1-5% (v/v) Triton X-100

Organic solvents were stirred or homogenised with extracts for 10min prior to centrifugation at 10,000×*g* to separate the phases. Triton X-100 was stirred with extracts (whether or not included during homogenisation) for at least 1h before or after centrifugation as above. Irrespective of treatment extracts were filtered through two layers of sterile muslin after the centrifugation step to remove residual plant débris.

2.8.5 Concentration

Virus concentration was achieved by one or two rounds of (a) poly(ethylene glycol) (PEG) 6000 precipitation or (b) ultracentrifugation, the latter sometimes through a sucrose cushion.

a) Clarified extracts were adjusted to 4% PEG and 0.4-4% NaCl by addition of solid and stirred at 4°C for 1.5h. Extracts clarified with organic solvents were also adjusted to 1% (v/v) Triton X-100 at the same time. PEG-precipitated virus was collected by centrifugation at 10,000×*g* for 15min. When a second round of PEG-precipitation was employed pellets were resuspended as below (2.8.6), centrifuged at 10,000×*g* for 10min and supernatants were reprecipitated as described.

b) Extracts were centrifuged at 30,000rpm in a Beckman Type 30 rotor at 4°C for 2.5h or 3h if a cushion was used. 5ml sucrose cushions (25% in extraction buffer) were underlayed in tubes prior to centrifugation. When used, a second round of ultracentrifugation was carried out as described after resuspension as below (2.8.6) and a clearing spin at 10,000×*g* for 10min.

2.8.6 Resuspension

Pellets were resuspended in 0.8ml/g plant tissue if a second concentration step was employed and 0.2ml/g if not.

Resuspension was achieved by gently swirling the pellets in cold resuspension buffer and incubating at 4°C for at least 1h and sometimes overnight. Vortexing and vigorous mechanical resuspension were avoided in view of the potential shearing forces involved. Occasionally seemingly

insoluble parts of pellets were broken up using a rubber 'policeman'.

Pellets were usually resuspended in extraction buffer with or without additives to aid solubilisation. The following additives were used alone or in combination:

1M urea

0.1% (v/v) 2-mercaptoethanol

1mM EDTA

0.1% (v/v) Triton X-100

2.8.7 Further Purification

Resuspended pellets from the first or second round of precipitation or centrifugation were subjected to a clearing spin at 10,000×*g* prior to further purification by (a) sucrose density centrifugation, (b) permeation chromatography on controlled-pore glass (CPG) columns, (c) sucrose/caesium sulphate step gradient centrifugation or (d) isopycnic density gradient centrifugation in CsCl or Cs_2SO_4.

a) Sucrose density gradient centrifugation was carried out as described in 2.12.3.

b) Chromatography on CPG columns was carried out essentially as described by BARTON (1977). CPG of 70nm pore size and 120-200mesh (CPG-700; Sigma) was suspended in extraction buffer, washed several times by decantation to remove "fines", resuspended in extraction buffer containing 1% PEG-20M and degassed for 15min on a vacuum line while shaking gently. Beads were then washed several times in extraction buffer to remove free PEG-20M. Columns were made by pouring the CPG suspension into glass columns (800×10mm) while vibrating the supporting clamp continuously with a Whirlimixer and allowing buffer to elute then the columns

were washed for 2h in buffer. Effective operation of the column was checked by running a sample of Blue Dextran 2000 and monitoring the eluate for a compact elution profile.

Partially purified virus preparations (≃1ml) were applied in and eluted using degassed extraction buffer at 4°C. Elution profiles were monitored at 254nm using a LKB Uvicord II Type 8303A and fractions were collected using a Gilson Microcol TDC80.

Columns were washed and sterilised with two void volumes of 2M HCl followed by re-equilibration with buffer.

c) Step gradients were initially prepared by layering in Beckman SW28.1 tubes 3ml each Cs_2SO_4 (densities 1.35 and 1.2g/cm^3) and 3ml 20% sucrose (all in extraction buffer) and overlaying 8ml virus preparation. Gradients were centrifuged at 26,000rpm overnight and opalescent zones were removed by hand. Fractions were monitored for presence of virus by EM and those containing virus were pooled and further purified by isopycnic centrifugation as below (2.8.7d). Sometimes particulate matter banded where virus zones were expected thus obscuring them. In this case the whole zone was removed, diluted once in buffer, centrifuged at 10,000×g and supernatants were used for isopycnic centrifugation as below.

Subsequently a modified protocol was introduced (see 2.8.8).

d) Virus preparations, either before or after step gradient centrifugation as above (2.8.7c), were adjusted to 1.3g/cm^3 with solid CsCl or to 1.28g/cm^3 with solid Cs_2SO_4 and centrifuged at 35,000rpm in a Beckman SW50.1 rotor for at least 24h. Opalescent bands were removed separately using syringes by puncturing the side of the tube with the needle, virus was located by refractive index measurement then EM and salt was removed by dialysis. If necessary virus was concentrated by ultracentrifugation.

2.8.8 Working Protocol

Plant material was frozen to -70°C, crushed manually and homogenised gently in 2vol buffer (20mM HEPES (pH7.4), 10mM DIECA, 5mM EDTA, 1mM PMSF, 0.1% (v/v) 2-mercaptoethanol: all added separately from concentrated stocks except DIECA which was added as a solid). The homogenate was filtered through two layers of sterile muslin and plant material was homogenised again at high speed in 1vol buffer until a fine breis was produced. The breis was squeezed through the same muslin and the combined filtrates were centrifuged at 10,000×*g* for 15min at 4°C. Supernatants were filtered through two layers of sterile muslin, adjusted to 0.5% Triton X-100 using a 20% stock in 20mM HEPES (pH7.4), 5mM EDTA, and stirred at 4°C for 1h. Extracts were poured into Beckman type 30 centrifuge bottles, underlayed with 5ml sucrose cushions (25% in 20mM HEPES (pH7.4), 5mM EDTA, 0.1% (v/v) 2-mercaptoethanol) and centrifuged at 28,000rpm for 3h at 4°C in a Beckman type 30 rotor. Pellets were resuspended as described above (2.8.6) in 0.15-0.2ml/g plant material 20mM HEPES (pH7.4), 1mM EDTA, 0.1% (v/v) Triton X-100 for at least 1h at 4°C. Extracts were centrifuged at 10,000×*g* for 5min and overlayed on step gradients prepared as follows: Cs_2SO_4 solutions, densities 1.0, 1.1, 1.2, 1.3 and 1.5g/cm^3, in 20mM HEPES (pH7.4), 1mM EDTA containing 20% sucrose, were carefully layered (1, 1, 1.5, 1.5, and 1ml respectively) into Beckman SW28.1 tubes (densities and volumes of steps were selected to maximise separation of virus from host components). Gradients were then centrifuged at 27,000rpm for 3h at 4°C and the opalescent band of virus, located just above the 1.3/1.5g/cm^3 interface, was removed using a syringe. Sometimes particulate matter banded where the virus was expected to be. In this case the whole zone was removed using a pipette, diluted with an equal volume of 20mM HEPES, 1mM EDTA, centrifuged at 10,000×*g* and the

supernatant was layered on another step gradient and recentrifuged as above. Cs_2SO_4 was removed by dialysis against 20mM HEPES (pH7.4), 1mM EDTA then virus, concentrated by ultracentrifugation if necessary, was stored at -20°C.

Crude BaYMV RNA could be prepared by resuspension of pellets from the sucrose cushion ultracentrifugation step in RNA Extraction Buffer (see 2.11.1). During virus purification pellets from clearing spins after this stage, which contained insoluble virus, were retained and treated in the same way.

2.9 Electron Microscopy (EM)

Transmission electron microscopy of negatively stained samples was carried out on Phillips 301 or AEI EM6B microscopes using formvar coated copper grids. Thin sections were prepared, viewed and photographed by K.Plaskitt (John Innes Institute).

2.9.1 Formvar Coating

New microscope slides were wiped with vellin tissue and coated with a thin layer of formvar by dipping in a formvar solution (0.6% in chloroform), withdrawing slowly and evenly, briefly holding in the vapour then drying under a bench lamp. Even withdrawal was important to ensure uniform thickness and the speed of withdrawal determined the thickness, the slower the speed the thinner the coating. An ideal thickness produced a silvery colour when viewed at an angle. Using a mounted needle a 5×2cm area was scored on the coated slide which was then breathed on and lowered at a 45° angle into a brimful bowl of double-glass-distilled H_2O. The formvar rectangle floated off the slide which was

then removed. Copper grids (200μm mesh) were placed dull side down on the film with a pair of forceps then the raft was picked up with a piece of copper gauze by inversion under water so that grids were trapped between the gauze and the film. After drying, coated grids were stored in a dessicator at room temperature and carefully picked off the gauze, formvar intact, immediately prior to use.

2.9.2 Leaf Dips

A small piece of leaf (ca. 1cm long) was ground in a 1.5ml Eppendorf tube with a few drops of cold dH_2O and a small amount of carborundum (Carborundum Co.) using a rounded pasteur pipette. Samples were centrifuged for 5min and a small amount of supernatant was removed to another Eppendorf tube on ice.

2.9.3 Sample Application and Negative Staining

A small drop of sample (virus or leaf dip preparation) (2-5μl) was placed on a formvar coated grid, held in forceps, and the excess liquid withdrawn by touching the sides of the grid with filter paper triangles. Grids were allowed to dry then a small drop of stain (2% phosphotungstate (PTA) or 2% methylamine tungstate (EMscope)) was applied and withdrawn in a similar fashion. Grids were stored on filter paper prior to viewing. Samples containing high salt (eg. Cs_2SO_4) were spotted onto grids sitting on wet filter paper and allowed to dialyse for 1h at 4°C prior to drying and staining as above.

2.10 Serological Studies

2.10.1 Immunosorbent Electron Microscopy (ISEM)

ISEM (DERRICK, 1973; MILNE & LUISONI, 1975) was carried out using formvar coated grids (2.9.1) activated with antiserum (kindly provided by Dr. W. Huth, Braunschweig, FRG) raised to a mixture of the strains BaYMV-M and -NM (HUTH *et al.*, 1984). Grids were incubated on drops of antiserum diluted 1:1000 in 0.1M sodium phosphate buffer (pH7.0) for 30min at 37°C then washed with 20 consecutive drops of buffer and dried. Virus particles were adsorbed by incubating the grids on droplets of virus or leaf dip preparations for 0.5-4h at 4°C. The shorter time was usually sufficient but longer incubations were necessary for dilute samples. Grids were washed with 30 consecutive drops of dH_2O then dried and stained as above (2.9.3) prior to viewing (2.9).

2.10.2 Gel Immunodiffusion Plates

An attempt to develop a serological test based on immunological double-diffusion (OUCHTERLONY, 1962) was made using Huth's antiserum (see 2.10.1). Plates were prepared by pipetting 7ml molten agarose, at 60°C, onto 4×4cm glass squares, allowing it to set, then cutting 6 wells in a hexagon (1.5cm long axis) round a 7th using a template and a Pasteur pipette on a vacuum line. Agarose (0.7%) was made up in PBS (10mM sodium phosphate, 0.9% NaCl) and sometimes adjusted to 0.5% SDS or 0.1% sodium deoxycholate (SDOC) prior to pouring. Antiserum was used at dilutions of 1:1, 10, 100 and 1000 (in PBS) and virus samples were treated, prior to dilution as above, in one of the following ways: with 0.5% SDS; with 0.1% SDOC; by 3 rounds of freezing at

-20°C and thawing rapidly at 60°C with vortexing; by sonication 8 times for 15s each; or with no treatment. Samples and antiserum were loaded into wells in different combinations and the plates were incubated at 37°C on damp filter paper in sealed petri-dishes for up to 3 days. Wells were periodically refilled with PBS to prevent drying and to aid diffusion.

2.11 RNA Extraction

2.11.1 Crude Viral RNA Extraction

It was found convenient to use extraction of RNA from crude virus preparations (pellets from the sucrose-cushion ultracentrifugation step; 2.8) and denaturing gel electrophoresis (2.6.1b) to assay not only different virus purification methods but also different RNA extraction methods. The latter included virus disruption by (a) the highly denaturing guanidinium method (BEVAN & CHILTON, 1982; DE GREVE *et al.*, 1982) and (b) by SDS-treatment in the presence of proteinase K and bentonite followed by solvent extraction and/or sucrose gradient centrifugation. RNA extracted in this way was designated crude BaYMV RNA.

a) Virus suspensions were mixed with 4vol guanidinium thiocyanate (GuSCN) buffer (4M GuSCN (Fluka), 25mM tris-HCl (pH7.0), 2% sarkosyl (Koch-Light), 0.03% (v/v) Antifoam A (Sigma), 10mM EDTA, 1% (v/v) 2-mercaptoethanol) for 30min at 4°C and centrifuged at 6,000rpm for 10min. The supernatant was decanted and nucleic acids precipitated with 0.025vol 1M acetic acid and 1vol ethanol at -20°C overnight. Nucleic acids were collected by centrifugation at 6,000rpm for 30min, dissolved in 0.5vol 7.4M guanidinium hydrochloride (GuHCl; Fluka), 25mM tris-HCl (pH7.0), and

reprecipitated with 0.025vol 1M acetic acid and 0.75vol ethanol at -20°C overnight. Precipitates were collected by centrifugation at 7,000rpm for 30min, dissolved in 0.5vol 25mM EDTA (pH8.0) and extracted with phenol/chloroform (1:1) and chloroform. The final aqueous phase was precipitated with 2vol ethanol at -20°C overnight.

b) Virus pellets were suspended in an RNA extraction buffer (20mM tris-HCL (pH9.0), 1mM EDTA, 1% SDS, 200µg/ml bentonite (2.5.2) and 200µg/ml proteinase K (Sigma) (added from frozen stock)), incubated at 37°C for 30min or at 4°C overnight and extracted once with an equal volume of tris-saturated phenol by emulsion for 10min followed by centrifugation at 10,000rpm for 5min to separate the phases. The aqueous phase was removed to another tube and the phenol phase was re-extracted with 0.5vol dH_2O then the combined aqueous phases were extracted with phenol/chloroform (1:1) repeatedly until the interface disappeared (usually 2-3 times) and finally with chloroform alone prior to ethanol precipitation.

Variations of this protocol included: omission of bentonite or proteinase K or both in the disruption buffer; omission of the initial phenol extraction (polyadenylated RNAs are sometimes sequestered at phenol-aqueous but not phenol/chloroform-aqueous interfaces; HARI *et al.*, 1979); and omission of the solvent extraction completely if sucrose gradient centrifugation purification (2.12.1) was to be performed (phenol extraction has been found to be unsuitable for purification of some potyviral RNAs; BRAKKE & VAN PELT, 1970).

2.11.2 Purification of Viral RNA

RNA was extracted from purified virus preparations by adjusting to 1×RNA extraction buffer using concentrated stocks and treating as described above (2.11.1b).

Alternatively, it was sometimes found to be more convenient to purify viral RNA from crude RNA preparations (2.11.1b) by sucrose gradient centrifugation (2.12.1) or elution from gels (2.11.7)

2.11.3 Total RNA Extraction from Plants

Total RNA was extracted from healthy and BaYMV-infected barley plants by the method of PARISH & KIRBY, (1966) as modified by R.Townsend (pers. comm.). Plant material was ground in a baked mortar in the presence of liquid nitrogen, transferred to a Waring blendor and homogenised with 1vol Kirby mix (1% tri-isopropylnaphthalene sulphonate (TNS), 6% sodium 4-amino salicylate, 50mM tris-HCl (pH8.3), 6% (v/v) tris-buffered phenol) intermittently for 30min at 4°C. 1vol phenol/chloroform was added and, after mixing for 5min, the emulsion was centrifuged at 5,000×g for 15min. The aqueous phase was removed, re-extracted twice with phenol/chloroform as above and nucleic acids were precipitated with 0.2M sodium acetate and 2.5vol ethanol at -20°C overnight. Nucleic acids were collected by centrifugation at 5,000×g for 10min, washed in 80% (v/v) ethanol/0.15M sodium acetate (pH6.5), dried *in vacuo* and dissolved in dH_2O. After addition of 3vol 4M lithium acetate (pH6.0) insoluble RNA was allowed to precipitate at 0°C for 1h then was collected by centrifugation at 15,000×g for 15min and washed twice with 3M lithium acetate. Drained pellets were dissolved in 50mM tris-HCl (pH7.6) containing 5mM $MgCl_2$ and incubated at 0°C for 30min with 25µg/ml RNase-free DNase I (see 2.5.3). After extraction with

phenol/chloroform and chloroform RNA was precipitated with 0.3M sodium acetate (pH6.5) and 2.5vol ethanol at -20°C overnight.

2.11.4 Selection of Polyadenylated RNA

Polyadenylated RNAs were isolated from total plant RNA by oligo dT-cellulose chromatography essentially as described by MANIATIS *et al.* (1982). A column was made in a 2ml disposable syringe using 0.5g oligo dT (Sigma) suspended in loading buffer (10mM tris-HCl (pH7.5), 500mM NaCl, 0.1% sarkosyl (Koch-Light)). The column was washed in 2 column volumes of 0.1N NaOH and then washed and equilibrated in loading buffer. 2mg total plant RNA (2.11.3) was dissolved in 1ml TE and heated to 65°C for 2min. An equal volume of 2× loading buffer containing 0.1% bromophenol blue was added at room temperature and the RNA was loaded onto the column (RNA was heated to 65°C in a relatively large volume and in the absence of salt to assist in its complete denaturation). The first column volume of eluate, monitored by the progress of the dye, was collected, passed through the column again, recollected and ethanol precipitated. This fraction was designated "poly A^- RNA". The column was washed with four further column volumes of loading buffer then "poly A^+ RNA" was eluted with elution buffer (5mM tris-HCl (pH7.5), 1mM EDTA, 0.1% sarkosyl). Fractions of 0.5ml were collected, OD's measured at 260nm using acid-washed cuvettes and uv-absorbant fractions pooled and ethanol precipitated in the presence of carrier tRNA (Sigma) (20µg/ml).

2.11.5 Double-Stranded RNA Extraction from Plants

Double-stranded (ds) RNA was extracted from plants mechanically-inoculated or naturally infected with BaYMV and from healthy plants by the method of ZELCER *et al.* (1981) and purified by the cellulose chromatography method of FRANKLIN (1966) as modified by (a) ZELCER *et al.* (1981) or (b) MORRIS & DODDS (1979). Batch purification (c) was carried out as in MORRIS *et al.* (1983).

For large-scale extractions up to 200g fresh or frozen leaf or root tissue was ground in a chilled mortar in the presence of liquid nitrogen, transferred to a Waring blendor, thawed, and further homogenised in the presence of 1vol GPS buffer (0.2M glycine, 0.1M Na_2HPO_4, 0.6M NaCl (pH9.6)), 0.1vol 10% SDS, 0.001vol 2-mercaptoethanol, 1vol GPS-saturated phenol and 1vol chloroform/isoamyl alcohol (25:1), intermittently for 30min at 4°C. The homogenate was centrifuged at 10,000rpm for 10min to separate the phases then the aqueous phase was decanted and passed through sterile muslin to remove any plant débris. dsRNA was either (a) further purified by digestion of DNA and precipitation of ssRNA with 2M LiCl prior to CF11-cellulose chromatography or (b) selected directly on CF11-cellulose.

a) Nucleic acid was precipitated for at least 3h at -20°C, collected by centrifugation at 10,000rpm for 20min, resuspended in 50mM tris-HCl (pH7.5) and dialysed overnight against 2l of the same buffer at 4°C to remove low molecular weight impurities such as solvent molecules, plant phenolics etc. The nucleic acid solution was then clarified by centrifugation at 10,000rpm for 5min, made 10mM with respect to $MgCl_2$ and incubated with 50µg/ml DNase I (2.5.3) for 40min at room temperature. RNA was precipitated with 2vol ethanol and 100mM sodium acetate (pH6.5) at -20°C for at least 3h, collected by centrifugation at 10,000rpm for 20min, dried *in vacuo* and

resuspended directly in 5-10ml 2M LiCl. After mixing for 10min the slurry was centrifuged for 20min at 10,000rpm and the salt-soluble nucleic acids were precipitated with 3vol ethanol at -70°C for 1h. The precipitate was collected by centrifugation for 20min at 10,000rpm, resuspended in STE buffer and adjusted to 17.5% ethanol. CF11-cellulose (Whatman) had previously been suspended in 0.1N NaOH, washed extensively with dH_2O to lower the pH and stored in STE. 5ml columns were made in syringe barrels with glass wool plugs, washed with STE then equilibrated with 17.5% ethanol/STE. Samples in 17.5% ethanol/STE were loaded onto the column at room temperature and the column was washed extensively with 17.5% ethanol/STE to elute residual DNA and ssRNA until the OD at 260nm of the eluate was less than 0.01. dsRNA was then eluted sequentially with 0.5ml aliquots of STE, the OD's of the different fractions monitored and the uv-absorbent fractions pooled and ethanol precipitated. Yeast tRNA at 5µg/ml was used as a precipitation carrier when necessary.

b) Alternatively dsRNA was purified directly from total nucleic acids as described in MORRIS & DODDS (1979) modified for large volumes. The nucleic acid solution was adjusted to 17.5% ethanol then CF11-cellulose, without pre-treatment, was added at the rate of 0.02g/g plant tissue. The mixture was stirred at 4°C for 30min then the cellulose was collected by centrifugation at 3,000rpm for 5min and washed four times with 17.5% ethanol/STE at room temperature (CF11-cellulose was washed and columns were eluted at room temperature since at 4°C some cellular ssRNA has sufficient secondary structure to bind to the cellulose in 17.5% ethanol). The cellulose was then packed into a column, washed with 17.5% ethanol/STE and dsRNA was eluted as in (a). Traces of DNA and ssRNA were removed by DNase I digestion and LiCl-fractionation as above.

c) dsRNA extractions of small amounts of tissue (0.5g) were carried out as described as in MORRIS *et al.* (1983). Tissue was powdered in liquid nitrogen in a small mortar and homogenised in 0.7ml 2×STE, 0.3ml 10% SDS and 0.1ml bentonite (40mg/ml, prepared as in 2.5.2). The homogenate was transferred to a 1.5ml Eppendorf tube, agitated for 10min at room temperature and clarified by centrifugation for 2min. The supernatant was transferred to a second tube containing 0.02g CF11-cellulose powder, adjusted to 17.5% ethanol and shaken for 10min. Cellulose was collected by centrifugation for 2min and washed by resuspending in 17.5% ethanol/STE five times. dsRNA was eluted with a final wash of 0.4ml STE and ethanol precipitated prior to analysis on gels.

The identity of DNase-resistant, 2M LiCl-soluble nucleic acids was further confirmed by RNase A sensitivity in high or low salt concentrations (ZELCER *et al.*, 1981). dsRNA is resistant to digestion by RNase A in 2×SSC but susceptible in 0.1×SSC. ssRNA is susceptible under both conditions. Incubations were at 37°C for 30min with RNase A at 0.5µg/ml. Following incubations RNase was digested with proteinase K (50µg/ml for 15min at 37°C), solutions were extracted once with phenol/chloroform and electrophoresed directly or ethanol precipitated.

2.11.6 Isolation of Polysomes

Polysomes were isolated from *Hordeum vulgare* plants by the method of JACKSON & LARKINS (1976) as modified by PALUKAITIS *et al.* (1983). Plant material was harvested from healthy and BaYMV-infected plants 42 days after inoculation (2.7.3a). Material from meristematic as well as young and old leaves was taken. All further manipulations were carried out at 4°C. 3.5g plant material was ground on ice

in a chilled mortar with 35ml buffer (200mM tris-HCl (pH9.0), 400mM KCl, 200mM sucrose, 35mM $MgCl_2$, 25mM ethylene glycol bis(2-aminoethyl ether) N,N'-tetracetic acid (EGTA; Sigma), 1% 2-mercaptoethanol (Sigma)) and the homogenate was squeezed through sterile nylon bolting cloth. Following centrifugation of the filtrate at 15,000rpm for 10min the supernatant was decanted and Triton X-100 was added to a final concentration of 1% (v/v). The polysomes were then pelleted through a 4ml layer of 1.75M sucrose (in 40mM tris-HCl (pH9.0), 200mM KCL,30mM $MgCl_2$, 5mM EGTA) at 50,000rpm for 60min in a Beckman Type 65 rotor. The cushion interfaces, then the pellets, were washed briefly with sterile dH_2O to remove non-polysomal material then the pellets were frozen at -20°C.

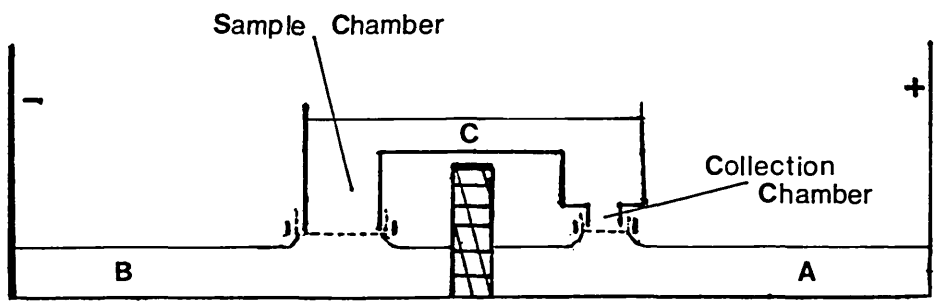
Pellets were thawed and treated in one of three ways. Total polysomal RNA was isolated as in 2.11.1b. Polysomes for use directly in *in vitro* translation (IVT) experiments were prepared by resuspension of pellets in 200µl of 8mM tris-HCl (pH8.5), 5mM KCl, 2mM $MgCl_2$. Insoluble material was removed by microcentrifugation for 5min and supernatants were either frozen at -20°C or used immediately in IVT incubations. Polysomal RNA, free from encapsidated viral RNA, was prepared by disruption of polysomes in 40mM tris-HCl (pH9.0), 15mM EDTA for 10min followed by high speed centrifugation as described above and phenol/chloroform extraction and ethanol precipitation of the supernatants (less the sucrose cushions). An aliquot of this supernatant , prior to solvent extraction, was tested by ISEM (2.10.2) for the presence of virus particles. Pellets from the second round of ultracentrifugation were washed as above and RNA extracted as in 2.11.1b. Thus three fractions of RNA were produced: total polysomal RNA; viral RNA-free polysomal RNA and polysomal RNA-free viral RNA.

2.11.7 Gel Purification of RNA

BaYMV RNA was purified by denaturing agarose gel electrophoresis and (a) electroelution, (b) extraction from low melting point (LMP) gels or (c) freeze/thaw treatment. Ds RNA was extracted from agarose and polyacrylamide gels by electroelution as in (a). Occasionally impurities were removed by DEAE-cellulose chromatography (d).

a) Electroelution was performed using an ISCO model 1750 sample concentrator (ALLINGTON *et al.*, 1978) as described by ZASSENHAUS *et al.* (1982) modified as follows. The design of the electroelution apparatus is illustrated below.

Dialysis membrane (indicated by dotted lines) was held in place with plastic compression rings at the bottom of the sample and collection chambers. Compartment A of the electrophoresis tank was filled with 3M sodium acetate/1×TAE, compartment B with 10×TAE and the electroelution cup (compartment C) contained TE. Excised gel slices were placed in the sample chamber and electroelution was carried out at 100V for 1h at 4°C. The eluate was withdrawn from the collection chamber after first carefully removing the rest of the buffer in the cup then the same buffer was returned to the cup and electroelution repeated. The two eluates were pooled, extracted with phenol/chloroform to remove residual agarose and ethanol precipitated.



b) RNA was extracted from LMP agarose by a modification of the method in (MANIATIS *et al.*, 1982). RNA was denatured with MeHgOH and electrophoresed in gels not containing MeHgOH (2.6.1e). After staining bands were sliced out of the gels, transferred to 1.5ml Eppendorf tubes and volumes were estimated. 2vol 1% SDS and 3vol phenol were added and mixtures were incubated at 65°C with occasional vortexing until a clear one phase solution was obtained (≈5min). Tubes were chilled on ice until white emulsions were formed (2-3min) then microcentrifuged for 10min. Supernatants were removed and interfaces were re-extracted with 0.5vol dH_2O at 65°C as described. Combined aqueous phases were extracted with phenol/chloroform repeatedly until there was no interface, once with chloroform and then RNA was ethanol precipitated.

c) RNA was eluted from gel slices ater denaturing agarose electrophoresis by a modification of the method of TAUTZ & RENZ (1983). Sterile 2ml disposable syringes were prepared by slicing off the nozzle with a hot scalpel leaving a small hole in its place. Gel slices were squeezed through this syringe into an eppendorf tube, mixed with 3vol TE containing 1M NaCl which had been used to wash the syringe and frozen at -70°C. Capless 0.5ml eppendorf tubes, the bottoms of which had been punctured, were packed with a small amount of baked, siliconised glass wool and suspended in capless 1.5ml eppendorf tubes. Gel slurries were thawed, transferred to these 0.5ml tubes and microcentrifuged for 30s. Eluates were collected in the 1.5ml tubes and agarose residues were re-extracted with 75µl each of the same buffer as described. Combined eluates were extracted with phenol/chloroform until the interface disappeared then once with chloroform and then RNA was ethanol precipitated.

d) DEAE-cellulose (Sigma) was washed by decantation in 0.5N HCl, 0.5N NaOH and finally in dH_2O to lower pH. "Fines" were concommittantly removed in this way. 0.2ml columns were made in 2ml sterile disposable syringes and equilibrated in 10mM tris-HCl (pH7.5), 0.3M NaCl. RNA was loaded and columns were washed in the same buffer then RNA was eluted with 100µl aliquots of 10mM tris-HCl (pH7.5) containing 7M urea and 1M NaCl. Eluates were monitored by spotting 2µl samples on agarose plates containing 0.5µg/ml ethidium bromide and viewing on a transilluminator. Fractions containing RNA were pooled and ethanol precipitated.

2.12 Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was used to fractionate RNA, cDNA and virus preparations. Linear gradients were made by layering four appropriate sucrose solutions (eg 5,10,15 and 20% solutions for a 5-20% gradient) in the relevant centrifuge tubes and allowing them to diffuse at 4°C overnight.

2.12.1 RNA

Crude viral RNA preparations were fractionated on 5ml 5-20% sucrose (RNase-free, Sigma) gradients made up in TES (10mM tris-HCl (pH7.5), 1mM EDTA, 100mM NaCl). RNA, prepared as in 2.11.1b, was treated with 50µg/ml DNase I (RNase-free, 2.5.3) at 37°C for 15min, precipitated with 2M LiCl for 4h at 0°C to remove some of the heterogeneous low molecular weight RNA, collected by microcentrifugation for 10min and washed twice with 70% ethanol. RNA pellets were dissolved in TES, 0.1% SDS (up to 10mg/ml total RNA), heated to 65°C for 2min, quick-cooled in ice-water and

microcentrifuged for 5min. Supernatants were layered onto gradients (<200µl/gradient) and centrifuged in a Beckman SW50.1 rotor at 40,000rpm for 4h at 10°C. Gradients were fractionated by hand into 0.25ml aliquots and 5µl samples of each fraction were electrophoresed in denaturing agarose gels (2.6.1b) to locate RNAs of interest. Fractions consisting of the same RNA species were pooled, diluted 3× and ethanol precipitated.

2.12.2 cDNA

Single-stranded cDNA was fractionated on alkaline sucrose gradients (HEIDECKER & MESSING, 1983) (a) and double-stranded cDNA on neutral gradients (b).

a) 16ml alkaline gradients (15-30%) were made up in 0.1N NaOH, 1M NaCl, 2mM EDTA. Samples of cDNA at the end of reverse transcriptase reactions (2.13.2) were adjusted to 0.3N NaOH, heated to 65°C for 20min, adjusted to 1M NaCl and 2mM EDTA then loaded onto alkaline gradients and centrifuged for 18h at 10°C in a Beckman SW28.1 rotor at 27,000rpm. Gradients were hand fractionated into 0.5ml aliquots, radioactivity was located using a mini-monitor and the sizes of cDNAs in each aliquot were determined by alkaline gel electrophoresis (2.6.1d), gel-drying (2.6.5) and autoradiography (2.15.3). Fractions containing cDNAs of desired size were pooled, neutralised with acetic acid, diluted 3× and ethanol precipitated.

b) Double-stranded products of 2nd strand synthesis reactions (2.13.2) were fractionated analogously on neutral 16ml 15-30% sucrose gradients made up in 50mM tris-HCl (pH7.4), 1mM EDTA except that samples were not denatured before centrifugation and fractions were not neutralised prior to ethanol precipitation.

2.12.3 Virus

Partially purified preparations of BaYMV were subjected to a 10min clearing spin at 8,000rpm then layered onto 36ml 10-40% gradients (2ml virus/gradient) made up in 0.05M sodium phosphate (pH7.3) and centrifuged in a Beckman SW28 rotor at 25,000rpm for 2.5h at 4°C. Gradients were fractionated using a Gilson Microcol TDC80 fractionator attached to a LKB Uvicord II type 8303A. Fractions were monitored for virus by EM (2.9.3) and virus containing zones were pooled, diluted with 5vol 0.05M sodium phosphate (pH7.3) and centrifuged at 30,000rpm for 2h in a Beckman Type 30 rotor at 4°C. pellets were resuspended in the same buffer and stored at 4°C or at -20°C.

2.13 cDNA Synthesis

2.13.1 Preparation of cDNA probes

[^{32}P]-labelled cDNA probes were prepared as described by GOULD & SYMONS (1977) and TAYLOR *et al.* (1976) using oligo dT- or random-priming and RNA purified as in 2.11.2. Random primer was prepared by partial hydrolysis of salmon sperm DNA (Sigma) with DNase I (Worthington). DNA was dissolved at 5mg/ml in 10mM tris-HCl (pH8.3) containing 10mM $MgCl_2$, DNase I was added to 70µg/ml and the mixture was incubated for 2h at 37°C followed by heating to 120°C in a paraffin bath to inactivate the DNase I. Primer was quick-cooled to prevent reannealling and stored at -20°C. For cDNA synthesis 10µCi [α-^{32}P]dCTP (1mCi/ml; 410Ci/mmol: Amersham) was dried *in vacuo*, taken up in a 25µl reaction volume containing 50mM tris-HCl (pH8.3), 10mM $MgCl_2$, 140mM KCl, 30mM 2-mercaptoethanol, 1mM each dATP, dGTP and TTP (Sigma), 1.25µg oligo dT_{12-18} (P-L Biochemicals) or 25µg primer DNA, 0.5-2µg RNA and 10-20u reverse transcriptase

("Super RT": Anglian) and the reaction mixture was incubated at 42°C for 90min. The reaction was terminated and RNA hydrolysed by addition of NaOH to 0.3M and incubation at 70°C for 1h or 37°C overnight.

Radioactive cDNA was separated from unincorporated nucleotides by the "spin-column" procedure of MANIATIS *et al.* (1982). A column was made by filling a 2ml disposable syringe barrel, plugged with a Whatman 3MM paper disc, with Sephadex G75 (Pharmacia) which had been suspended and autoclaved in TE. The column was suspended in a 15ml Corex tube, centrifuged at 1,000rpm for 2min in a bench-top centrifuge and washed with 50µl TE by centrifugation as above. The cDNA reaction mix was loaded onto the centre of the column and the first fraction was collected in a capless 1.5ml Eppendorf tube by centrifugation at 1,000rpm for 1min. Further fractions were collected in separate tubes by sequential addition of 50µl TE and centrifugation as above. The radioactivity in each tube was measured with a mini-monitor and fractions constituting the first elution peak were pooled. Radioactivity was measured by counting a 2µl sample, spotted and dried onto a Whatman 3MM paper disc, in 8ml scintillant containing 2vol toluene to 1vol Triton X-100 (Rose) with 0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis[2-(5-phenyloxazolyl)]benzene (both Sigma) using a Packard "Tri-Carb" liquid scintillation spectrometer.

2.13.2 ds cDNA Synthesis

ds cDNA was synthesised for cloning in M13 bacteriophage vectors (2.18) by (a) the "loop-back" method (MANIATIS *et al.*, 1982) or (b) the RNase H method (WATSON & JACKSON, 1985). In both cases first strand synthesis was carried out as above (2.13.1) except that reaction volumes were 50µl and contained, in addition, 25µM dCTP and 50u of RNasin (P&S).

a) This method relies on transient hair-pin formation at the ends of the single-stranded cDNA providing primers which can be extended by the "Klenow" fragment of *E.coli* DNA Pol I. The product is ds cDNA with a loop at one end which must be removed by S_1 nuclease or restriction endonuclease treatment.

1st strand cDNA was fractionated on alkaline sucrose gradients (2.12.2a). Fractions consisting of DNA of ≥200 bases (as determined by alkaline gel electrophoresis; 2.6.1d) were pooled, ethanol precipitated and resuspended in 20µl dH_2O. 10µCi [α^{32}P]-dCTP was dried *in vacuo*, taken up in a 50µl reaction volume containing 1st strand cDNA, 100mM HEPES (pH6.9), 10mM $MgCl_2$, 70mM KCl, 2mM DTT, 0.5mM each dATP, dGTP and TTP, 125µM dCTP and 5u "Klenow" fragment (Amersham) and incubated at 15°C overnight. The reaction was terminated by phenol/chloroform extraction and products were ethanol precipitated.

b) In this method RNA-cDNA hybrids are nicked with RNase H then nick-translated by *E.coli* DNA Pol I. "Ragged ends" are filled in by T4 DNA polymerase.

RNA-cDNA hybrids were fractionated on Sephadex G75 as above but omitting the RNA hydrolysis step. Eluted hybrids were extracted with phenol/chloroform and chloroform, ethanol precipitated, dried *in vacuo* and resuspended in 25µl dH_2O. 10µCi [α^{32}P]-dCTP was dried *in vacuo* and taken up in a 100µl reaction volume containing 1st strand cDNA, 100mM HEPES (pH7.6), 4mM $MgCl_2$, 70mM KCl, 0.13% (v/v) 2-mercaptoethanol, 0.2mM each dATP, dGTP and TTP, 5µM dCTP, 30u *E.coli* DNA Pol I (Amersham) and 1u RNase H (P-L)/µg cDNA. The reaction mix was incubated for 1h at 14°C then for 1h at room temperature. The reaction was terminated by addition of EDTA to 5mM and products were fractionated on Sephadex G75, deproteinised and ethanol precipitated as above. Ragged ends were filled in by resuspending the ds cDNA in 30µl of the following reaction mixture: 33mM tris-

acetate (pH7.9), 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT, 0.1mM each dATP, dCTP, dGTP and TTP, 0.1mg/ml BSA (nuclease-free; Pharmacia) and 9u T4 DNA polymerase (Anglian) and incubating at 37°C for 30min. The reaction was terminated by phenol/chloroform extraction and then products were ethanol precipitated.

2.14 Nick-Translation of M13 RF DNA

Radioactive DNA probes were prepared by the method of RIGBY *et al.*, (1977).

10µCi [α^{32}P]-dCTP was dried *in vacuo* and taken up in a 100µl reaction volume containing 100mM tris-HCl (pH7.4), 20mM 2-mercaptoethanol, 10mM $MgCl_2$, 15µM each dATP, dGTP and TTP, 0.03mg/ml BSA (nuclease-free) and 1µg M13 RF DNA. DNaseI was added to 4ng/ml and the reaction was initiated by the addition of 2u *E.coli* DNA Pol I. After incubation at 16°C for 90min the reaction was terminated by the addition of 10µl 1M NaOH and 2µl 0.5M EDTA. Labelled DNA was fractionated as in 2.13.1.

2.15 Blotting and Hybridisation

A number of different transfer procedures, transfer membranes and hybridisation protocols were used depending on availability and convenience.

2.15.1 Blotting/Transfer

a Dot-Blots

Dot-blots were used to detect BaYMV in plant tissues as described in MAULE *et al.*, (1983) and BAULCOMBE, (1984). Samples of healthy and infected tissue were ground in

Eppendorf tubes using a small amount of carborundum and a rounded Pasteur pipette with 2ml/g 50mM sodium phosphate (pH7.5). Homogenates were adjusted to 100µg/ml proteinase K, incubated at 65°C for 10min and microcentrifuged for 5min. Supernatants and purified viral RNA (used as a control) were serially diluted in phosphate buffer and applied as 5µl spots to either nitrocellulose (Bio-Rad) which had been air-dried following presoaking in 20×SSC or GeneScreen™ without pretreatment (1cm²/spot). Membranes were air-dried then baked at 80°C for 2-4h prior to hybridisation.

Recombinant M13 clones were screened by dot-blotting using a slightly different procedure. M13 RF dsDNA was prepared by the "mini-prep" method (2.18.7) and 5µl spots of DNA (0.1µg/µl) were applied to untreated nitrocellulose (1cm²/spot). Control spots of viral RNA or extracts from healthy and infected plants (prepared as above) were spotted onto air-dried 5µl spots of 20×SSC on the same membrane. Membranes were the dried and baked as above.

b <u>"Northern Blots" (RNA transfer)</u>

Denatured ss and dsRNA were transferred to GeneScreen *Plus*™ membranes according to the manufacturers recommended protocols after methylmercury gel electrophoresis (2.6.1b). After staining and photographing (2.6.4) wells were cut away and gels were treated with 50mM NaOH for 30min then were neutralised in 1M tris-HCl (pH7.0) for 30min. Meanwhile the GeneScreen *Plus*™ membrane was cut to size and soaked in water for 5min then in 10×SSC for 15-20min. A sheet of Whatman 3MM paper was placed over an elevated glass plate in a glass baking dish containing 10×SSC so that the ends of the paper were in the solution. The paper was then wet with 10×SSC and the gel was placed in the middle and surrounded with Parafilm® strips. The transfer membrane was positioned on the gel, concave side (side B) down, excluding all air bubbles then 5 gel sized sheets of

Whatman 3MM, followed by a 3-6cm stack of absorbant towels, was laid on top of the membrane. The whole structure was covered in 'cling-film', a large weight (≈800g) was placed on top of the paper and capillary transfer of RNA was allowed overnight. After transfer the membrane was washed briefly in 2×SSC, air-dried and baked at 80°C for 1h.

After non-denaturing agarose electrophoresis (2.6.1a), dsRNA was transferred to GeneScreen *Plus*™ in a similar fashion.

c **"Southern Blots" (DNA transfer)**

Recombinant M13 RF dsDNA, linearised by restriction endonuclease digestion (2.18.2), was electrophoresed in non-denaturing agarose gels (2.6.1a) and transferred to GeneScreen *Plus*™ membranes by recommended protocols. After staining wells were cut away and gels were soaked in 0.25M HCl for 15min to depurinate the DNA, in 0.2M NaOH, 0.6M NaCl for 30min to denature the DNA and then in 0.5M tris-HCl (pH7.5), 1.5M NaCl for 30min. After transfer of DNA as described in 2.15.1b. membranes were soaked in 0.4M NaOH for 60s then in 0.2M tris-HCl (pH7.5), 2×SSC for 60s, air-dried and baked at 80°C for 1h.

d **Plaque Lifts**

Plaque lifts of recombinant M13 clones were performed using Pall® Biodyne™ A and Colony/PlaqueScreen™ membranes using identical protocols. Autoclaved 82mm membrane discs without prewetting were placed (convex side down for Colony/PlaqueScreen™) on plates of M13-infected *E.coli*, generated as in 2.18.6 and precooled to 4°C. Plates and membranes were marked asymmetrically in three places for subsequent orientation then incubated at 4°C for 5min. Each membrane was removed and placed plaque-side up on a 1ml drop of denaturing solution (2.5M NaCl, 0.5M NaOH) on a stretched sheet of Saran Wrap for 5min and then on a 1ml

drop of neutralizing solution (3M sodium acetate, pH5.5) for 5min then air-dried and baked at 80°C for 1h.

2.15.2 Hybridisation

DNA and RNA, bound to transfer membranes as above, were detected by hybridisation to radioactive DNA probes (2.13.1, 2.14) and autoradiography (2.15.3). Membranes were prehybridised in heat-sealed plastic bags containing 10-15ml hybridisation solution (without probe) per 100cm² for at least 4h to block any non-specific binding sites. Probes were denatured by boiling for 5min, quick-cooled in an ethanol/ice bath at -20°C and added to the hybridisation solution at 10^{5}-10^{6} cpm/ml. A number of different hybridisation conditions were employed:

a GeneScreen™ Membranes

Hybridisation was for 18-24h at 42°C in 50% formamide (deionised), 0.2% PVP, 0.2% BSA, 0.2% ficoll, 0.05M tris-HCl (pH7.5), 1M NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulphate, 100µg/ml sheared and denatured salmon sperm DNA (Sigma).

Membranes were washed in 100ml of each of the following: 2×SSC for 5min at room temperature; 2×SSC, 0.5% SDS for 30min at 60°C (twice); 0.1×SSC for 30min at room temperature. After briefly drying, membranes were wrapped in 'Saran Wrap' and autoradiographed.

b Nitrocellulose Membranes

Membranes were hybridised by the method of SINGH & JONES (1984), for 18-24h at 65°C in 4×SET (1×SET= 0.1M NaCl, 0.03M tris-HCl (pH7.4), 2mM EDTA), 10% dextran sulphate, 0.2% SDS, 0.1% sodium pyrophosphate, 500µg/ml heparin (sodium salt; Sigma).

Washing was in 100ml each of the following: 2×SET for 5min at room temperature; 2×SET, 0.2% SDS for 30min at 65°C (twice); 1×SET, 0.2% SDS for 30min at 65°C; 2×SET at room temperature for 5min. After briefly drying membranes were wrapped in Saran Wrap and autoradiographed.

c **GeneScreen *Plus*™ Membranes**

Membranes were prehybridised at 42°C for 4h in 50% formamide, 1M NaCl and 10% dextran sulphate. Sheared and denatured salmon sperm DNA was boiled with the probe and added to the hybridisation solution at 100μg/ml then hybridisation was at 42°C for 18-24h.

Membranes were washed as described for GeneScreen™.

2.15.3 Autoradiography

Membranes were autoradiographed in a light-proof cassette with Kodak X-Omat S film at -70°C for an appropriate length of time (1-14 days). Intensifying screens were used when necessary. Films were processed in a Kodak X-Omat automatic processor.

2.15.4 Rehybridisation

Probes were stripped from blots, prior to rehybridisation with different probes, by the following methods: nitrocellulose membranes were washed three times for 10min each in dH_2O at 100°C; GeneScreen™ membranes were washed in 5mM tris-HCl (pH8.0), 0.2mM EDTA, 0.05% sodium pyrophosphate, 0.002% PVP, 0.002% BSA, 0.002% ficoll for 1-3h at 65°C with constant agitation; GeneScreen *Plus*™ membranes were washed in 0.4N NaOH at 42°C for 30min and then in 0.2M tris-HCl (pH7.5), 0.1×SSC, 0.1% SDS at 42°C for 30min.

2.16 *In Vitro* Translation

In vitro translation of viral RNA, polysomal RNA and polysome preparations was carried out using (a) message-dependent rabbit reticulocyte lysate (MDL) (P&S, Amersham) and (b) wheat germ extract (a gift from M.Brisco, John Innes Institute).

a The MDL had been prepared by a modification of the method described by PELHAM & JACKSON (1976) in which endogenous mRNA is destroyed by treatment with a calcium-dependent nuclease. *In vitro* translations were performed according to manufacturers instructions. 12.5µCi L-[4,5-^{3}H]leucine (1mCi/ml, 55-128 Ci/mmol; Amersham) was used per final reaction volume of 25µl. Occasionally 20µCi L-[^{35}S]methionine (12.7mCi/ml, 1330 Ci/mmol; Amersham) was used. The appropriate volume of labelled amino acid was dried *in vacuo* in a 0.5ml Eppendorf tube. If MDL from P&S Biochemicals was employed the radioactivity was resuspended in the following mixture per reaction volume: 16µl MDL, 1µl [2M KCl, 10mM $MgCl_2$] (P&S), 1µl amino acids mixture (19 amino acids, 1mM each; P&S), 1µl sterile dH_2O, 1µl 0.2M phosphocreatine (P&S). Alternatively, if Amersham MDL was used, the radioactivity was simply resuspended in 20µl MDL per reaction volume. Aliquots of 20µl were then dispensed into pre-chilled 0.5ml Eppendorf tubes and made up to 25µl by addition of 5µl RNA (0.1-1.0mg/ml) or sterile dH_2O. The contents of the tubes were thoroughly mixed and incubated at 30°C for 60min.

Duplicate 1µl samples were withdrawn at various times throughout the incubation, digested with RNase A, spotted onto 1.5cm squares of Whatman 3MM paper and dried under a lamp. The proteins were precipitated and unincorporated radioactivity removed by washing the squares in three changes of 5% trichloroacetic acid (TCA) containing 110mM sodium pyrophosphate (10ml/square) for 10min each. Finally

the squares were rinsed in methanol and dried. Their radioactivity was measured in 8ml scintillant as in 2.13.1. The remainder of each translation mixture was RNase-treated and stored at -20°C until analysed on discontinuous polyacrylamide gels (2.6.3b) and by fluorography (2.17).

b Wheat germ S-30 fraction was prepared as in DAVIES (1979). 20µCi [^{35}S]methionine was dried down as above and resuspended in the following mixture per reaction volume of 25µl: 12.5µl wheat germ extract, 2.5µl "Mix 6" (3.75mM GTP, 19 amino acids (-met), 0.375mM each, 25mM K_2ATP, 90mM phosphocreatine, 0.1mg/ml creatine phosphokinase), 2.5µl "HKMS" (200mM HEPES (pH7.6), 400mM potassium acetate, 4mM magnesium acetate, 4mM spermidine), 2.5µl sterile dH_2O. The wheat germ extract, "HKMS" and "Mix 6" were gifts from M.Brisco (John Institute). Aliquots of 20µl were dispensed into pre-chilled 0.5ml Eppendorf tubes then incubations, sampling, filter washing, scintillation counting and translation product analysis were carried out as described for MDL (2.16a).

2.17 Fluorography

Radioactively labelled proteins were detected in polyacrylamide gels by fluorography as described by CHAMBERLAIN, (1979). Gels were usually stained with Coomassie Brilliant Blue R-250 as in 2.6.4 prior to fluorography. After staining gels were soaked in about 20vol dH_2O for 30-60min to raise the pH and prevent precipitation of salycylic acid. Gels were then soaked in 10vol 1M sodium salycylate (pH5-7) at room temperature for 30min before gel drying (2.6.5). Dried gels were exposed to Kodac X-ray film at -70°C and films were developed using a Kodac X-omat film processor.

2.18 cDNA Cloning in Bacteriophage M13

2.18.1 Cloning Strategy

Attempts were made to "shotgun"-clone fragments of DNA complementary to the BaYMV genome into bacteriophage M13 vectors to provide a source of probes. Due to difficulties in obtaining sufficient quantities of BaYMV RNA and its apparent resistance to reverse transcription the cloning strategy involved synthesis of cDNA to crude BaYMV RNA preparations, enrichment by size fractionation on alkaline (ss cDNA) and neutral (ds cDNA) sucrose gradients and differential screening of clones by plaque-lift, dot- and Southern-blot hybridisation. Since the restriction map of BaYMV is not known "blunt" and "sticky" ended fragments were generated with a variety of "four-cutters" (restriction endonucleases with recognition sequences of 4bp) and ligated into the multi-cloning sites of M13 mp8 and mp18 vectors linearised with an appropriate restriction enzyme. Insertion or deletion within this site destroys β-galactosidase activity within cells infected with the recombinant bacteriophage and hence production of the blue dye on metabolism of X-gal. The following combinations of enzymes were used:

cDNA cut with	M13 cut with	Notes
Sau3A (Biolabs)	BamH1 (Amersham)	"Sticky"
Mbo1 (P-L)	BamH1	"Sticky"
Taq1 (Amersham)	Acc1 (P-L)	"Sticky"
HpaII (P-L)	Acc1	"Sticky"
EcoR1* (Amersham)	EcoR1	"Sticky" (see 2.18.2)
HaeIII (Anglian)	SmaI (Anglian)	"Blunt"
uncut	SmaI	"Blunt"

2.18.2 Restriction Enzyme Digestions

Digestions were carried out in volumes of 10-20µl containing <150µg/ml DNA and 2-5u enzyme/µg DNA in appropriate buffers and at optimum temperatures (summarised in the Biolabs catalogue, 1985) in the relevant salt concentration (MANIATIS *et al.*, 1982).

All reactions were carried out in Medium Salt Buffer (MSB) at 37°C except:

TaqI MSB at 65°C

HpaII & EcoR1* appropriate buffer at 37°C.

The buffers used were:

Medium Salt Buffer: 10mM tris-HCl (pH7.5), 50mM NaCL, 10mM $MgCl_2$, 1mM DTT, 10mM spermidine.

HpaII Buffer: 10mM tris-HCl (pH7.5), 10mM KCl, 10mM $MgCl_2$, 5mM 2-mercaptoethanol.

EcoR1* Buffer: 10mM tris-HCl (pH7.5), 10mM $MgCl_2$, 1mM DTT, 10mM spermidine, 20% glycerol.

(In the absence of salt and in the presence of 20% glycerol EcoR1 becomes a four-cutter, recognising the sequence N↓AATTN. This activity is known as EcoR1*).

Digestions were for 1-2h and whenever possible were monitored by agarose gel electrophoresis. Reactions were terminated by phenol/chloroform extraction then DNA was ethanol precipitated, dried *in vacuo* and resuspended in a minimal volume of TE.

2.18.3 Ligation

The amount of insert DNA available for ligation was low and difficult to quantify so the desired 2-3 fold molar excess of insert over vector DNA was not usually achieved. A standard 10ng of vector DNA per ligation reaction was found to be appropriate for a convenient plating density.

For each set of ligations two control reactions were carried out in the absence of insert DNA, one with and one without ligase. On transformation of competent cells (see below) these controls allowed estimation of the efficiency of restriction cleavage of vector, the efficiency of ligation and the proportion of "false whites" (white plaques produced by deletion rather than insertion within the M13 *lac* operon). Otherwise reactions contained insert DNA, 10ng vector DNA (cut with the appropriate enzyme), 50mM tris-HCl (pH7.7), 10mM $MgCl_2$, 5mM spermidine, 1mM DTT, 1mM rATP and 2u T4 ligase (Anglian) for "sticky" or 10u for "blunt" end ligations and were incubated at 12°C overnight. For "sticky" end ligations T4 ligase was diluted to 2u/µl prior to use in 20mM sodium phosphate (pH7.6), 1mM EDTA, 5mM DTT, 60mM KCl, 50% glycerol.

2.18.4 Growth and Maintenance of Bacteria

Escherichia coli strain JM101 was subcultured monthly on minimal medium plates supplemented with its auxotrophic requirement and stored sealed at 4°C.

Minimal Agar

500ml	3% Bactoagar (Difco)
500ml	2×M9 salts
1ml	0.1M $CaCl_2$
1ml	1M $MgSO_4$
1ml	20% glucose

These components were autoclaved separately and combined prior to pouring the plates.

Supplement: 0.5ml 1% Thiamine-HCl (Vitamin B_1) (filter-sterilised)

M9 salts

6g	Na_2HPO_4
3g	KH_2PO_4
0.5g	NaCl
1g	NH_4Cl
dH_2O	to 500ml

Frozen stocks of cells were stored in minimal broth containing 20% (v/v) glycerol at -70°C, to be replated when required.

Overnight cultures were grown by inoculating 2ml 2TY broth in a 10ml Sterilin tube with a single colony of *E.coli* JM101 taken from a minimal agar plate and shaking overnight at 37°C.

2.18.5 Preparation of Competent Cells

Transformation-competent *E.coli* JM101 cells were prepared by the method of MANIATIS *et al.* (1982). 7.5ml 2TY broth in a 10ml Sterilin tube was inoculated with 25µl of overnight culture and shaken vigorously at 37°C until the OD_{550} reached 0.3 (≈4h) so that cells were in log phase. Cultures were chilled on ice for 5min, centrifuged at 3,500×g for 5min at 4°C and supernatants were discarded. Cells were resuspended in 10ml cold, sterile 50mM $CaCl_2$ with gentle mixing, stored on ice for 20min then centrifuged again as above. Supernatants were discarded and

cells were resuspended in 1ml cold 50mM $CaCl_2$ and used for transformations within 48h.

2.18.6 Transformation

For each set of transformations three controls were carried out, one using uncut vector DNA and the other two using the control ligation reactions (see 2.18.3 above). The uncut vector control allowed estimation of transformation efficiency.

2-20ng DNA, in a minimum volume (≤10µl), was added to 200µλ of competent cells and incubated on ice for 40min. During this incubation tubes of 3ml molten H top agar were cooled to 42°C along with 20µl 24mg/ml isopropyl-β-D-thio-galactopyranoside (IPTG; BRL), 30µl 20mg/ml (in dimethyl formamide) 5-bromo-4-chloro-3-indoyl-β-galactoside (X-gal; Anglian) and 200µl overnight culture. 50µl aliquots of transformation mix were added to these agar tubes and heat-shocked at 42°C for 3min then immediately poured onto prewarmed (37°C) bottom agar plates (1.5% Bactoagar in 2TY). When the top agar had set plates were incubated, inverted, at 37°C overnight.

2.18.7 M13 RF "Miniprep"

Individual white plaques were 'toothpicked' into 10ml Sterilin tubes containing 1ml 2TY broth and 100µl overnight culture and tubes were then shaken vigorously at 37°C for 6h. Cultures were poured into 1.5ml Eppendorf tubes and microcentrifuged for 5min. Supernatants were removed and cells were resuspended in 100µl 50mM glucose, 25mM tris-HCl (pH8.0), 10mM EDTA, 4mg/ml lysozyme and incubated at room temperature for 5min. 200µl 0.2N NaOH, 1% SDS were added with gentle mixing and tubes were incubated on ice for

5min. Bacterial DNA was precipitated by addition of 150µl 3M potassium acetate (pH4.8), incubation on ice for 5min and microcentrifugation for 5min. Supernatants were removed and extracted with phenol/chloroform and chloroform then nucleic acids were precipitated with 2vol ethanol at room temperature for 2min and collected by microcentrifugation for 5min. Pellets were dried *in vacuo*, resuspended in 50µl TE and treated with 40µg/ml RNase A for 15min at room temperature then stored at -20°C..

2.19 Extraction of Soluble Plant Proteins

Soluble proteins were extracted from healthy and BaYMV-infected barley seedlings by the low pH method of COUTTS (1978) or from intercellular fluid (IF) by the method of PARENT & ASSELIN (1984).

2.19.1 Low pH Method

Leaf samples (1-5g) were ground in a mortar at 4°C with 1ml/g MacIlvaine buffer (84mM citric acid, 32mM disodium hydrogen phosphate (pH2.8)) containing 14mM 2-mercaptoethanol. Extracts were centrifuged at 45,000×*g* for 30min and supernatants were either analysed directly on polyacrylamide tube gels (2.6.3c) or stored at -20°C prior to electrophoresis.

2.19.2 IF Method

Leaf samples (1-5g) were cut into 4cm long pieces and infiltrated *in vacuo*, with gentle agitation for three periods of 30s, with a large excess (≈30ml/g) of the following mixture at 4°C: 25mM tris-HCl (pH7.8), 0.5M

sucrose, 10mM $MgCl_2$, 10mM $CaCl_2$, 5mM 2-mercaptoethanol and 5mM PMSF. Pieces were gently blotted dry and placed in a disposable 10ml syringe barrel plugged with a disc of Whatman 3MM filter paper, suspended over a capless 1.5ml eppendorf tube in a 50ml Sterilin tube. After centrifugation at 2,500rpm in a bench centrifuge for 10min the extract was recovered in the eppendorf and analysed by PAGE on tube gels (2.6.3c) or stored at -20°C.

3 RESULTS & DISCUSSION

3.1 Virus Transmission and Propagation

a) Sap Transmission

Barley Yellow Mosaic Virus was successfully inoculated into and propagated in *Hordeum vulgare* cvs. Maris Otter and Tipper by the method described earlier (2.7.3). Results were not, however, consistent and the infection rates achieved by FRIEDT (1982) were rarely obtained.

Symptoms appeared after as little as 2 and as much as 7 weeks post-inoculation. Variation may have been due to day-length and/or growth rates of host plants as the earliest symptom expression was found in plants grown in growth cabinets with 14h day lengths and longest delays in symptom expression were found in plants grown in an unheated greenhouse without supplementary lighting over the winter months (day-lengths ca.8-10h). Under the growth cabinet conditions described most infected plants showed symptoms after 3-5 weeks.

Symptoms appeared first as chlorotic streaks in the youngest leaves and subsequently spread to new leaves (Figs.3-5). Secondary growth of plants whose leaves had been harvested showed uniform obvious symptoms. As plants aged leaves of Tipper plants developed an orange-yellow discolouration while in Maris Otter plants chlorotic streaks became necrotic particularly at the leaf tips (Fig.6).

Fig.3 Yellow mosaic symptoms in BaYMV-infected barley

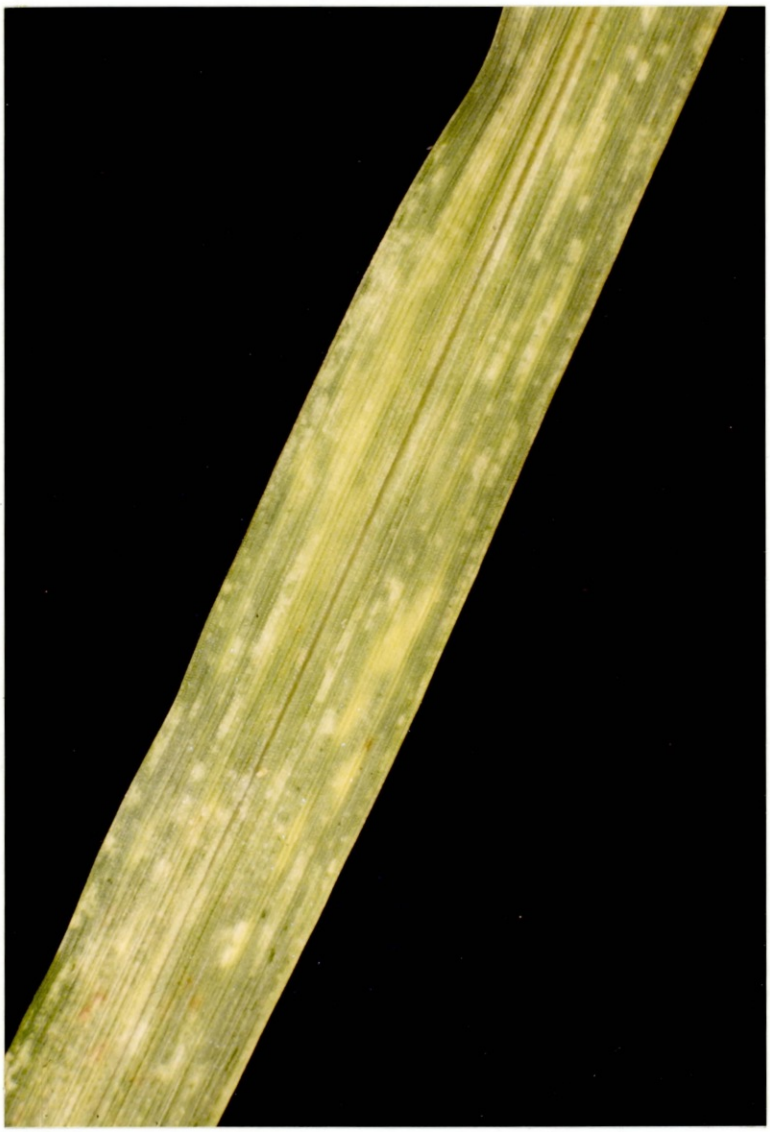


Fig.4 Yellow mosaic symptoms in BaYMV-infected barley

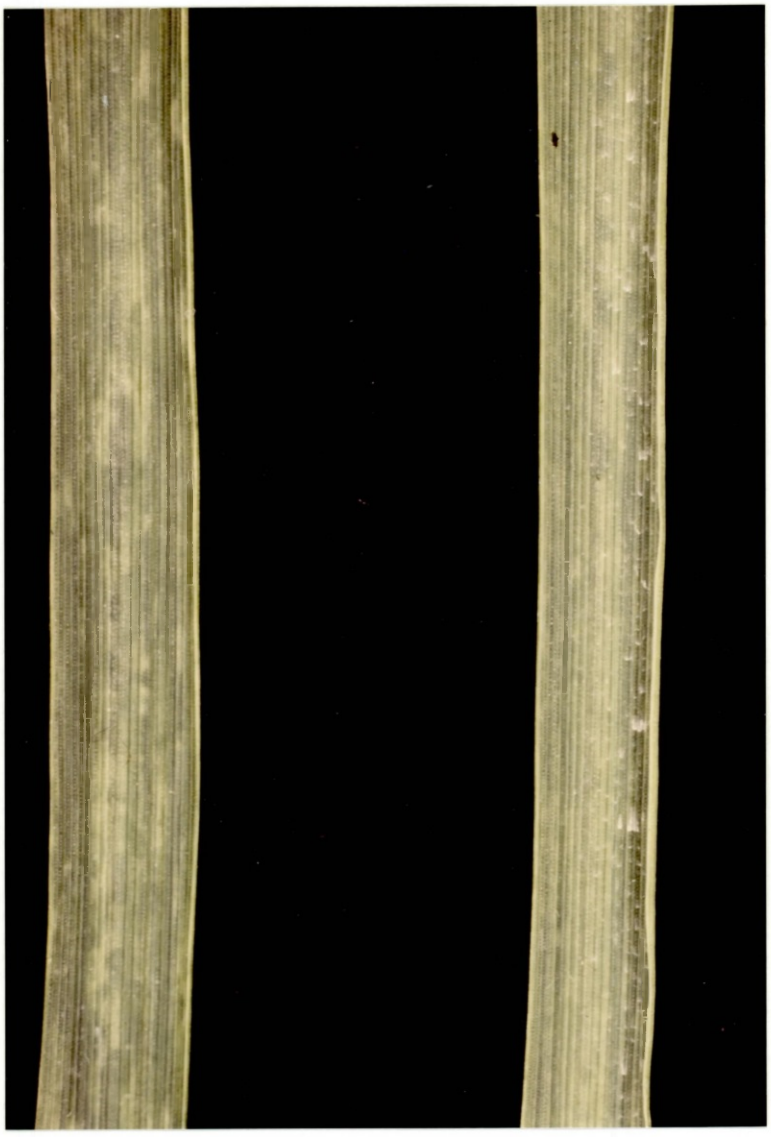


Fig.5 Yellow mosaic symptoms in BaYMV-infected barley

Fig.6 Necrosis in BaYMV-infected barley cvs Tipper (T) and Maris Otter (MO) leaves

Efficiencies of infection commonly varied between 20 and 75%. Some of this variation can be ascribed to inoculum infectivity as infection rates tended to drop in successive pots treated with the same batch of inoculum. Thus no more than six pots were ever inoculated with one batch of inoculum. There was further variation in infection rates that could not be explained in this way and could have been due to inconsistent inoculation technique, genetic variability of host plants or slight variation in growth conditions. Apart from the requirement for low temperatures very little is known about optimal conditions for infection and symptom expression.

Differences between these results and those of FRIEDT (1982) were not due to the slight variations in protocol as higher infection rates were not obtained using the buffers or double inoculation as described in this report; in fact a second inoculation resulted in higher mortality and poorer growth rates. The results may reflect differences in inoculation technique or may be due to a strain-specific phenomenon. The Japanese isolate of BaYMV is said to be 'not readily' transmissible (INOUYE & SAITO, 1975) and variations in sap-transmissibilty have been reported for BaYMV strains in Germany (EHLERS & PAUL, 1986). The latter authors suggested that mechanical-transmissibility depends on virus coat protein stability. The poor transmission and absence of any but a 30K protein in preparations of the Little Clacton isolate may thus be linked (see 3.2.7).

Attempts to find a local-lesion or high-titre propagation host for BaYMV among 19 species in 11 genera all proved unsuccessful. None of the species listed in 2.7.1 except *Hordeum vulgare* developed symptoms after inoculation nor did they contain virus particles detectable by EM in leaf-dip preparations regardless of growth conditions. Analogously restricted host ranges have been found for WYMV, RNMV, OMV and WSSMV, viruses that share

many characteristics with BaYMV (see 1.7). Viruses of monocotyledonous plants characteristically have restricted host ranges but all infect more than one graminaceous genus and some also infect dicotyledonous plants. Even barley stripe mosaic virus (BSMV) which is virtually confined to barley as a host in nature (TIMIAN, 1974) can be transmitted to other graminaceous hosts and some *Chenopodiaceae* and *Solanaceae* (ATABEKOV & NOVIKOV, 1971). Other viruses of monocots can have quite unrestricted host ranges: brome mosaic virus (BMV) infects fifty genera in the *Gramineae* and a few genera in six dicotyledonous families (LANE, 1977). Potyviruses of monocotyledonous hosts do not in general infect dicotyledonous plants but none have such restricted host ranges as BaYMV.

b) RNA Inoculation

It is not known whether BaYMV is a mono- or multi-partite virus as there have been no conclusive infectivity studies using purified particles or RNAs. USUGI & SAITO (1976) reported that after sucrose density centrifugation the upper of two virus containing zones consisted mainly of short particles and was much less infectious than the lower zone which consisted of a mixture of long and short particles. Since the particles in the upper zone could have been fragmented and the longer particles could have been aggregates of shorter particles the results do not distinguish between the possibilities that infectivity is associated with long particles, short particles or a mixture of the two. The bimodal length distribution of BaYMV (INOUYE & SAITO, 1975, HUTH *et al.*, 1984) and the presence of two ssRNAs in purified virions (HUTH *et al.*, 1984) argue in favour of a bipartite genome organisation though the possibility of a packaged subgenomic RNA cannot be eliminated.

Infectivity experiments using RNA extracted from purified and crude virus preparations and from whole plants were carried out with a view to characterising this aspect of the BaYMV genome. However, while 50% of control sap-inoculated plants showed symptoms after 25d, none of the RNA samples described in 2.7.3b proved infectious under the conditions used even at RNA concentrations of 30µg/ml, a level sufficient for infectivity of most plant viral RNAs. This was true (a) for RNAs extracted with or without the use of proteinase K which might have degraded an essential 5' VPg (proteolytic degradation of VPgs destroys infectivity of some e.g. Nepoviral but not other e.g. Comoviral RNAs; WIMMER, 1982), (b) for a mixture of the two virion RNAs extracted from gels or purified by sucrose-gradients, (c) for total virion RNA (thus allowing for the presence of smaller, undetected genomic or packaged subgenomic RNAs) and (d) for total infected plant RNA which would also contain non-virion subgenomic RNAs. Some plant viruses (eg AlMV: BOL, 1971) require the presence of coat protein for genomic RNA infectivity. However, coinfection with subgenomic RNA 4 (normally packaged in virions) abolishes this requirement (ALBLAS & BOL, 1978). If BaYMV genomic RNA infectivity depends on coinfection with coat protein or subgenomic coat protein message (packaged or not) RNA samples (c) or (d) should have been infectious unless a factor in or the huge excess of plant RNA in (d) was inhibitory. Infectivity tests using purified RNAs singly and in combination were thus not possible.

The possible explanations for the failure of these experiments are inadequacies of technique, use of below-threshold concentrations of RNA, presence of inhibitors or an absolute requirement for a soluble protein. BaYMV RNA would not be infectious if it were of negative polarity but this is unlikely in view of its ability to direct *in vitro* protein synthesis (3.8) and of virus particle morphology, all negative strand plant viruses being bacilliform. Non-

infectivity of viral RNA led to the suggestion that tomato spotted wilt virus RNA was of negative polarity (VAN DEN HURK *et al.*, 1977) but *in vitro* translation and transcription experiments (VERKLEIJ *et al.*, 1982) later showed this to be untrue.

3.2 Virus Purification

A method is presented (see 2.8.8) for the purificatiion of barley yellow mosaic virus. This method was developed independently and differs in a few important ways from published protocols (USUGI & SAITO, 1970, 1975, 1976; HUTH *et al.*, 1984) which proved unsatisfactory. This method suffers from the same disadvantage as the published ones, i.e. considerable loss of virus during low-speed centrifugation because of insolubility, and is not presented as the best possible protocol. Because of the nature of the project a method that maximised yield of full-length RNA was sought (even at the expense of capsid) and the protocol presented was developed from the first experiments to yield any full-lenth RNA (published methods were ineffective in this respect). The step that requires most further investigation is the resuspension of the virus pellet after ultracentrifugation through a sucrose cushion. Any method of disaggregating virus clumps without causing disruption of virions would greatly increase yields.

3.2.1 Plant Material and Homogenisation

Since no other propagation host was available BaYMV was purified from barley plants which are relatively fibrous so ideally only young tissue was used. Tissue homogenisation was aided by manually crushing at -70°C and the use of at least 3vol buffer. Grinding in liquid nitrogen was not used

routinely but may increase efficiency of extraction slightly. Use of buffer:tissue ratios greater than 3:1 (ml:g) did not increase the extraction efficiency sufficiently to affect final yields.

3.2.2 Extraction Buffers

The final choice of extraction buffer was influenced by the first succesful purification method. This was based on the method of DOUGHERTY & HIEBERT (1980), who used HEPES buffer and PEG-precipitation to purify pepper mottle virus and tobacco etch virus, modified to include a sucrose-caesium sulphate step gradient. HEPES (20mM, pH7.4) was subsequently shown to be superior to citrate (0.1M, pH7.0) by EM (2.9) and RNA extraction of ultracentrifugation pellets (2.11.1).

The effect of divalent cations was investigated by incubating partially purified virus samples (after one high speed centrifugation step) at 4°C overnight in the presence and absence of Mg^{++} (1 & 10mM) or EDTA (1 & 5mM) followed by a clearing spin and EM and RNA extraction of pellets and supernatants. No requirement of divalent cations for particle stability was found in that EM showed no evidence of increased fragmentation in the presence of EDTA at either concentration, nor was there any decrease in extractable full-length RNA. Mg^{++} at 10mM (but not appreciably at 1mM) caused clumping of particles and host components, as seen by EM, and an increase in the proportion of RNA found in clearing spin pellets. Similar behaviour has been reported for AlMV which is precipitated by Mg^{++} concentrations greater than 1mM (HULL & JOHNSON, 1968). In view of these results and the fact that initial extracts have higher levels of potentially deleterious divalent cation-dependent enzymes EDTA at 5mM was included in extraction buffers. The higher level also ensured

disruption of ribosomes so that following RNA extraction of ultracentrifugation pellets there was no ribosomal RNA contamination to interfere with viral RNA purification by sucrose gradients or gel extraction.

Other additives were included as precautionary measures, standard to most virus purification protocols. Both 2-mercaptoethanol and DIECA are reducing agents, the latter also being an inhibitor of polyphenol oxidase through its copper-chelating activity. The combination of these two lowers the chances of free radical damage. PMSF is an inhibitor of serine proteases so prevents cleavage of coat protein at susceptible peptide bonds that might destabilise virion structure or expose RNA. Despite the use of this protease inhibitor the only protein found consistently in virus preparations in this study was a 30K protein. Thus if, as proposed by EHLERS & PAUL (1986), the 30K protein arises by proteolytic degradation of a 35K capsid protein the enzyme involved is probably not a serine-protease. In this recent German study 20mM sodium iodoacetate, an inhibitor of cysteine-proteases, was included in the extraction buffer in addition to PMSF and EDTA.

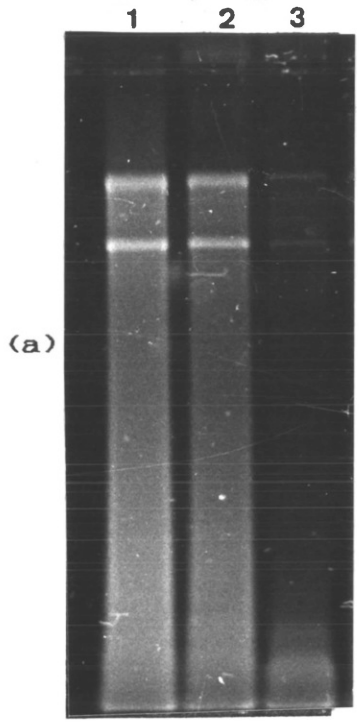
3.2.3 Clarification

Solubilisation of chloroplast and other plant cell membranes with Triton X-100 after filtration through muslin and low speed centrifugation was the preferred method of clarification of crude plant extracts for the following reasons:

(a) leaf-dip EM and ISEM showed that very few virus particles were sedimented during the low-speed centrifugation step even when this step was carried out before membrane solubilisation, indicating that BaYMV is not intimately associated with membranous structures within

the cell and that buffer:tissue ratios of 3:1 (ml:g) were sufficient for dispersal of virus and host material;
(b) centrifugation prior to membrane solubilisation allowed removal of nuclei and some other subcellular organelles at an early stage;
(c) treatment with any of the organic solvents listed in 2.8.3 as compared with Triton X-100 resulted in fewer and more fragmented particles after the concentration step as determined by EM;
(d) no difference in aggregation or fragmentation of particles was detectable by EM between crude samples incubated at 4°C overnight in 0.5% (v/v) Triton X-100 and in buffer alone;
(e) clarification with CCl_4 as compared with Triton X-100 resulted in a 30-50 fold decrease in extractable full-length RNA after concentration (Fig.7a,b, Lanes 1 & 3).

Although clarification with Triton X-100 results in greater yields of intact virus particles, organic solvent clarification removes more plant protein so may be preferable for the preparation of virus suitable for antiserum production. After clarification with CCl_4 and two rounds of PEG-precipitation but no further purification the only protein detectable in virus preparations by SDS-PAGE is the 30K protein (Lane 3, Fig.8). After Triton X-100 clarification and analogous concentration a less pure preparation is produced (Lane 1, Fig.9). Yields of the 30K protein are similar in both cases.



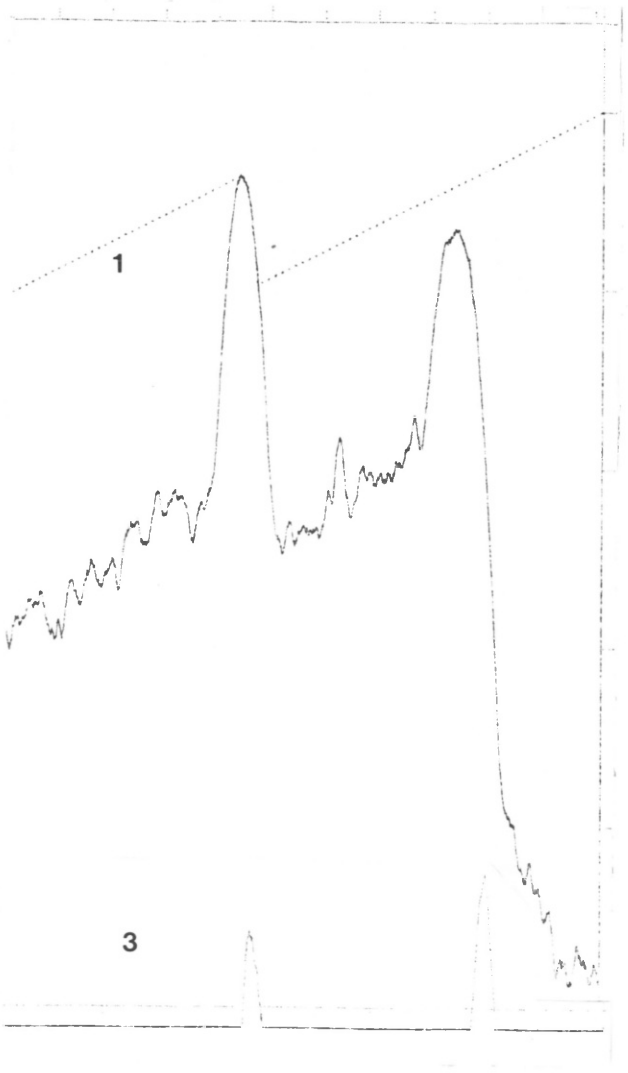
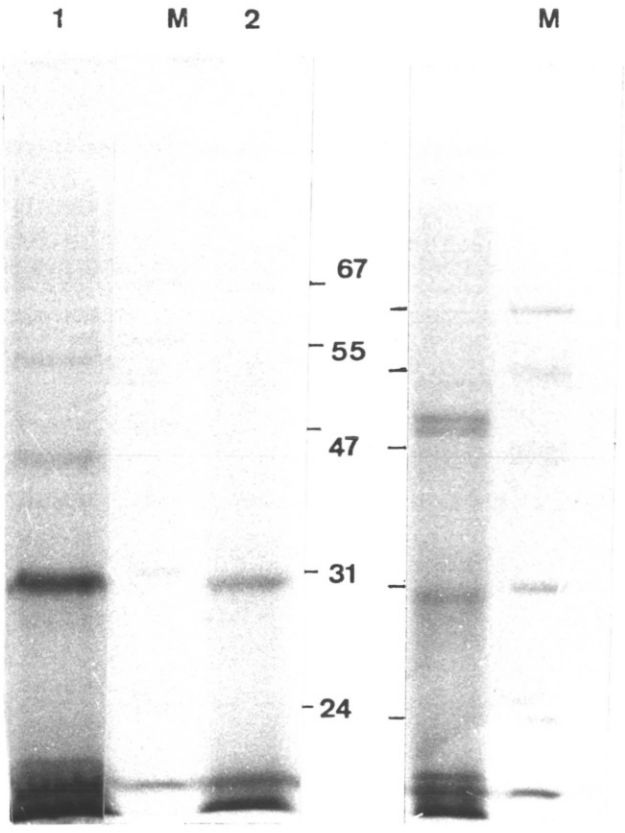


Fig.7a Effects of clarifying and deproteinising methods on RNA yield. **Lane 1**; BaYMV RNA extracted from ultracentrifugation pellets as in 2.11.1. **Lane 2**; As Lane 1 but with phenol/chloroform replacing initial phenol extraction. **Lane 3**; As Lane 1 except RNA was extracted from virus preparations clarified with 0.2vol CCl_4 instead of Triton X-100. RNA equivalent to 0.85g plant material per lane.

Fig.7b Densitometer scan of Fig.7a.



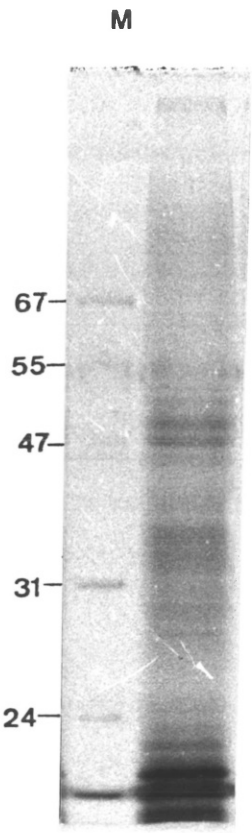


Fig.8 Fig.9 Fig.10

Figs.8-10 5-15% Gradient SDS-PAGE of virus extracts prepared by purification protocols differing only in clarification and concentration methods . Each lane represents extracts from 5g plant tissue

Fig.8 CCl_4-clarified BaYMV preparations after two rounds of (1) ultracentrifugation and (2) PEG-precipitation

Fig.9 Triton X-100-clarified BaYMV preparation after two rounds of PEG-precipitation

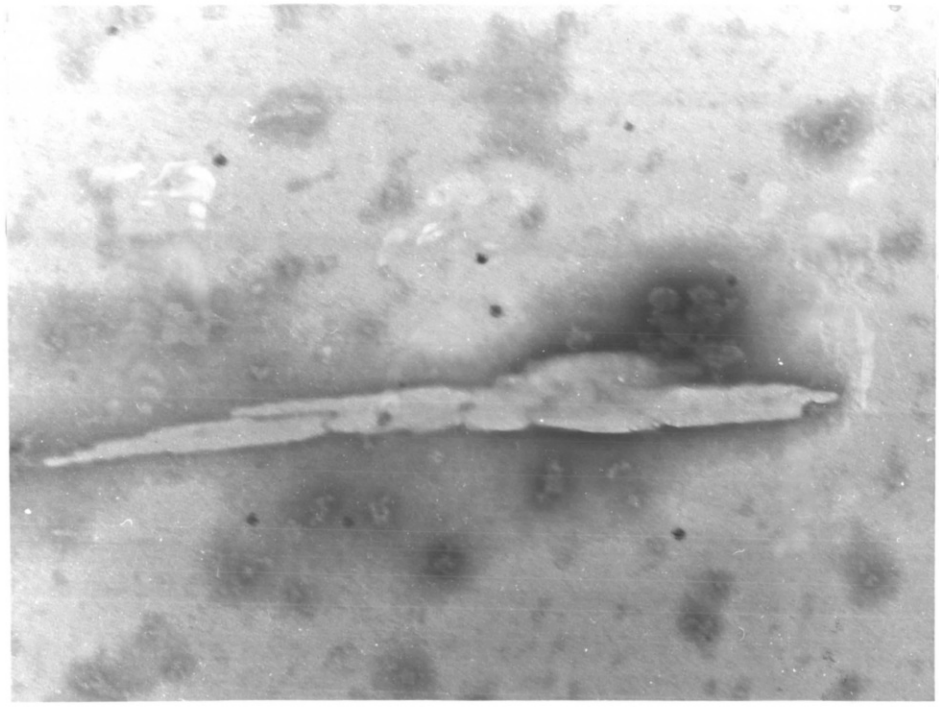
Fig.10 Healthy extract after Triton X-100-clarification and two rounds of PEG-precipitation

Lane M-Marker proteins (molecular weights are indicated)

3.2.4 Concentration

In view of the low levels of BaYMV in barley sap and hence the large volumes usually handled practical considerations favoured the use of PEG-precipitation as a concentration method. Furthermore the low centrifugation forces needed in this method were deemed less likely to be deleterious to the relatively fragile virions than those used in ultracentrifugation. PAGE of virus preparations after two rounds of PEG-precipitation or ultracentrifugation (without a sucrose cushion) showed that the former produced a purer preparation with respect to the 30K protein though yields were lower (see Fig.8). Neither of these preparations contained extractable intact RNAs. EM revealed a high degree of fragmentation in both cases , individual particles nearly all being short (<200nm), and in PEG-purified preparations aggregates were present in which individual particles were hardly distinguishable (see Figs.11a,b). It is probable that larger aggregates of this type would have been lost during low speed centrifugation leading to the lower yields found. Close inspection of these aggregates shows that they consist generally of small fragments. Whether or not the fragmentation is a result of PEG-induced aggregation is not clear but it does explain the absence of intact RNAs. Thus PEG-precipitation does not seem to be suitable for the purification of viral RNA unless conditions can be found that minimise loss through aggregation. The use of HEPES buffer, Triton X-100 clarification and only one round of PEG-precipitation allowed the purification of some intact virions by sucrose-caesium step (Fig.12) and isopycnic gradient centrifugation but yields were too low to be satisfactory. The RNA loaded in track 2 of Fig.13 was extracted from virions purified in this way from 25g infected tissue.

(a)



(b)

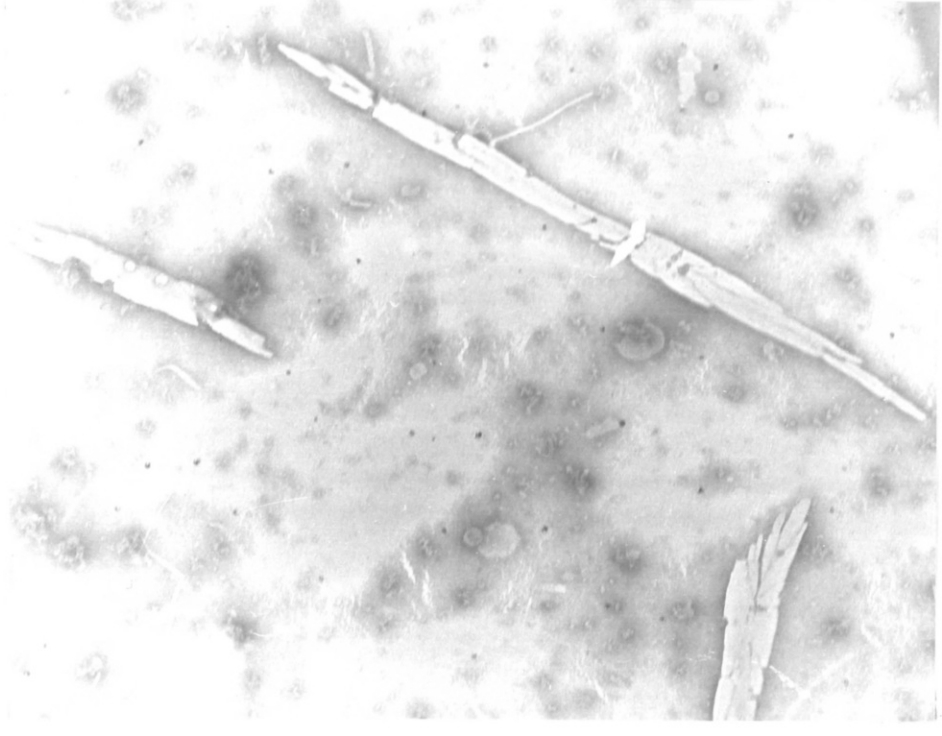


Fig.11 Electron micrographs of BaYMV aggregates after PEG-precipitation. Magnification; (a) ×54,000 (b) ×27,000

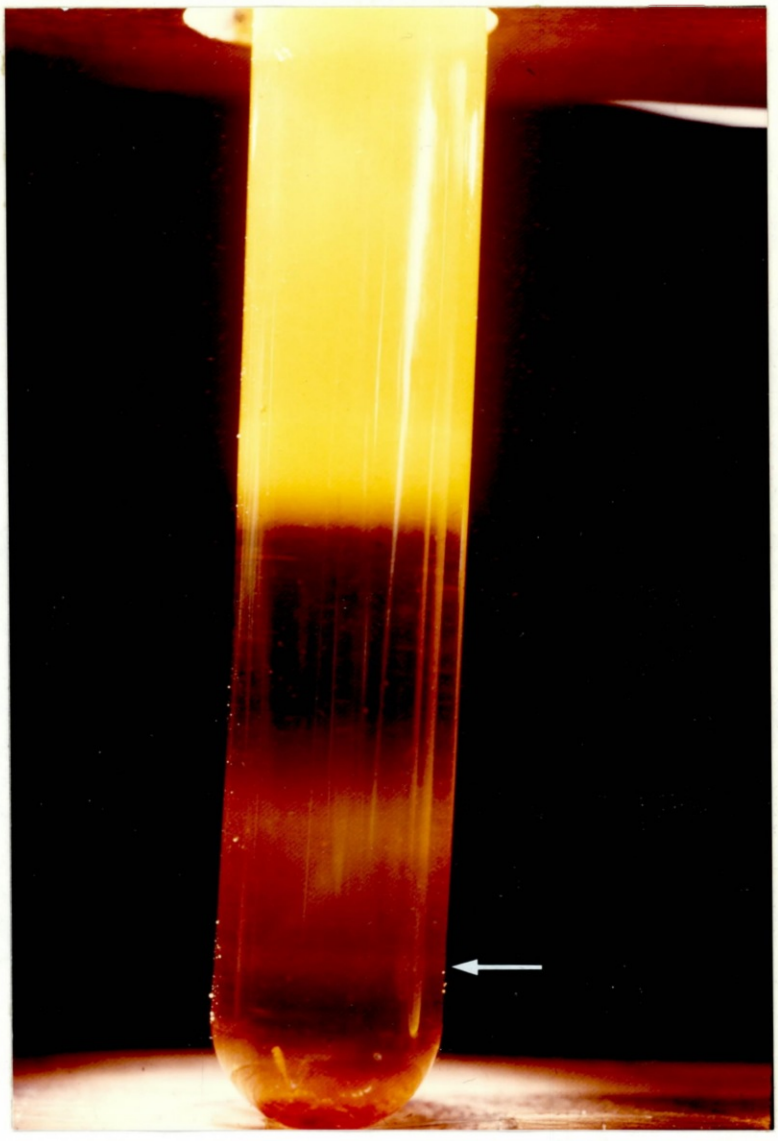


Fig. 12 Sucrose/caesium sulphate step-gradient purification of BaYMV

(Arrow indicates opalescent virus zone)

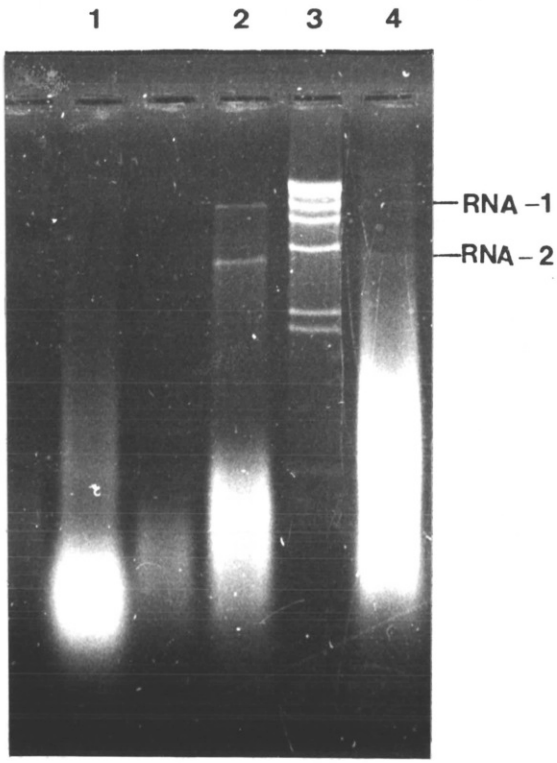


Fig.13 Methylmercury gel electrophoresis of BaYMV RNA extracted from three opalescent zones in an isopycnic caesium sulphate gradient (2.8.7d)

Lane 1; Upper zone

Lane 2; Middle zone

Lane 3; λ *Hind* III digest

Lane 4; Bottom zone

Ultracentrifugation pellets are dense and hard to resuspend and high centrifugal forces result in compaction and mechanical breakage of BaYMV virions. These limitations can be avoided, at least partly, by using a sucrose cushion, which concommitantly adds a crude fractionation step. EM of such resuspended pellets shows that a high proportion of the particles are long (Fig.14) and, although associated with each other and plant material, are not aggregated to the degree of those concentrated by PEG-precipitation. Direct extraction of RNA from these pellets, denaturing gel electrophoresis and estimation of viral RNA concentration from the gel (eg Fig.15) shows that 100-150µg intact RNA is extractable from each 100g plant tissue processed.

Assuming negligible fragmentation (unlikely) and 5% RNA content (INOUYE & SAITO, 1975) pellets must contain in excess of 3mg virus/100g tissue and plants even more. Low yields thus result from manipulations after this step in purification procedures.

3.2.5 Resuspension

Resuspension of the virus pellet after the concentration step is probably the most important stage in the purification of BaYMV in terms of yield loss. Unfortunately no entirely satisfactory method for this was found. No less than 25% and as much as 75% of extractable RNA is found in the pellet of a low speed clearing spin after resuspension. Consequently this centrifugation step was often ommitted prior to fractionation on sucrose-caesium step gradients. However in these cases comparable amounts of extractable RNA sedimented during the low speed centrifugation step necessary to remove particulate matter banding at the same part of the gradient as BaYMV (see 3.2.6 below). Neither of these procedures consistently produced greater yields of purified virus than the other.

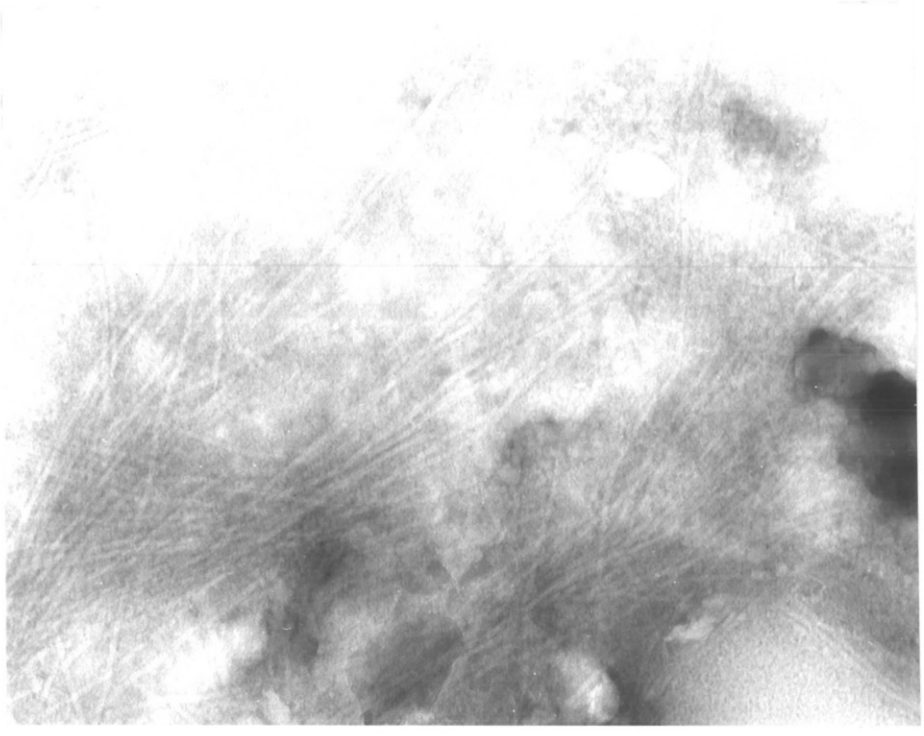


Fig.14 Electron micrograph of BaYMV after ultracentrifugation through a sucrose cushion as in 2.8.8. Magnification; ×65,000

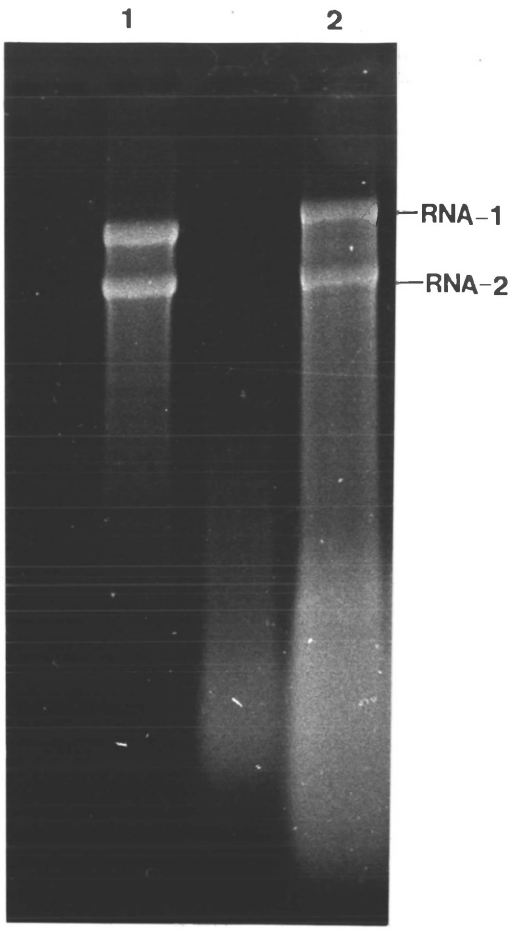


Fig.15 Crude BaYMV RNA extracted from ultracentrifuge pellets as in 2.11.1b

Lane 1; 2μg CPMV RNA

Lane 2; Crude BaYMV RNA equivalent to 2g plant material

By analogy to potyviruses (eg VAN OOSTEN, 1972; THOUVENEL *et al.*, 1976) urea and 2-mercaptoethanol were tested for their ability to solubilise BaYMV particles. Urea at 1M, on its own or in the presence of 0.1% (v/v) 2-mercaptoethanol in fact had the opposite effect causing aggregation resembling that resulting from PEG treatment and concommitant loss of all extractable full-length RNA. Figs.16a, b & c show the effects of incubation overnight at 4°C in 0.1M tris-HCl, pH8.0, with no additives, in the presence of 1M urea and with both 1M urea and 0.1% 2-mercaptoethanol respectively. Aggregation is apparent in the last two. Alone, 2-mercaptoethanol had no effect on aggregation or RNA state above control levels and was ommitted from resuspension buffers.

Triton X-100 at 0.1% and EDTA at 1mM were included in resuspension buffers to aid dissociation of virus from any remaining membranous host components (pellets were sometimes pale green).

3.2.6 Further Purification

a) Sucrose Gradient Centrifugation

Sucrose gradient centrifugation of virus preparations after one or two concentration steps did not result in any further purification, only causing dilution which thereby necessitated another round of ultracentrifugation or PEG-precipitation. Uv profiles of gradients showed no distinct peaks and EM of fractions revealed that virus was dispersed throughout the gradient and not separated from any but the slowest sedimenting host components. This effect has also been found in another study (HUTH *et al.*, 1984) and probably reflects the heterogeneous sedimentation properties caused by aggregation and fragmentation.

(a)

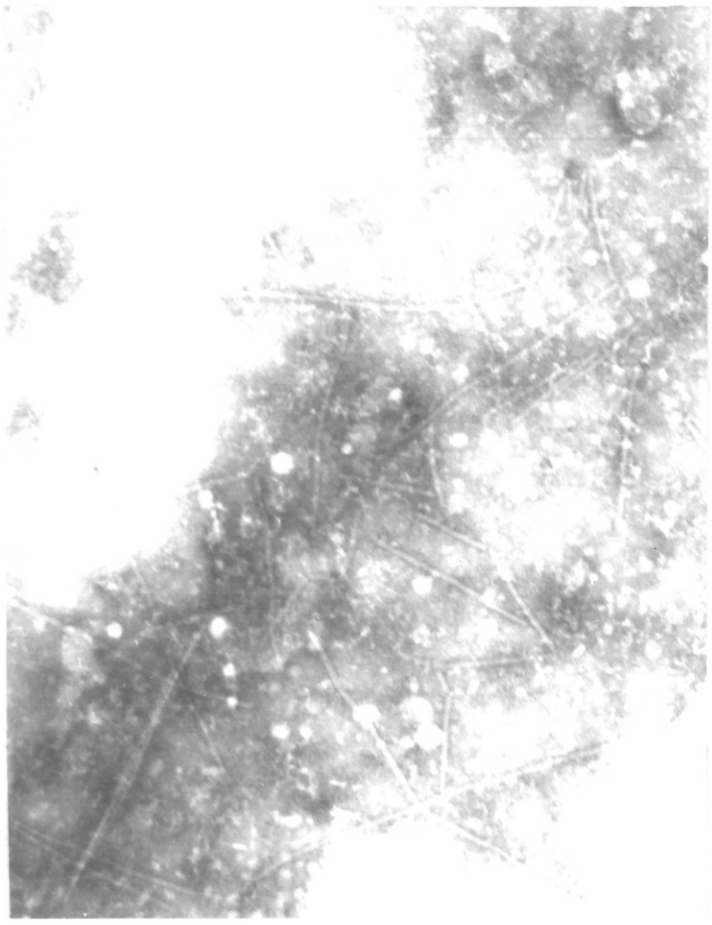
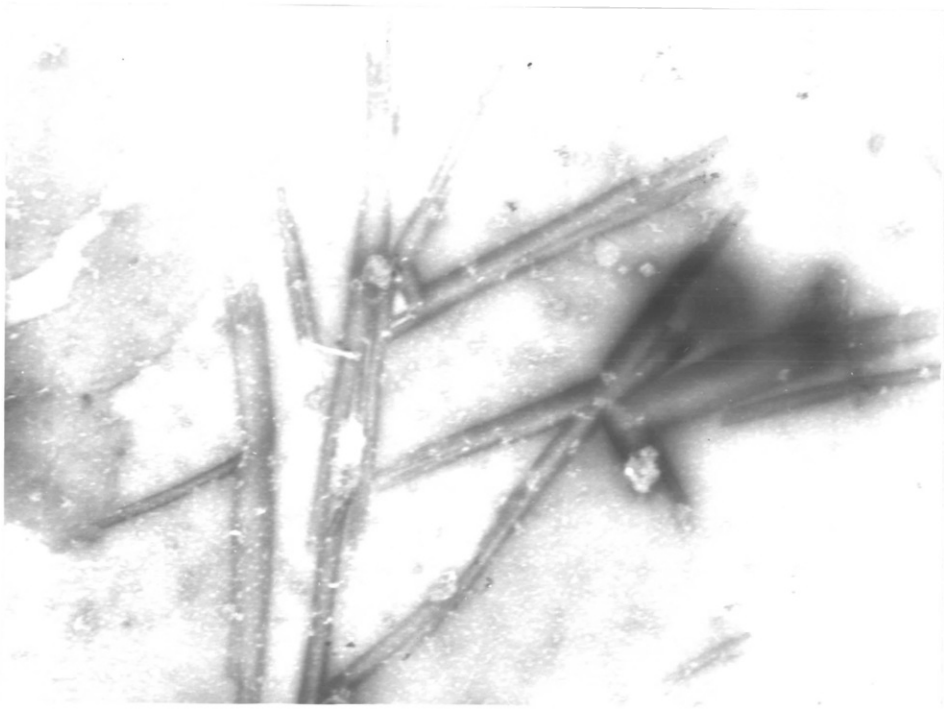


Fig.16 Effects of urea and 2-mercaptoethanol on BaYMV aggregation. Electron micrographs of BaYMV after resuspension of ultracentrifugation pellets in tris-HCl, pH8.0, and incubation at 4°C overnight (a) with no additives, (b) in the presence of 1M urea and (c) in the presence of 1M urea and 0.1% 2-mercaptoethanol.
Magnification; (a) ×54,000 (b) ×10,000 (c) ×27,000

(b)

(c)



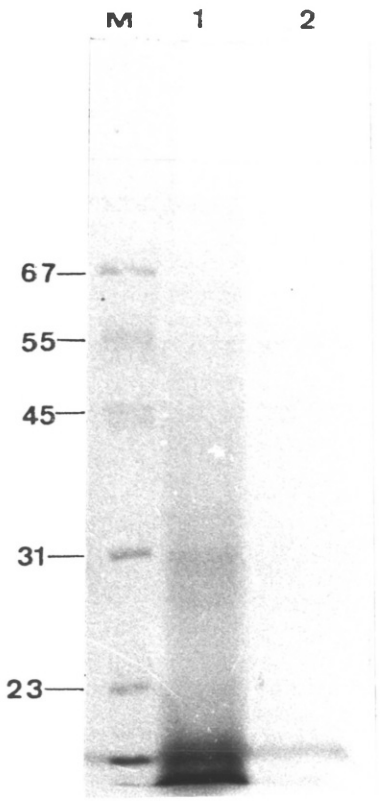


Fig. 17 5-15% gradient SDS-PAGE of CPG-column eluates. First (1) and second (2) uv-absorbing peaks produced by CPG-column chromatography of BaYMV, prepared by CCl_4-clarification and one round of ultracentrifugation from 10g plant material/lane.

Lane M - Marker proteins (molecular weights are indicated)

b) CPG Column Chromatography

Permeation chromatography on controlled pore glass columns was carried out using virus preparations after one and sometimes two rounds of concentration. Samples clarified with CCl_4 and concentrated once produced two uv absorbing peaks of varying sizes though the first was always equal in size to or larger than the second. After a second round of concentration only one peak, the first, eluted. Samples clarified with Triton X-100 only ever produced one peak. This was true for samples from healthy and infected plants. EM showed that, while no or very few virus particles were found in second peak eluates, first peak eluates always contained plant material as well as virions. SDS-PAGE of the two eluates confirmed this, showing partial purification of the 30K protein in the first peak & its removal from the second (Fig.17). No attempt was made to characterise the host component(s) excluded from the column so it is possible that modifications of the protocol could lead to its removal at an earlier stage and hence allow virus purification on the column. However CPG eluates never contained extractable full-length RNA. These results in conjunction with the large dilution effect of the column led to the abandonment of this purification method.

c) Caesium Gradients

Purification methods using centrifugation in caesium salts proved the most successful but suffered from three main drawbacks. The first is that the virus is not very stable in high salt concentrations for long periods. This effect is greater in CsCl than Cs_2SO_4. After overnight incubation at 4°C in 28% (w/w) CsCl partially purified virus preparations contained no extractable RNA whereas after a similar incubation in Cs_2SO_4 about 10% of the

control amount (incubated in buffer alone) remained. A similar salt sensitivity was reported by USUGI & SAITO (1975) who found that incubation in CsCl for two days completely destroyed infectivity. The greater effect of CsCl is probably due to the fact that the chloride ion is more chaotropic than than the sulphate ion. Differential stability in these two salts has been reported for other viruses (HULL, 1976). Losses can be minimised by the use of step gradients which can be run for much shorter times than isopycnic gradients since the gradient is partially formed and does not need to reach equilibrium to achieve adequate separation. Initially gradients were formed by overlaying 3ml each 20% sucrose, Cs_2SO_4 of density 1.2g/cm^3 and Cs_2SO_4 of density 1.35g/cm^3 and centrifugation was carried out for 5h. Fig. 12 shows the result of such a run, the orange colouration of the upper half of the tube being a characteristic of preparations isolated using partially chlorotic/necrotic tissue and PEG-precipitation. It is apparent from the photograph that the opalescent virus containing zone is not well separated from host proteins so contamination is a problem on fractionation. Virus must be further purified by isopycnic gradient centrifugation as in 2.8.7d with its concomitant yield losses. Subsequently four Cs_2SO_4 steps, of densities 1.0, 1.1, 1.2 and 1.3g/cm^3, were used to shorten run times and the steps of density above and below that of the virus were increased in size to improve separation. An example of such a gradient is shown in Fig.18. The final improvement was to include a fifth step of density 1.5g/cm^3 to raise the opalescent band off the bottom of the tube and thus make its removal simpler. Sometimes the virus zone had a flocculent as well as opalescent appearance though this did not affect particle integrity or purity. The reason for this is not understood but on dilution and dialysis the flocculence disappeared.

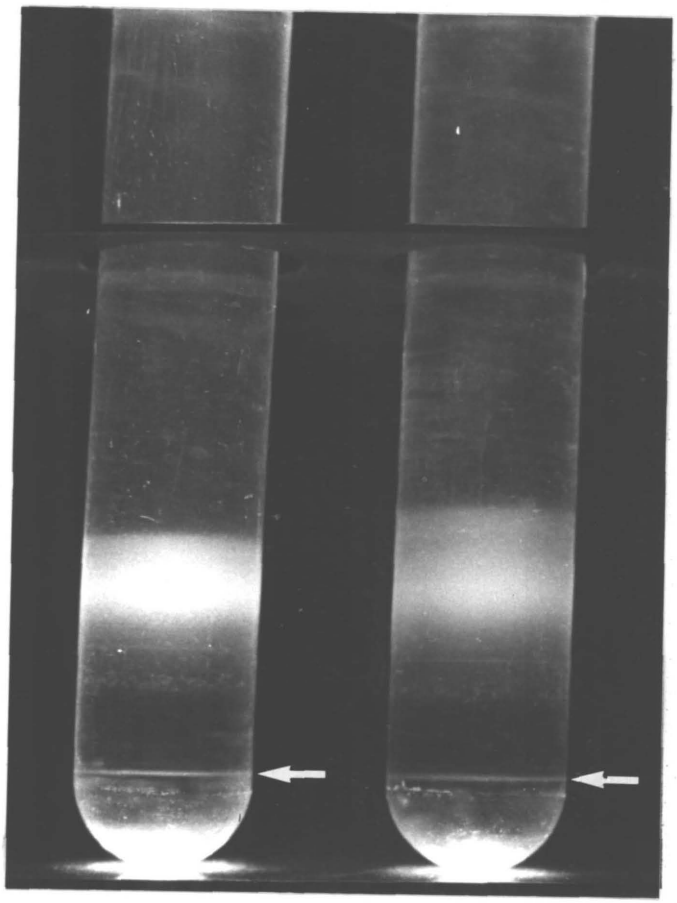


Fig. 18 Sucrose/caesium sulphate step-gradient purification of BaYMV

(Arrows indicate opalescent virus zones)

The other two shortcomings of this method are connected and are involved with insolubility and contamination. Virus purified by caesium gradients is characteristically in a fairly aggregated state, probably as a simple result of high concentration. The largest of these aggregates tend to be insoluble. This would not be a problem if the virus was pure as soluble RNA and capsid protein could be extracted by denaturation of insoluble aggregates. However, in many preparations plant derived particulate matter banded in the same part of the gradient as virus. As detailed in 2.8.8 this could be removed by dilution and low speed centrifugation and the virus repurified on another step gradient but insoluble aggregates are lost during the clearing spin. The nature and origin of the contamination is unknown but it is possibly formed by interaction between the high salt concentration and plant proteins such as ferritin or other compounds such as polyphenols. The occurrence of the contamination did not correlate with the age or chlorotic/necrotic state of the starting material as suggested by HUTH *et al.*, (1984). It remains to be seen whether steps can be taken to remove the contamination at an earlier stage.

3.2.7 BaYMV Coat Protein and Encapsidated RNAs

Contrary to other reports (HUTH *et al.*, 1984, EHLERS & PAUL, 1986) only one protein, of ca.30K, was consistently detected by SDS-PAGE of purified virus preparations (see eg Fig.19). No evidence of the 35K protein reported by the German workers was found. This may or may not be due to proteolytic degradation but in this study the 30K protein will be referred to as BaYMV coat or capsid protein.

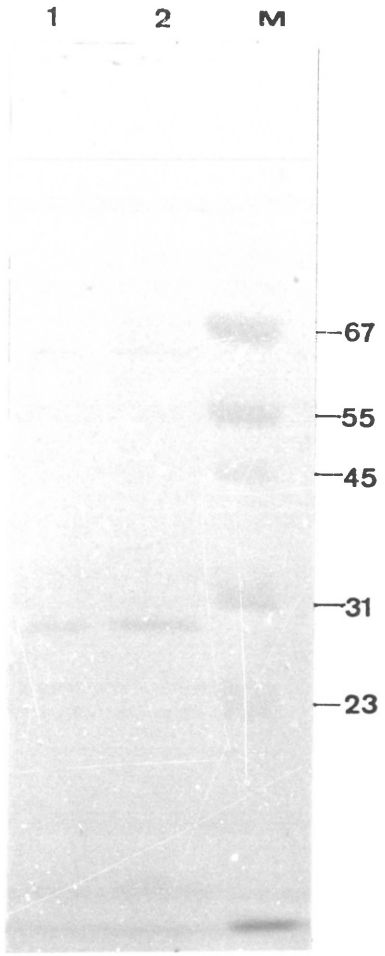


Fig. 19 SDS-PAGE of BaYMV purified as in 2.8.8 from (1) field-infected plants from Little Clacton and (2) mechanically-inoculated plants

M - Marker proteins (molecular weights are indicated)

In agreement with an earlier report (HUTH *et al.*, 1984) two RNA species, of molecular weights 2.7-8 and 1.3-4 × 10^6, were consistently found in virus preparations (see eg Fig.13). These will be referred to as BaYMV RNA-1 and RNA-2 in this study. There is no evidence that these two RNAs are sufficient or necessary for infection (see 3.1(b) above).

3.2.7 Summary

The purification protocol presented in 2.8.8 was developed independently of published protocols and differs from them in choice of buffer and additives, method of clarification and form of final purification step. Choice of buffer and method of clarification have been shown to be superior in terms of yield of intact particles from which full-length RNA could be extracted. Sucrose/caesium sulphate step gradients have the advantage over isopycnic gradients of shorter run times thus reducing the deleterious effects of high salt concentrations which are further ameliorated by the use of sulphate rather than chloride salts of caesium. Numerous attempts to purify BaYMV using published procedures never yielded intact virus particles from which full-length RNA could be extracted.

The virus appears to have no requirement for Mg^{++} ions. This allows the use of EDTA at levels that ensure disruption of ribosomes thus preventing their co-sedimentation on centrifugation. After RNA extraction of ultracentrifugation pellets ribosomal RNA would obscure viral RNA bands on gels and prevent sucrose gradient purification on account of the huge excess of rRNA and the rough equivalence in size of 18S rRNA and BaYMV RNA-2.

Greatest yield losses occur at all concentration steps because of the resultant insolubility of large aggregates. Any method of redispersal of such aggregates, physical or chemical, would greatly increase yields. Yields of virus at

the stage of the ultracentrifugation pellet could be as high as 3mg/100g plant tissue but after resuspension and one step gradient yields were between 0.4 and 0.8mg/100g. If two step gradients were required yields were rarely above 100µg/100g. The proportion of intact particles also decreased with each stage so that yields of intact RNA were in the order of 100, 10-20 and 0.5-5µg/100g tissue for the same stages. Characterisation of the particulate matter that obscures virus zones in step gradients and its removal at an earlier stage would ensure consistent and reasonable yields.

Caesium gradient centrifugation provided no evidence of different strains such as that reported by HUTH *et al.*, (1984). Occasionally more than one opalescent zone was seen in isopycnic gradients but these seem to have been due to empty or RNA-depleted particles (zones of lower density) or an association with host DNA (zones of greater density). Fig.13 shows the nucleic acid extracted from three such opalescent zones. RNA extracted from the upper zone (Lane 1) is completely degraded, that from the middle zone (Lane 2) contains full-length viral RNA and that from the lower zone (Lane 4) is associated with a large DNA species which was not degraded on prolonged *in situ* RNase digestion. Free DNA would have pelleted under centrifugation conditions used so a fairly stable interaction is implied between virus and DNA leading to an intermediate buoyant density. The existence of strains is not, however, eliminated by these findings.

3.3 Electron Microscopy

Electron microscopy was found to be a useful tool in diagnosis of infection (3.1a) and monitoring purification protocols (2.8.1). In addition, information was obtained about particle morphology and cellular location which may be relevant to virus classification.

Negatively stained leaf-dip preparations revealed some loose side-by-side aggregation (Fig.20a,b) but generally particles were not strongly associated with each other or cellular components. This was true even for preparations that were not microcentrifuged briefly before they were applied to the grids and implies that the phenomenon of massive aggregation (Fig.21) is caused by some aspect(s) of the purification method; most probably concentration and removal of the structured cellular environment. End-to-end aggregation was also apparent (Fig.22) though this is more likely to interfere with modal length measurements than purification. In some micrographs it was possible to discern particle polarity and a faint central canal which was more obvious at the concave end (Figs.22). More efficient penetration of stain here may be due to partial stripping of particles and/or loss of RNA. Correlation of particle polarity with RNA polarity will depend on assembly/disassembly experiments such as in BUTLER (1974) and PERHAM & WILSON (1976). It is not possible to speculate on intermolecular forces between adjacent capsid proteins further than to say that cation salt bridges between carboxyl groups are not an absolute requirement for particle stability since EDTA at 5mM does not cause disruption.

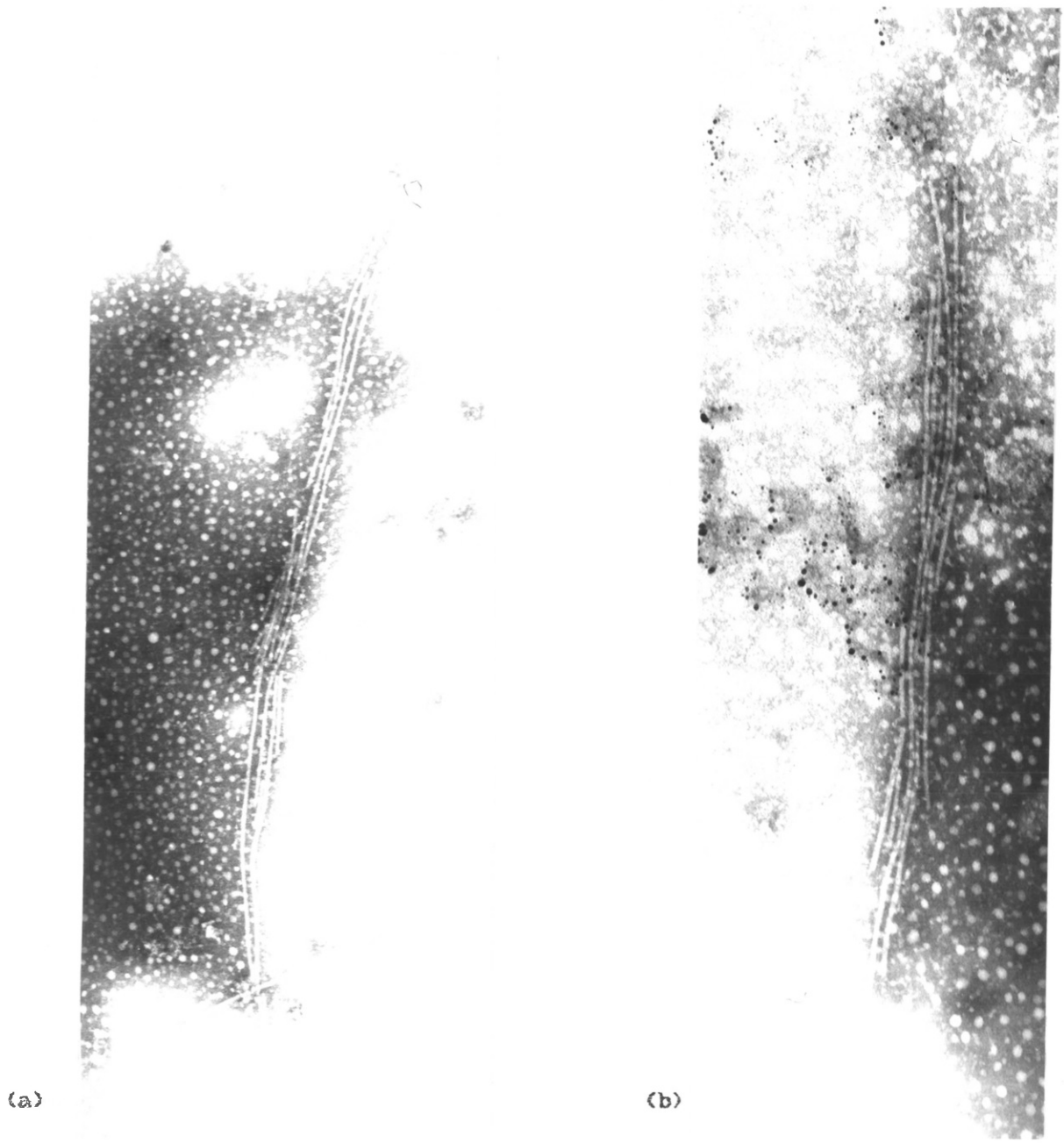


Fig.20 Electron micrographs of leaf-dip preparations of BaYMV showing side-to-side association
Magnification; (a) ×44,000 (b) ×56,000

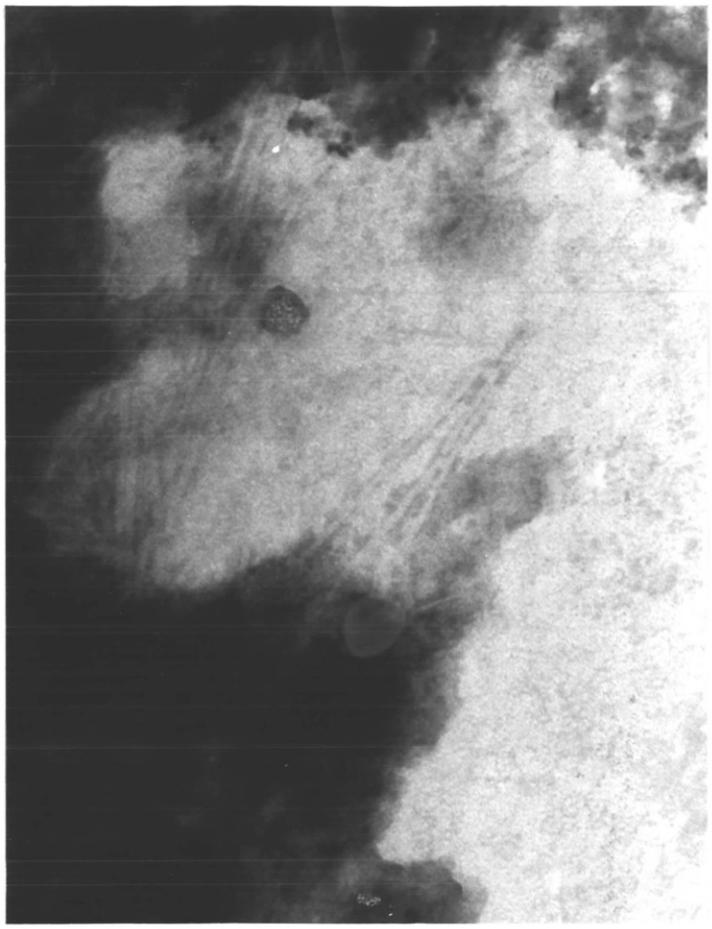


Fig.21 Electron micrograph of the edge of a massive aggregate of BaYMV resuspended from a clearing centrifugation step to remove insoluble material in a sucrose/caesium sulphate step-gradient (×100,000)

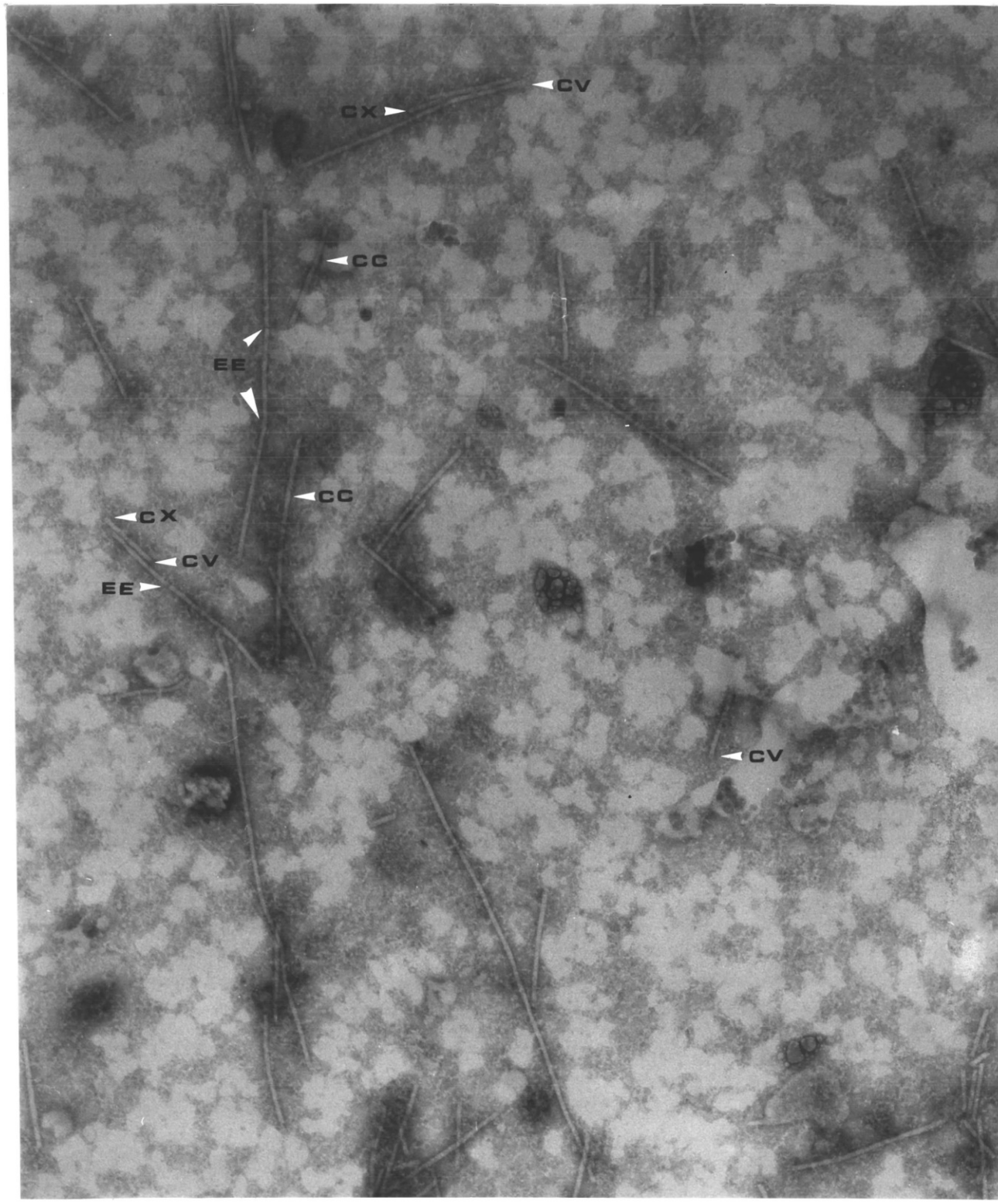


Fig.22 Electron micrograph of BaYMV prepared by leaf-dip ISEM (×60,000). Examples of end-to-end association (EE), a faint central canal (CC) and concave (CV) and convex (CX) ends are indicated.

Micrographs of thin-sections of both mechanically-inoculated and field-infected plants (Fig.23a-f) revealed the large filamentous aggregates and pinwheel inclusions also reported by HUTH *et al.* (1984) and MAROQUIN *et al.* (1982) though the latter were less well formed and association with ER was not apparent. This could be due to the angle of sectioning although the different appearences of filamentous aggregates in Fig.23b and Fig.23e indicate that more than one angle was viewed. Differences in inclusion morphology could reflect infection stage or environmental influences, factors that could also explain the absence of "chain-mail" membranous structures found by other authors (HUTH *et al.* (1984) and MAROQUIN *et al.* (1982). No evidence of scroll formation was found here or in the other studies.

Pinwheel inclusions are typical of the potyvirus group but not diagnostic unless in combination with other properties such as transmissibility in a non-persistent manner by aphids (HOLLINGS & BRUNT, 1981). However, HUTH *et al.* (1984) noted that by comparison with potyvirus inclusions the presence of pinwheels but absence of scrolls would put BaYMV in subgroup II (as described by EDWARDSON, 1974). Studies of sequential developement of potyviral inclusions (LAWSON *et al.*, 1971) showed that they change in size, shape, number and location during the infection and at a fairly late stage incomplete inclusions predominate over intact pinwheels. Again by comparison this would explain differences in inclusion morphology found in this study and by HUTH *et al.* (1984) and MAROQUIN *et al.* (1982).

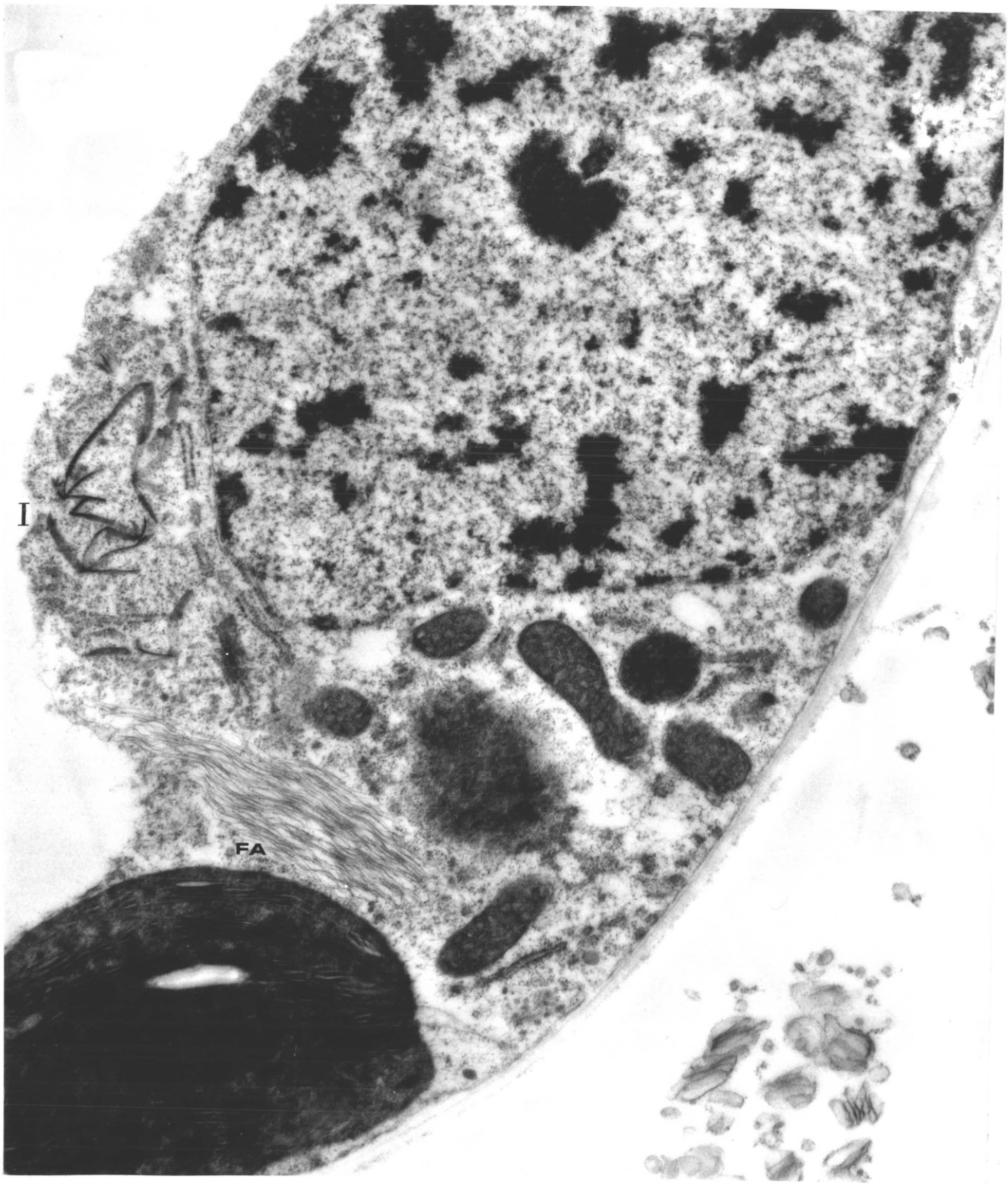


Fig.23a (×21,000)

Fig.23a-f Electron micrographs of thin sections of barley naturally infected (a-c) and mechanically-inoculated (d-f) with BaYMV
Filamentous aggregates (FA) and inclusions (I) are indicated.
(Thin-section micrographs courtesy of K.Plaskitt, John Innes Institute)

Fig. 23b (×70,000)

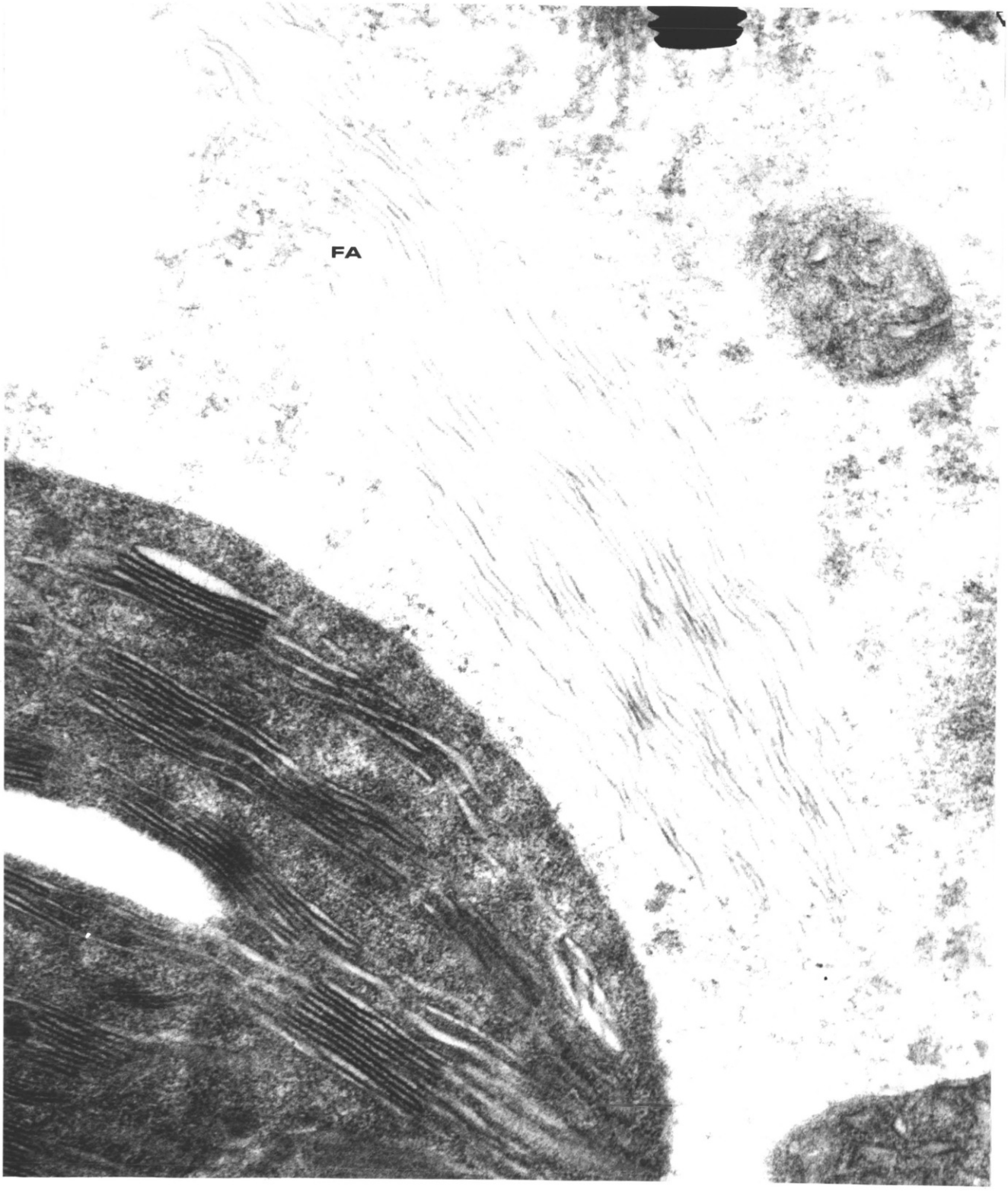


Fig.23c (×70,000)

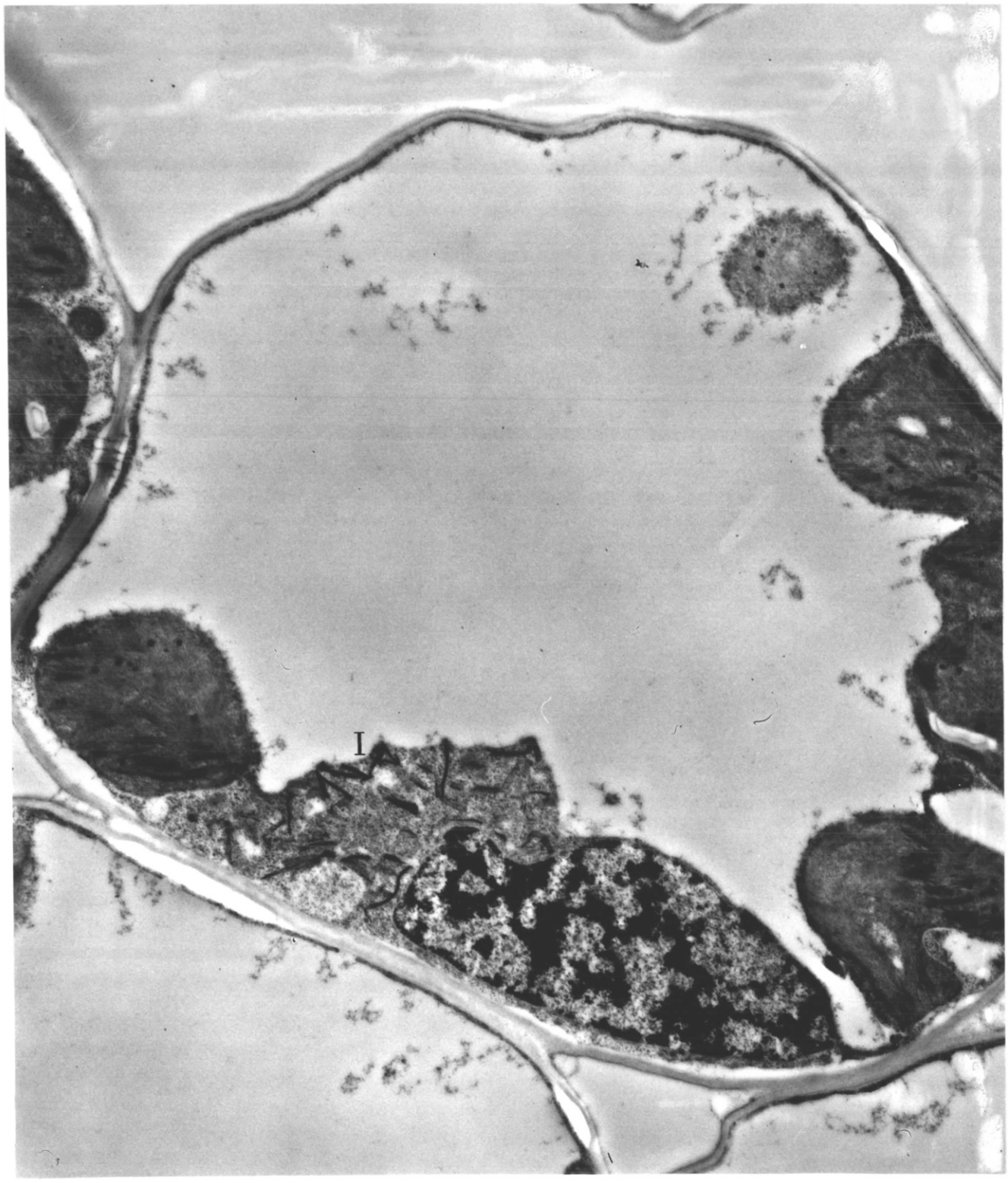


Fig.23d (×10,500)

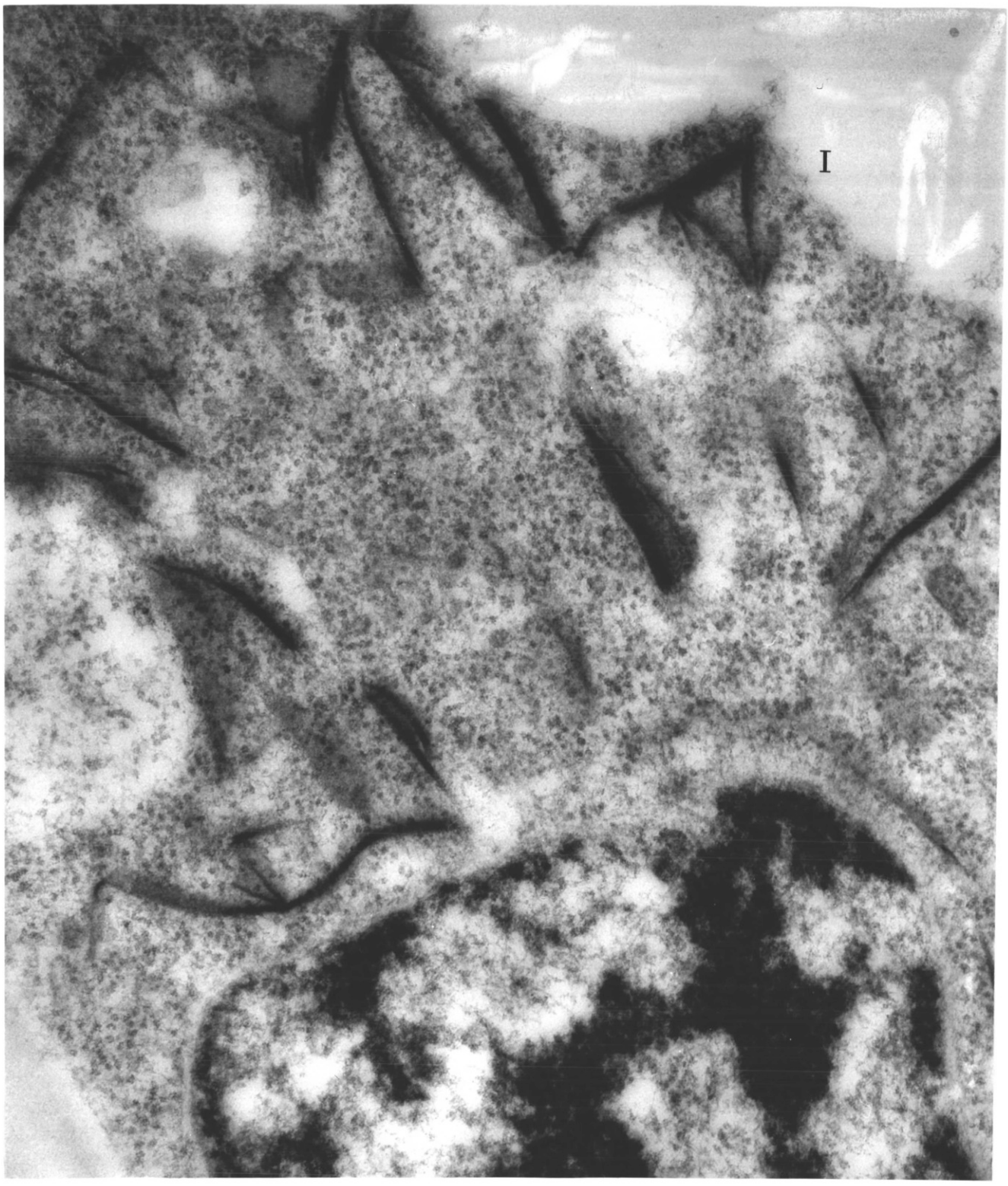


Fig.23e (×70,000)

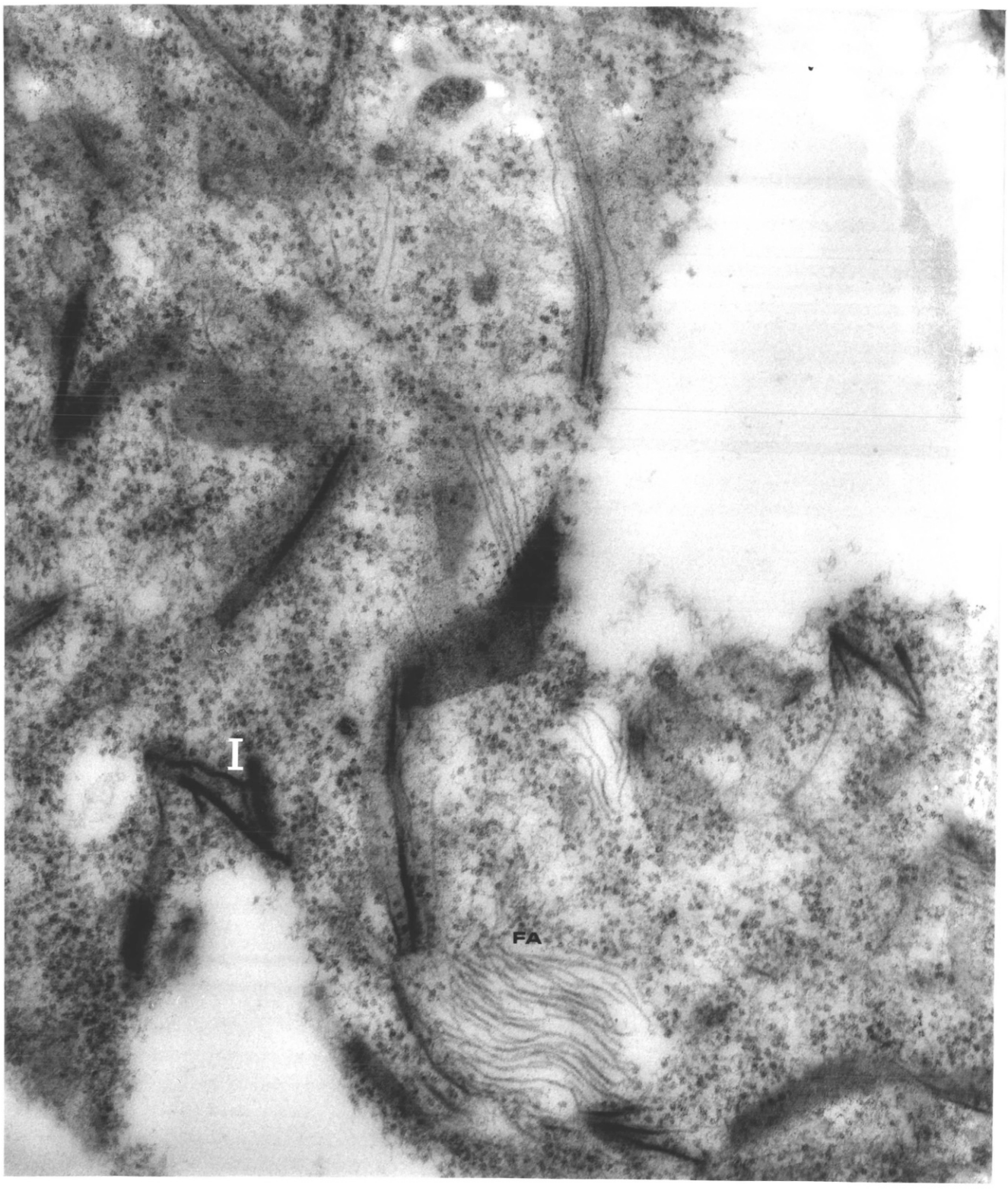


Fig.23f (×70,000)

3.4 Serology

Using grids coated with antiserum raised to a mixture of the M and NM BaYMV strains, ISEM revealed a close serological relationship between at least one of the German isolates and at least one strain from the Little Clacton site. Virus particles were adsorbed from sap of plants infected with BaYMV-M/NM (provided by W.Huth), plants infected naturally at Little Clacton and plants infected by mechanical inoculation. In decoration tests all particles reacted positively. Because of the nature of the antiserum, it is only possible to establish a serological relationship between one German strain and a sap-transmissible English strain. No evidence for the existence of strains in England was provided. However if the absence of a 35K protein in virus preparations is a result of coat protein lability then the Little Clacton isolate is probably reacting with antibodies to BaYMV-NM. Its sap-transmissibility would then indicate a closer relationship with BaYMV-So. Further studies using BaYMV-So antisera would be interesting.

All double-diffusion tests proved unsuccessful. No precipitin lines were seen in agar plates irrespective of antiserum concentration or virus pretreatment. The most probable explanation of this result is that intact particles did not diffuse sufficiently through the gel to be detected by this method and that capsid protein denatured by SDS or SDOC was not recognised by the antiserum which was raised to native virions (HUTH, 1984). Virions disrupted by sonication or repeated freezing and thawing might not have possessed uniform diffusion characteristics. Whatever the explanation it appears that the double-diffusion method is not suitable for the investigation of serological relationships of BaYMV nor as a means of detection in the absence of an electron microscopic or other sensitive techniques such as ELISA or dot-blot hybridisation.

3.5 BaYMV Single-Stranded RNA

3.5.1 Extraction from virions

Using the crude RNA extraction assay described in 2.11.1 two standard RNA extraction methods were compared and the more successful one optimised. The guanidinium method yielded no full-length BaYMV RNA on any occasion indicating that either virions were not being disrupted or that the released RNA was not stable under the conditions employed. This method was therefore abandoned. In contrast virus disruption in the presence of 1% SDS did yield intact RNA. The inclusion of bentonite in the disruption buffer (as used in extraction of potyviral RNA, BRAKKE & VAN PELT, 1970) did not appreciably increase yields whereas the use of proteinase K resulted in two-fold increases. This enzyme concomitantly afforded protection against RNase activity and was found by SDS-PAGE and Coomassie Blue staining to digest all proteins present in these extracts. Brief incubation of crude virus samples with RNase A prior to disruption, attempted with a view to digesting unencapsidated host RNA and hence isolating viral RNA in a purer form, resulted in degradation of viral RNA as well. Thus the virion structure seems to be 'loose' enough to allow penetration of small proteins at least at some sites. This may in part explain the instability of BaYMV infectivity in extracts of plants. These results are summarised in Fig.24. Disruption buffer containing SDS, bentonite and proteinase K gave greatest yields of full-length RNA so was used thereafter.

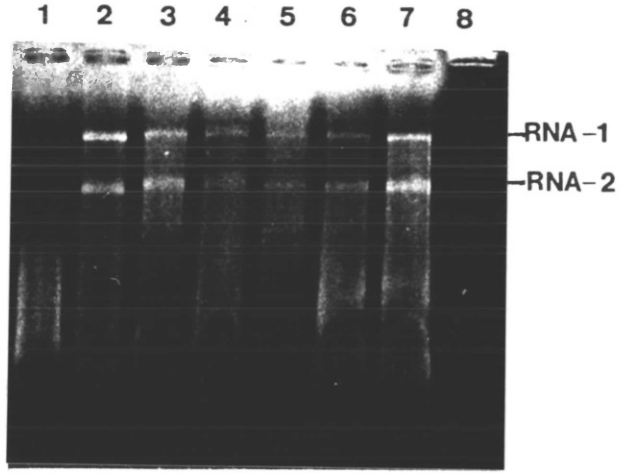


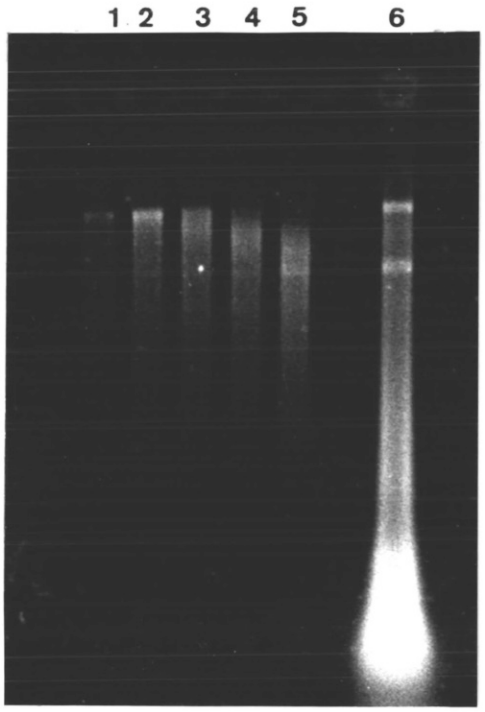
Fig.24 Methylmercury gel showing effects of different RNA extraction methods on BaYMV RNA yield. Each lane contains RNA from equivalent to 2g healthy (Lane 1) or BaYMV-infected (Lanes 2-8) plants. RNA extraction was as described in 2.11.1b except Lanes 3 & 7 (bentonite omitted), Lanes 4 & 5 (proteinase K omitted) and Lane 6 (bentonite and proteinase K omitted). Lane 8 shows the effect of incubating the crude virus sample with RNase A (0.5µg/ml) for 5min prior to RNA extraction.

Phenol extraction was found to be an effective means of deproteinising samples in contrast to results for some potyviruses (HARI *et al.*, 1979). Disruption of virions followed by sucrose gradient purification with or without a prior phenol extraction resulted in equivalent yields. Use of phenol/chloroform in place of phenol for the first extraction did not affect yields either (Fig.7, Lanes 1 & 2) which indicates that BaYMV RNAs do not have long polyadenylate tracts . For practical reasons an initial extraction with phenol alone was preferred since, being less dense than the solvent mixture, it allowed the sedimentation of bentonite and some other insoluble matter which otherwise formed a thick interface between aqueous and phenol/chloroform phases.

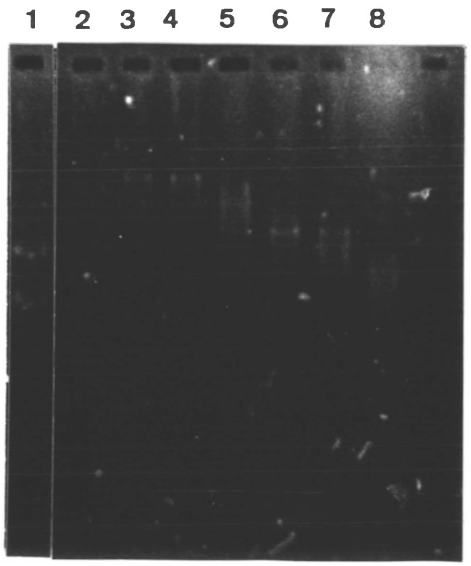
As mentioned above (3.2.8) yields of intact RNA from caesium gradient purified virus preparations rarely reached 15µg and were often as low as 0.5-5µg/100g tissue extracted. Yields in crude samples, however, could be as high as 100µg/100g. Consequently methods were developed to purify BaYMV RNA from these crude mixtures. BaYMV RNA-1 is the largest RNA species found in these mixtures (see eg. Figs.7, 15) and equivalent extracts from healthy plants apparently contained no RNAs as large as RNA-2 so BaYMV RNAs could potentially be purified by sucrose gradient centrifugation. In practice only RNA-1 could be obtained essentially pure (Fig.25a-d), RNA-2 fractions sometimes having contaminating fragments derived from RNA-1 degradation. However, radiolabelled probes made from mixtures of these fractions did not usually hybridise to plant RNA. The major disadvantage to this method is that because of loading constraints of the sucrose column the fractions contained RNA at concentrations of about 10µg/ml and the inefficiency of ethanol precipitation from sucrose led to yields of about 20%. However, its simplicity and reproducibility relative to virus purification make this a preferable RNA purification technique.

Fig.25.1a-d Methylmercury gels of BaYMV RNA fractionated on sucrose gradients as described in 2.12.1.

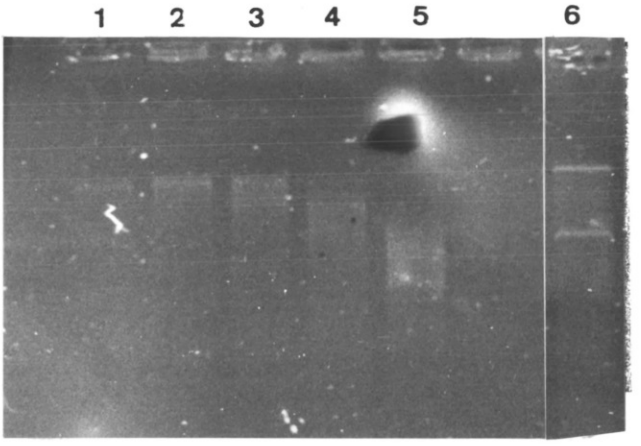
(a) Lanes 1-5; Fractions 7-11
Lane 6; Crude BaYMV RNA



(b) Lane 1; *E.coli* rRNA
Lanes 2-8 Fractions 4-10

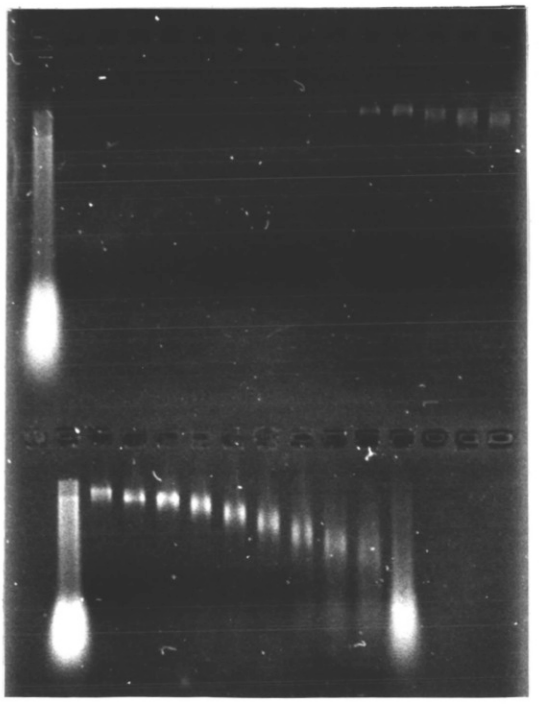


(c) Lanes 1-5; Fractions 5-9
Lane 6; Crude BaYMV RNA



(d) Fractions across an entire gradient

Top half of gel;
crude BaYMV RNA & fractions 1-14
Bottom half of gel;
crude BaYMV RNA & fractions 15-24



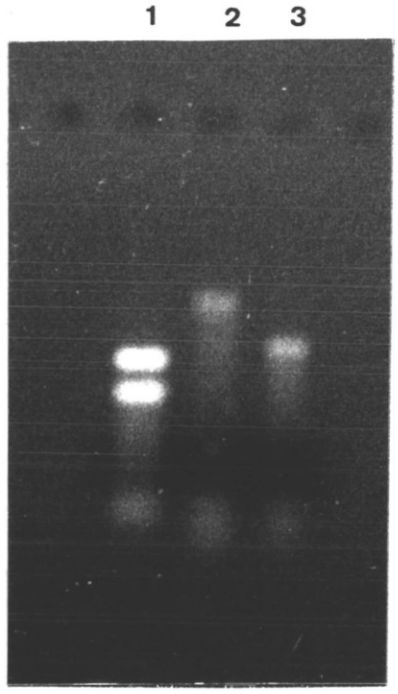


Fig.25.2 Methylmercury gel of BaYMV RNAs purified from gels as described in 2.11.7c.

Lane 1; *E.coli* rRNA

Lane 2; BaYMV RNA-1

Lane 3; BaYMV RNA-2

All gel elution methods were useful for RNA purification though each had its disadvantages. The freeze/thaw method produced relatively intact RNA (Fig.26) but with low yield (≃10%); electroelution gave higher yields (20-30%) but was prone to RNase contamination which sometimes led to loss of all RNA; extraction from low melting point agarose at 65°C gave best yields, approaching quantitative, with a small degree of degradation but suffered from greater agarose contamination which inhibited enzymes used in *in vitro* reactions. DEAE-cellulose chromatography removed these contaminants but losses were high (50-80%) for μg quantities of RNA. The simplicity of these methods again make them preferable to virus purification for RNA isolation.

3.5.2 RNA Structure & Sequence Homology

The absence of a poly(A) tail on BaYMV RNAs, suggested by RNA extraction data (3.5.1), was further indicated by the inability of oligo-dT to prime cDNA synthesis in a reverse transcriptase reaction under conditions in which CPMV but not TMV cDNA synthesis was primed. Furthermore, BaYMV RNA did not bind to oligo-dT-cellulose columns. These results are also incompatible with the presence of an internal polyadenylate sequence such as that found in BSMV RNA.

Scarcity of RNA precluded any further investigations of structures such as VPgs, caps and tRNA-like sequences at 5' and 3' ends.

The possibility of RNA-2 being a subgenomic of RNA-1 was eliminated by Northern blotting of crude BaYMV RNA and hybridisation with cDNA from gel purified RNAs. Fig.26 demonstrates that there is no extensive sequence homology between RNA-1 and RNA-2, though short common sequences may exist.

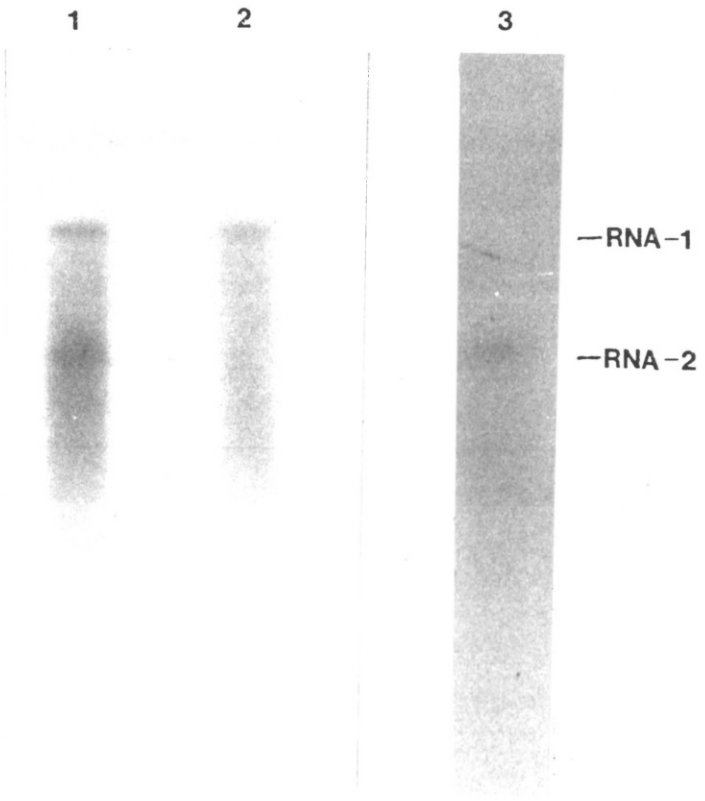


Fig.26 Investigation of RNA-1 and -2 cross-hybridisation. Crude BaYMV RNA, after methylmercury gel electrophoresis, was transferred to GeneScreen *Plus*™ and hybridised to cDNA from gel purified RNAs.

Lane 1; cDNA from a mixture of RNA-1 & -2

Lane 2; cDNA from RNA-1 alone

Lane 3; cDNA from RNA-2 alone

3.5.3 Single-Stranded RNA in Plants

BaYMV encapsidated and unencapsidated RNA constitutes less than 0.5% total plant RNA in infected tissue so cannot be detected by electrophoresis and ethidium bromide staining. Theoretically detectable levels are masked by the extreme overloading of plant RNAs, chiefly rRNAs. Figs.27a & b show agarose gels of glyoxalated and non-denatured total plant RNA from healthy and infected plants, between which no differences are apparent. Higher levels of loading result in smearing which would obscure any minor bands (cf. Lane 5, Fig.27b).

Selection of polyadenylated RNA does not lead to enrichment for viral RNA bands. This is in agreement with the results in 3.5.2.

Polysomal RNA is also chiefly rRNA so no viral messenger RNA can be detected by ethidium bromide staining (Fig.28a, Lane 3, Fig.28b, Lane 2). However, viral RNAs can be detected in polysomal RNA preparations by denaturing agarose electrophoresis, Northern transfer and hybridisation with BaYMV RNA specific probes. Autoradiographs of Northern blots of these gels (Lane 7, Fig.28a and Lane 4, Fig.28b) show essentially the same features: BaYMV RNAs-1 & -2 and two other minor bands. RNA-2 comigrates with the 23S rRNA band (both have Mr $\simeq 1.35\times10^{6}$) so has a bubble-like appearance (see below for evidence that this is not two bands). Both minor bands comigrate with ribosomal RNA bands so may be artefactual (cf. 1.11). If not these could represent subgenomic RNAs of Mr $\simeq 0.7$ & 0.38×10^{6}. The first blot (Fig.28a, Lanes 7 & 8) was stripped of probe and rehybridised with cDNA made to a mixture of BaYMV and host RNA template (Fig.28a, Lanes 9 & 10). Comparison shows comigration of RNA-2 with 23S rRNA, the large amount of which probably explains the strange appearance of the former in Lane 7, Fig.28a.

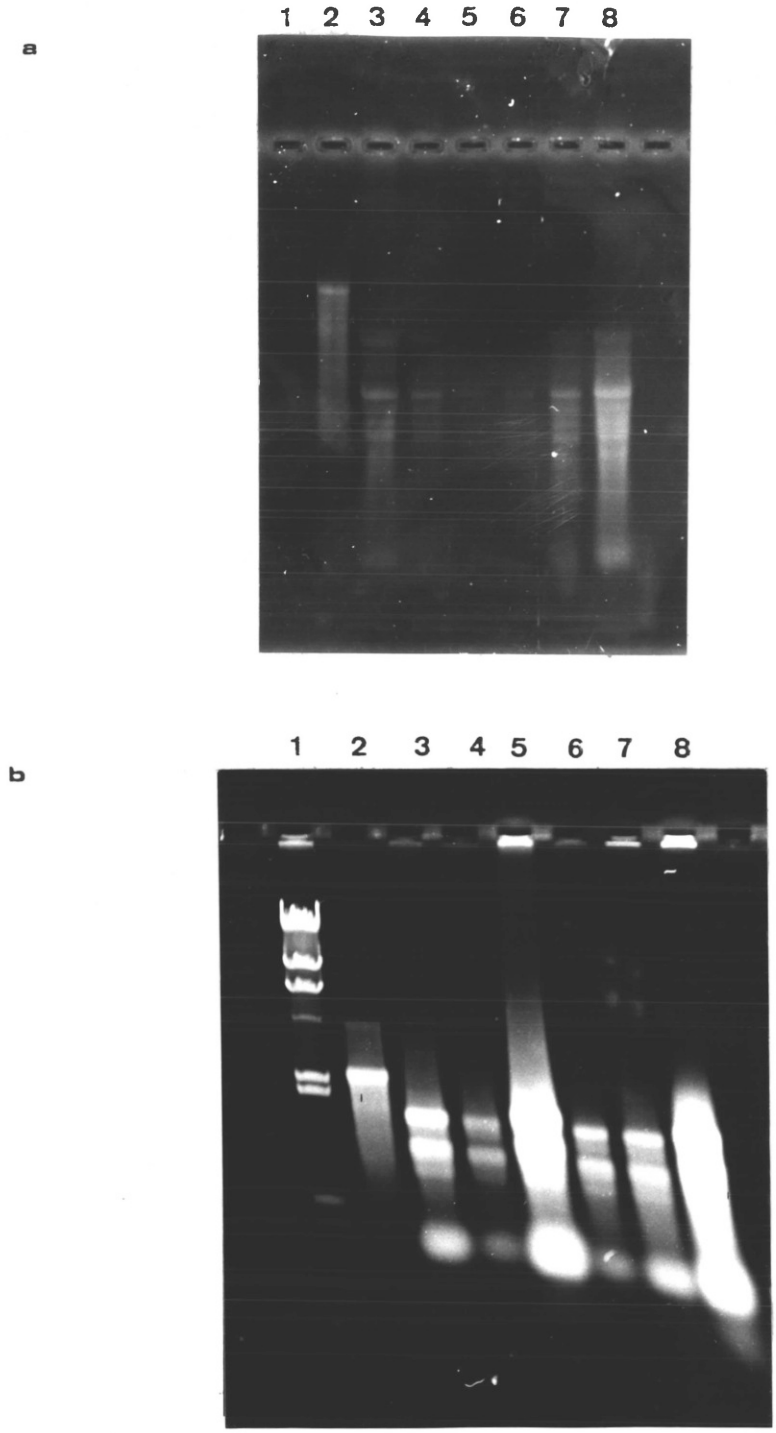


Fig.27a,b Agarose gel electrophoresis of (a) glyoxalated and (b) native total RNA from BaYMV-infected (Lanes 3-5) and healthy (Lanes 6-8) barley plants.

Loadings: (a) Lanes 5,6; 0.2µg. Lanes 4,7; 1µg. Lanes 3,8; 5µg.
(b) Lanes 4,6; 0.2µg. Lanes 3,7; 1µg. Lanes 5,8; 5µg.

Markers: Lane 1; λ *Hind* III. Lane 2; TMV.

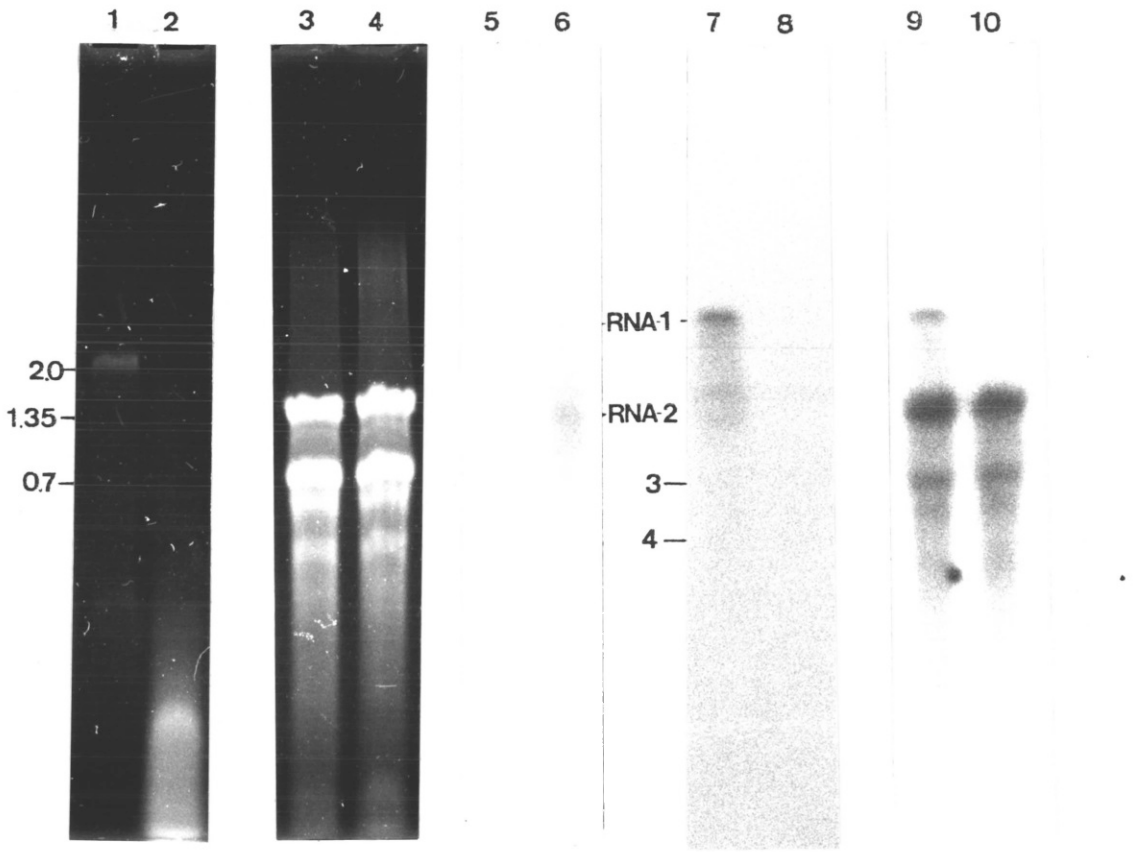


Fig.28a Methylmercury gel (Lanes 1-4) and Northern blot (Lanes 5-10) of polysomal RNA from healthy (Lanes 4 , 8 & 10) and BaYMV-infected (Lanes 3 , 7 & 9) barley plants. RNA was transferred to GeneScreen *Plus*™ and hybridised to cDNA to sucrose-gradient purified BaYMV RNA (Lanes 5-8) then rehybridised to a mixture of BaYMV RNA and barley rRNA (Lanes 9 & 10) after stripping of probe.

Lanes 1 & 5; TMV RNA

Lanes 2 & 6; crude BaYMV RNA

Lanes 3,4,7,8,9 & 10; polysomal RNA

(RNA-1 & -2 and minor hybridising bands 3 & 4 are indicated)

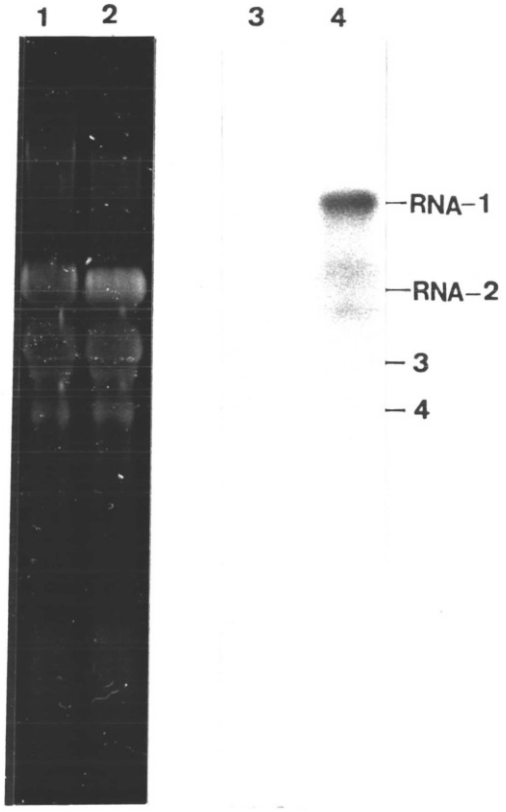


Fig.28b As Fig 28a except hybridised once only.
Lanes 1 & 3; polysomal RNA from healthy plants
Lanes 2 & 4; polysomal RNA from BaYMV-infected plants

The presence of BaYMV RNAs in polysomal RNA does not, however, imply a role as functional messenger RNAs since electron microscopy of polysomal preparations showed the presence of virus particles. Disruption of ribosomes with EDTA and recentrifugation allowed a theoretical separation of particles and polysomal RNAs. However, probing of both the pellet (virus particles) and the supernatant (polysomal RNA) fractions demonstrated an absence of viral RNA in either, probably as a result of degradation. Thus it is not possible to say whether viral bands in total polysomal RNA were due to contaminating virus particles or true translational activity. This question can only be resolved by further experiments of this nature.

The state of BaYMV RNA in crude extracts from ultracentrifuge pellets is also illustrated in Fig.28a. Here lane 2 shows that the majority of RNA is of heterogeneous low molecular weight and probably degraded. However, lane 5 shows that none of the RNA hybridises to BaYMV cDNA, demonstrating its plant origin. The presence of a large proportion of BaYMV RNA that is intact indicates that virus particles are not severely damaged by ultracentrifugation through a sucrose cushion.

3.6 Double-Stranded RNA

In view of the difficulties experienced in purification of virus and viral RNAs an investigation of the dsRNAs found in BaYMV-infected plants was initiated. The value of dsRNA analysis in detection and diagnosis of plant viral infection, genome characterisation and provision of viral RNA sequences has been discussed above (1.11).

3.6.1 Plant Material

Plants used for dsRNA analysis were barley cvs. Tipper and Maris Otter. Plants naturally infected with BaYMV were collected from the Little Clacton site in early spring of 1984 (LC84) and 1985 (LC85). Healthy plants (H) and those infected by mechanical inoculation (In) were grown from the same seed stocks sown at the Little Clacton site and the latter were inoculated with virus from the same site.

3.6.2 Extraction and Yields

DsRNA extraction was initially carried out by the method of ZELCER *et al.* (1981) (2.11.5a) but since the modified protocol of MORRIS & DODDS (1979) (2.11.5b) gave similar yields and was much simpler this method was adopted. DsRNA extracted in this way was often contaminated by some ssRNA and a discrete DNA species (see eg. Fig.39) but these could usually be removed by 2M LiCl precipitation and digestion with RNase A and DNase I under high salt conditions. The small scale batch method of MORRIS *et al.* (1983) never produced sufficient dsRNA to visualise by gel electrophoresis, indicating that levels in plants are low. In fact 20-40g of tissue was usually required for detectable amounts of RNA (the smallest quantity was 5g and the greatest 60g (see legends of Figs. 29-41)). This variability probably reflected the infection stage of plants and the presence of non-infected tissue in the samples.

3.6.3 Gels and Molecular Weight Standards

Three gel systems were used to analyse dsRNA extracts and the electrophoretic behaviour of dsDNA, dsRNA and ssRNA

was different in each. The published molecular weights of nucleic acid species used as markers are listed in Tables 8 & 9. Approximate molecular weights of BaYMV dsRNA bands were estimated from plots of log (molecular weight) vs. distance migrated of marker bands. Viral origin of dsRNA species was investigated by Northern transfer and hybridisation to BaYMV cDNA probes.

In 4% polyacrylamide gels maize rough dwarf virus (MRDV) and *Gaeumannomyces graminis var. tritici* virus 3b1a RNAs exhibited slightly different migration properties; log plots of MRDV RNAs were invariably linear whereas those of 3b1a were linear only over limited size ranges.

In non-denaturing 1% agarose gels log plots of dsRNA mobilities were roughly equivalent for all markers used (Figs.40,44) but not equivalent to those of dsDNA for which mobility was greater for a given molecular weight (Figs.39,40).

SsRNA migrates more slowly than dsRNA of equivalent size in polyacrylamide gels (Fig.34) but much more quickly than dsRNA of equivalent size in non-denaturing gels (Figs.30,44).

In denaturing agarose gels log plots for ssRNA and dsRNA mobilities were not equivalent (eg. Figs.42,43). ssRNA migrated more quickly than denatured dsRNA of equivalent size: 25S rRNA ($Mr=1.35\times10^6$) comigrates with MRDV RNA-6 (ssRNA $Mr=0.88\times10^6$) in Fig.42, CPMV RNA-2 ($Mr=1.37\times10^6$) comigrates with MRDV RNA-4 (ssMr=1.17×10^6) in Fig.43. Thus it seems that denatured dsRNAs cannot be used as valid ssRNA markers.

For the sake of uniformity BaYMV dsRNA molecular weights below 3×10^6 are given with respect to MRDV RNAs and those above, with respect to 3b1a unless stated otherwise but in view of data above these molecular weights must be regarded as approximate. Accurate sizing of the largest dsRNA species was not possible due to the unavailability of molecular weight standards.

3.6.4 Double-Stranded RNA Characterisation

Analysis of dsRNAs from BaYMV-infected plants proved to be far more complex than expected. Up to 14 different dsRNA species of Mr $\geq 0.1 \times 10^6$ were found. Electrophoretic patterns produced were often similar but quantitative and qualitative differences were found between different batches of material from the same source as well as between batches from different sources. Patterns were generally less complex for dsRNAs extracted from mechanically inoculated material but this was not always the case. DsRNAs of the same size as ssRNA-1 and -2 were not always observed and when present were not necessarily the most abundant species. Figs.29-41 illustrate the different electrophoretic patterns found and Tables 1-7 summarise the data.

Figs.29-32 illustrate one of the electrophoretic patterns of dsRNA produced in agarose gels and the data are summarised in Table 1. All extracts contained much heterogeneous low molecular weight nucleic acid despite 2M LiCl precipitation but nuclease digestions (Fig.29) illustrated the dsRNA nature of the extracts. Yields were high; as little as 7.5g material was needed to detect dsRNAs (Fig.30). Fig.30 also suggests that much of the dsRNA was nicked as no bands are visible following glyoxalation, though this is partly explained by the poorer ethidium bromide binding of glyoxalated RNA. Resolution in Fig.32 is not good but the figure shows that the only dsRNA in healthy plants is of heterogeneous low molecular weight. It is clear that, while there are major dsRNA species of the same sizes as BaYMV dsRNA-1 & -2, there are other more predominant bands (eg. bands designated Groups 1 & 2, see Table 1).

Table 1

Summary of data from Figs.29-32

Band	Mr ($\times 10^{-6}$)	Comment
A	>5	Probably RNA-1 RF
B	≈3.5	Faint
C	2.35-50	Probably RNA-2 RF
D	1.8-9	Faint
E	1.65-75	)
F	1.43-48	) Group 1
G	1.18-25	)
H	1.00-13	)
I	0.76-80	)
J	0.70-75	) Group 2
K	0.55-65	)

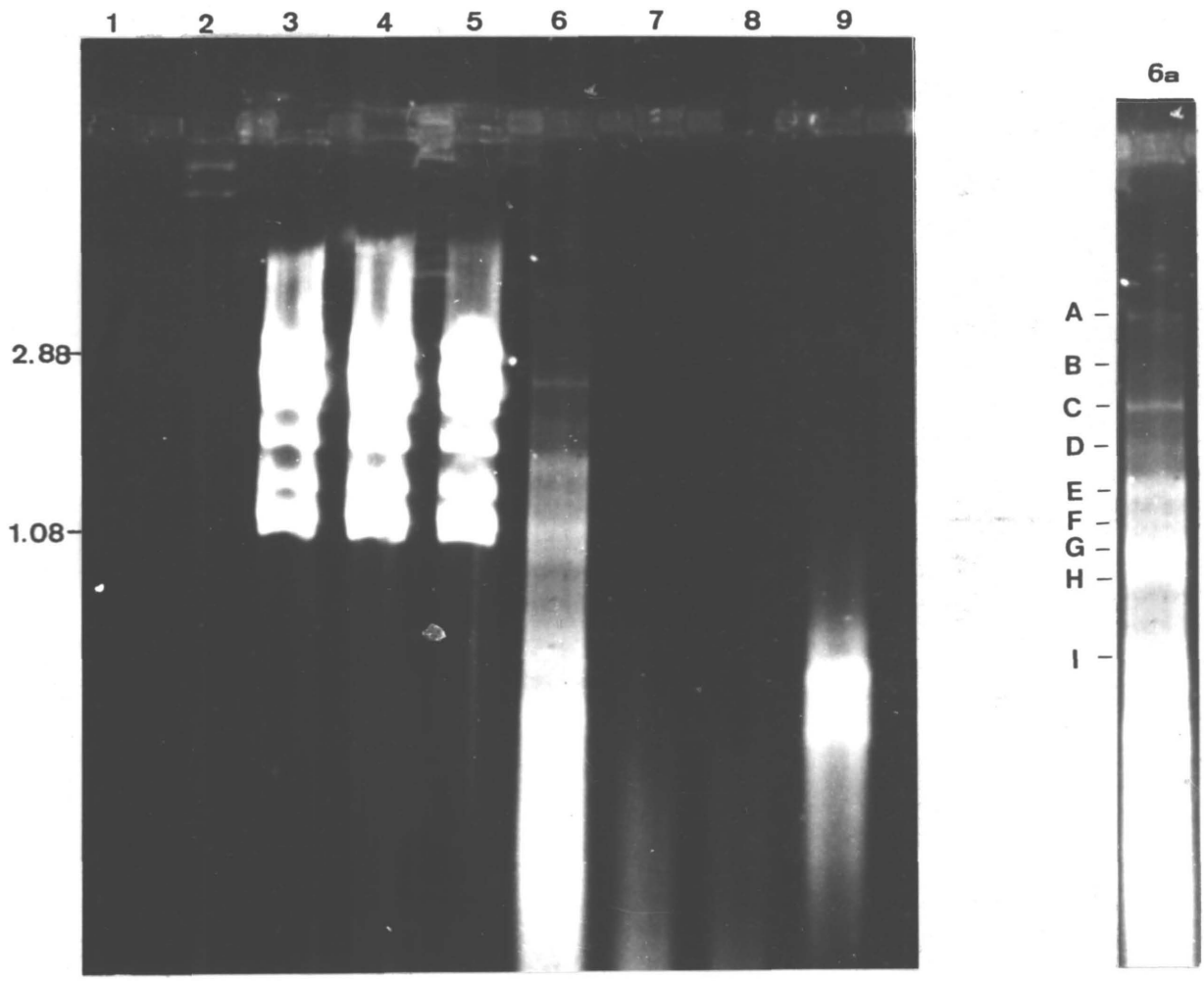


Fig.29 Nuclease digestions of BaYMV dsRNA. Agarose gel electrophoresis of nucleic acids digested with DNase-I (Lanes 1,3,6), RNase-A in 2×SSC (Lanes 4,6,8), RNase-A in 0.1×SSC (Lane 7) and untreated (Lanes 2,5,9).

Lanes 1 & 2; plasmid DNA

Lanes 3, 4 & 5; MRDV dsRNA

Lanes 6 & 7; BaYMV dsRNA from 10g LC84

Lanes 8 & 9; BSMV ssRNA

Lane 6a; as Lane 6, illustrating band nomenclature

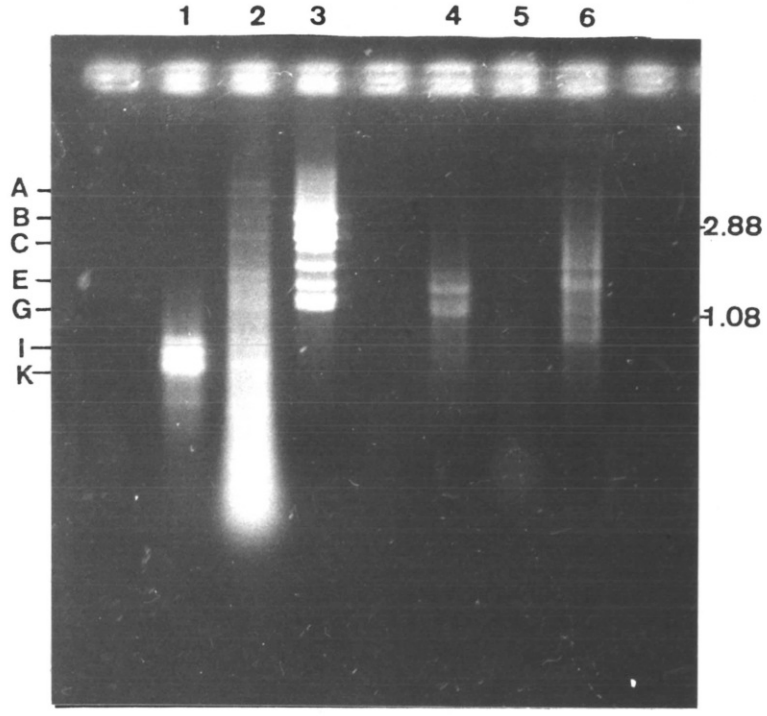


Fig.30 Agarose gel electrophoresis of native (Lanes 1-3) and glyoxalated (Lanes 4-6) RNAs.

Lanes 1 & 4; BSMV ssRNA

Lanes 2 & 5; BaYMV dsRNA from 7.5g LC84

Lanes 3 & 6; MRDV dsRNA

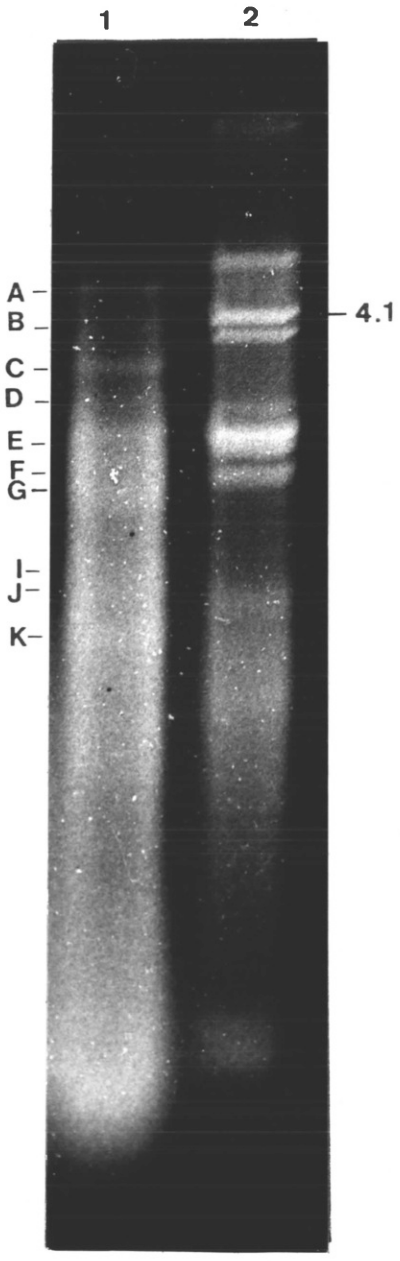


Fig.31 Agarose gel electrophoresis of BaYMV dsRNA.

Lane 1; BaYMV dsRNA from 25g LC84

Lane 2; 3b1a dsRNA

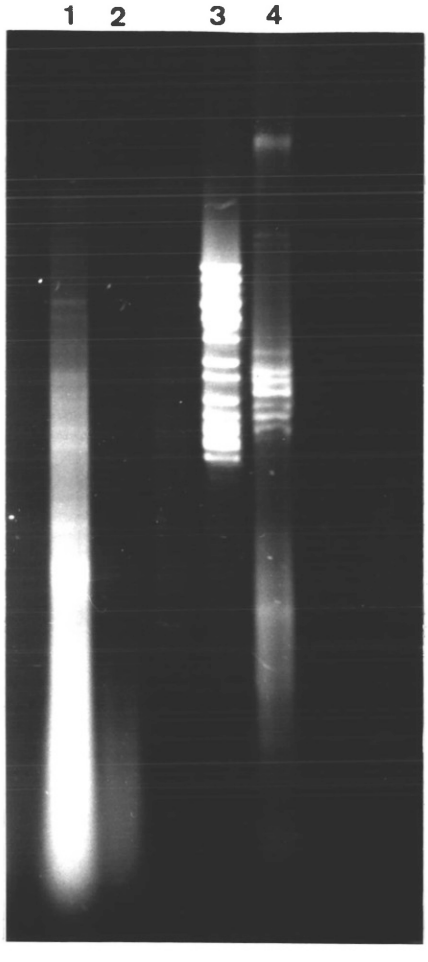


Fig.32 Agarose gel electrophoresis of dsRNA from healthy and BaYMV-infected plants.

Lane 1; BaYMV dsRNA from 12.5g LC84

Lane 2; dsRNA extracted from 25g healthy barley

Lane 3; MRDV dsRNA

Lane 4; 3b1a dsRNA

The next batch of material analysed was harvested from a different part of the Little Clacton site in 1984. Yields of dsRNA were much lower and the electrophoretic pattern was different (Figs.33-4). This is partly concerned with the greater resolving power of acrylamide gels but quantitative differences and the absence of a putative RNA-1 RF cannot be explained in this way. Therefore bands are labelled using a different nomenclature and comparisons with the earlier pattern are made in Table 2. Because of the low yields the dsRNA analysed on one gel represents extracts from many more leaves of many more plants so possibility of extracting a mixture of strains and/or defective RNAs is increased. This might explain the appearance of some of the minor bands and doublet bands.

Analysis of infected tissue harvested from the same site in 1985 revealed a similar electrophoretic pattern (Fig.35, Table 3) and comparably low yields though there are qualitative and quantitative differences. At least one new dsRNA species (designated β) is seen and three other bands (g,h & o) are absent. The band designated i' may be novel or it may be band i migrating slightly anomalously. Unfortunately undigested DNA partially obscures the top of the gel so it is not possible to say if high molecular weight species such as RNA-1 RF are present.

Table 2

Summary of data from Figs.33-4

Band	Mr ($\times 10^{-6}$)	Comment
b	3.10-15	Doublet in Fig.34. Possibly analogous to B (Table 1)
c	2.7	May be same as C (ie RNA-2 RF)
d	2.2	May be same as D
e	1.84(80)	Doublet)
f	1.7	) Probably equivalent to
g	1.6	Faint) Group 1; differences
h	1.5	Faint) from Figs.29-32 due to
i	1.31-34	) greater resolution in
j	1.25	Faint) acrylamide
k	1.2	Faint)
l	0.70-79	)
m	0.59-62	) Probably equivalent to Group 2;
n	0.56-58	) molecular weights approximate in
o	o.50-52	) this range

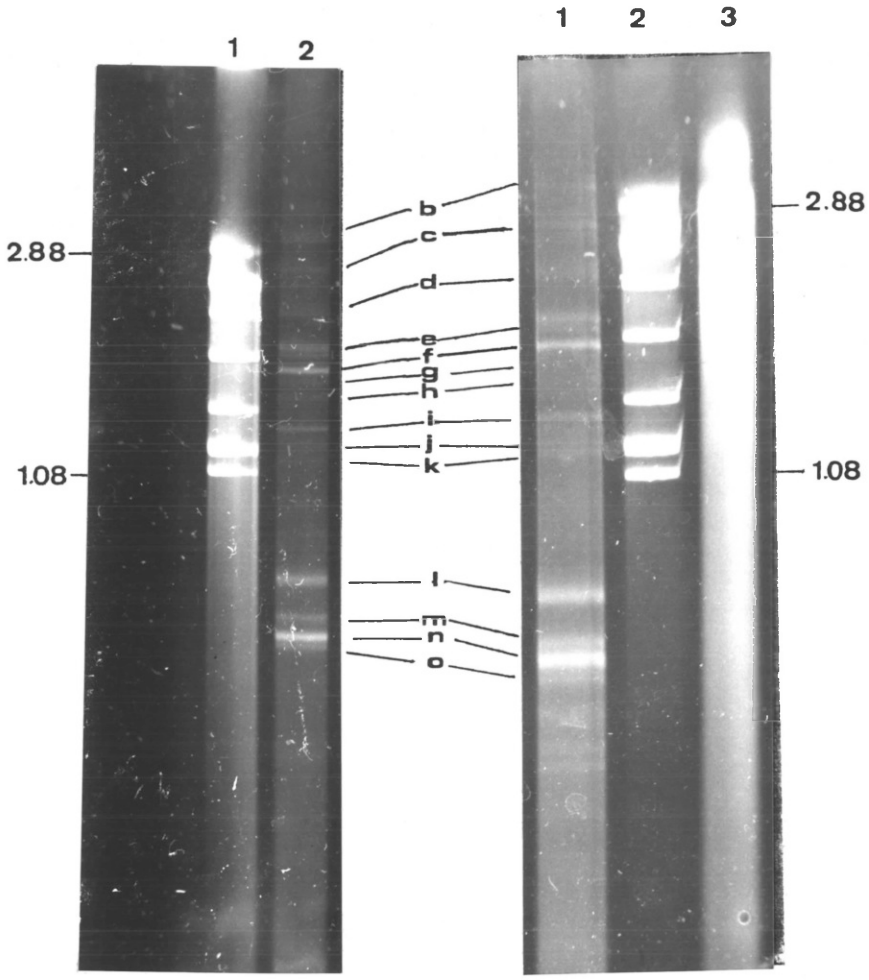


Fig.33

Fig.34

Fig.33 PAGE of BaYMV dsRNA from naturally infected plants.

Lane 1; MRDV dsRNA

Lane 2; BaYMV dsRNA from 30g LC84

Fig.34 PAGE of BaYMV dsRNA from naturally infected plants.

Lane 1; BaYMV dsRNA from 50g LC84

Lane 2; MRDV dsRNA

Lane 3; BSMV ssRNA

Table 3

Analysis of data in Fig.35

Band	Mr ($\times 10^{-5}$)	Comment
b	3.05(20)	Doublet as in Fig.34
c	2.75(80)	Doublet. RNA-2 RF?
β	2.35	Not seen in LC84 gels
d	2.2	Higher levels relative to LC84 gels
e	1.85	Not doublet (as opposed to LC84 gels)
f	1.7	)
i'	1.4	) Group 1
j	1.25	)
l	0.7	)
m	0.66	) Group 2
n	0.58	)

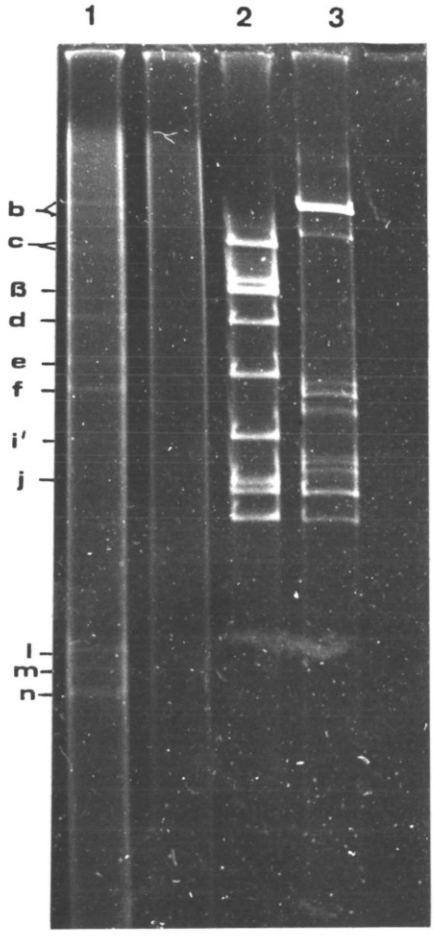


Fig.35 PAGE of BaYMV dsRNA from naturally infected plants.

Lane 1; BaYMV dsRNA from 40g LC85

Lane 2; MRDV dsRNA

Lane 3; 3b1a dsRNA

Analysis of dsRNA in mechanically inoculated plants showed that symptom expression preceded detectable dsRNA synthesis. Usually fewer dsRNA species were observed as compared with field infected material so initially some of the bands in the latter were ascribed to co-infection (eg. with a cryptic virus). However, on one occasion multiple species were isolated from inoculated plants (Fig.37) and later hybridisation studies showed bands in field infected material to be BaYMV-specific (see below).

Figs.36-9 illustrate electrophoretic patterns produced by dsRNA extracts from inoculated plants. Bands are labelled by gel type and are compared in Tables 4 & 5. Interestingly no bands corresponding in size to RF of RNA-1 were found on any occasion and a putative RNA-2 RF was seen only once (Fig.37). It is possible that replication is temporally or spatially confined and was not active in the tissue processed. The one band (or doublet) that is common to all extracts is a species of Mr 2.0-2.3 which was also the only band visible in another extract of inoculated plants. The other two dsRNA species seen in more than one extract are of sizes 3.0-3.5 and ≈1.3. The greater complexity of the pattern in Fig.37 may be a reflection of the amount of plant material processed as above.

Table 4

Analysis of Figs.36-7

Band	Mr ($\times 10^{-6}$)	Comment
b	3.05	Lower band of doublet in Figs.34,5
c	2.55(70)	Doublet; migrates faster than Fig.35
β	2.32	One of two major bands in Fig.36
d	2.2	Possibly doublet in Fig.36
e	1.8	
f	1.7	
h	1.5	
i	1.3	One of two major bands in Fig.36
k	1.2	

Table 5

Analysis of Figs.38-9

Band	Mr ($\times 10^{-6}$)	Comment
B	3.0-3.5	Seen in Fig.39 but not 38. (Mr from 3b1a)
D	2.0-2.1	Doublet in Fig.38. May be β or d of Fig.36
G	1.25-30	Not seen in Fig.39. Probably same as i in Fig.36
J	0.7	) May be present in Fig.39 also but
K	0.5	) impossible to size.

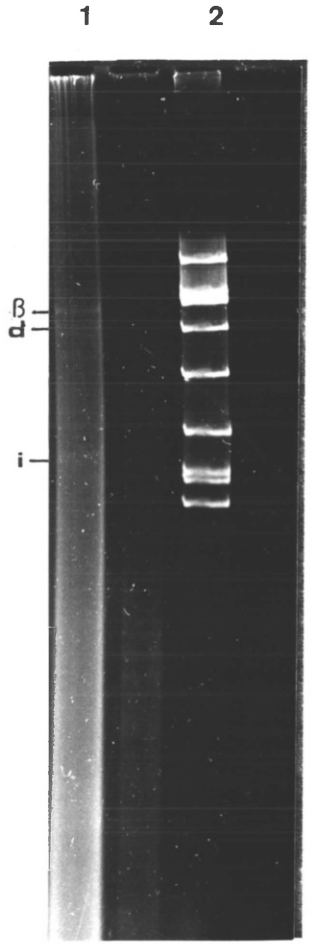


Fig.36

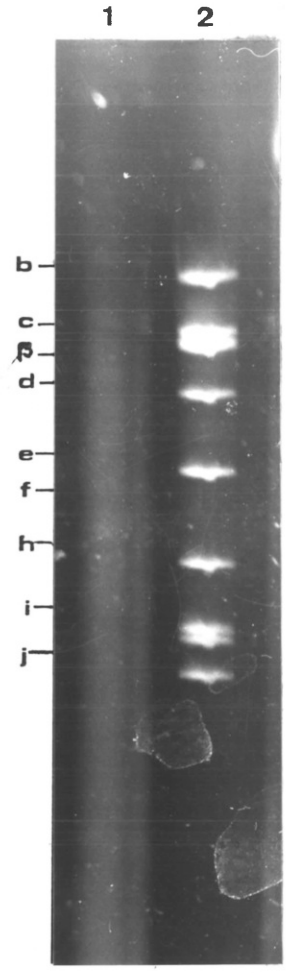


Fig.37

Fig.36 PAGE of BaYMV dsRNA from inoculated plants.
Lane 1;. BaYMV ds RNA from 25g In. (75 days post-inoculation)
Lane 2; MRDV dsRNA

Fig.37 PAGE of BaYMV dsRNA from inoculated plants.
Lane 1; BaYMV dsRNA from 60g In. (52 days post-inoculation)
Lane 2; MRDV dsRNA

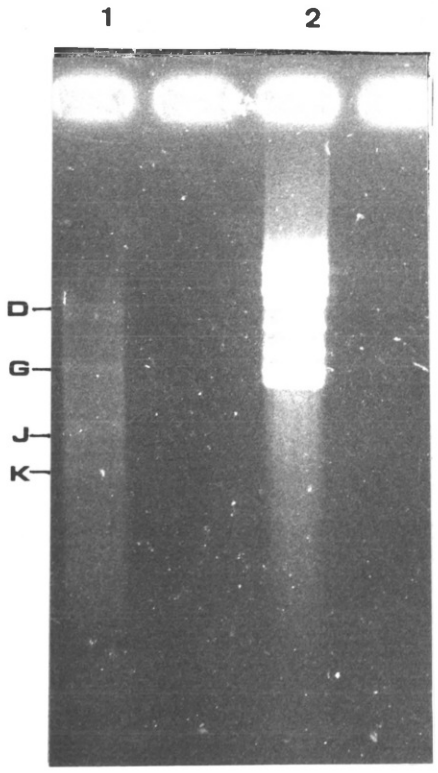


Fig.38 Agarose gel electrophoresis of BaYMV dsRNA from inoculated plants.

Lane 1; BaYMV dsRNA from 20g In. (42 days post-inoculation)

Lane 2; MRDV dsRNA

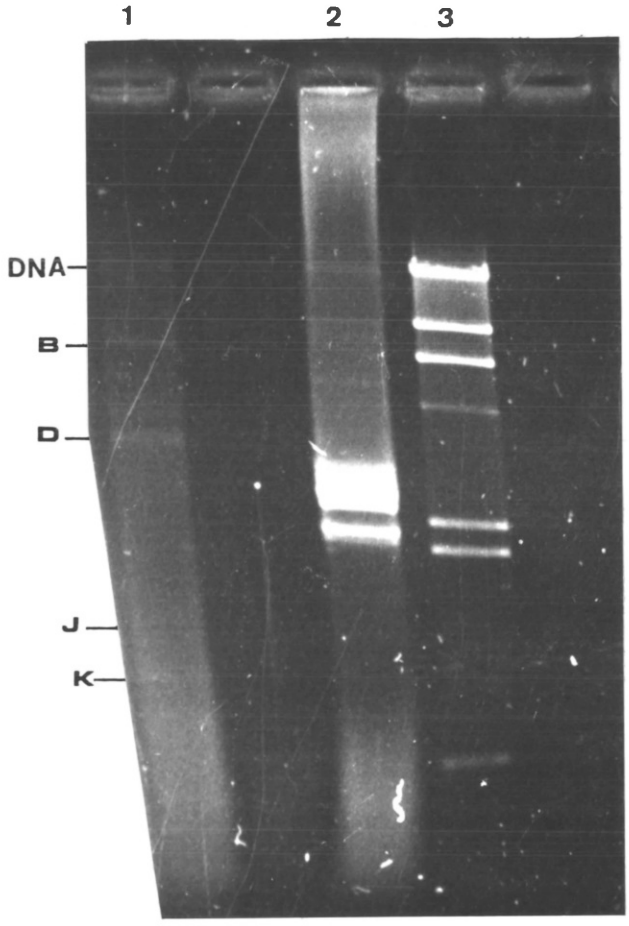


Fig.39 Agarose gel electrophoresis of BaYMV dsRNA from inoculated plants.

Lane 1; BaYMV dsRNA from 10g In. (41 days post-inoculation)

Lane 2; 3b1a dsRNA

Lane 3; λ *Hind* III digest

DsRNA extracted from a further batch of LC85 material (see Fig.35) was further analysed on non-denaturing gels prior to Northern transfer and hybridisation by the method of ZELCER *et al.* (1981). One such gel is shown in Fig.40 (Table 6) which illustrates a basically similar pattern to that in the acrylamide gel, given the greater resolving power of the latter, with a few differences. Doublets b(B) and c(C) are seen in both gels; β is probably running with doublet C in view of the compression of MRDV bands 2-5 in the agarose gel; d(D) is faint, obscured by the heterodisperse dsRNA smear, and migrates with an apparently lower molecular weight; e and f run as E; i and j are probably G and H, though more abundant in this batch of material; and l,m and n are compressed into I. Band A is not seen in Fig.35, but is possibly obscured by the undegraded DNA, and neither is band L.

All attempts to transfer RNA from non-denaturing gels to hybridisation membranes were unsuccessful. Such behaviour is well documented and is a consequence of RNA secondary structure interfering with binding. In the method used RNA was subjected to partial alkaline hydrolysis prior to transfer but hybridisation with cDNA probes failed to detect any RNA on blots under conditions in which RNA was detected after transfer from denaturing gels (see below).

Table 6

Analysis of Fig.40

Band	Mr(×10⁻6)	Comment
A	>4.5	Faint. Possibly obscured in Fig.35. RNA-1 RF?
B	3.15(30)	Doublet as b in Fig.35.
C	2.60(40)	Doublet as c in Fig.35. RNA-2 RF?
D	1.95	May be d
E	1.70	Probably equivalent to e and f.
G	1.30	) Probably i' and j though more
H	1.20	) abundant here
I	≈0.7	Probably k, l' and m poorly resolved
L	≈0.25	Not seen in any other material

Table 7

Analysis of Fig.41

Band	Mr(×10⁻6)	Comment
B	3.30	Faint relative to Fig.40.
C	2.65	Probably RNA-2 RF
D	2.10	Faint as in Fig.40
E	1.65	
G/H	1.25	Bands not resolved here
I	0.65-75	Probably 2 or 3 bands
L	≈0.25	

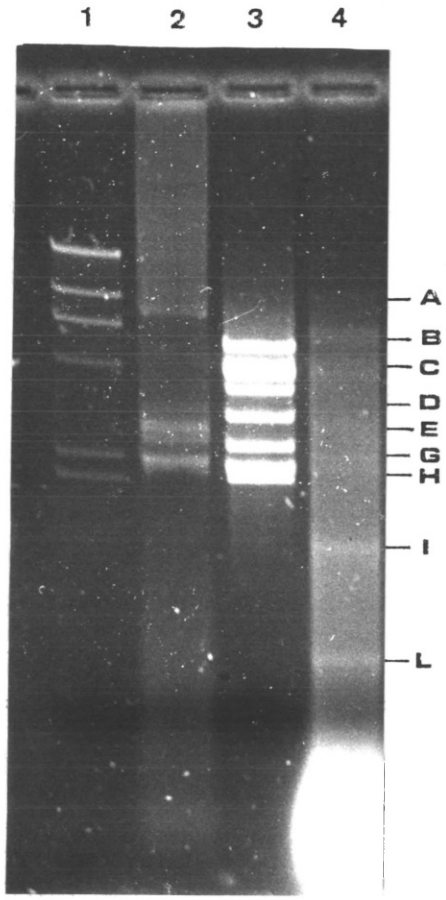


Fig.40 Agarose gel electrophoresis of BaYMV dsRNA from naturally infected plants.

Lane 1; λ *Hind* III DNA

Lane 2; 3b1a dsRNA

Lane 3; MRDV dsRNA

Lane 4; BaYMV dsRNA from 40g LC85

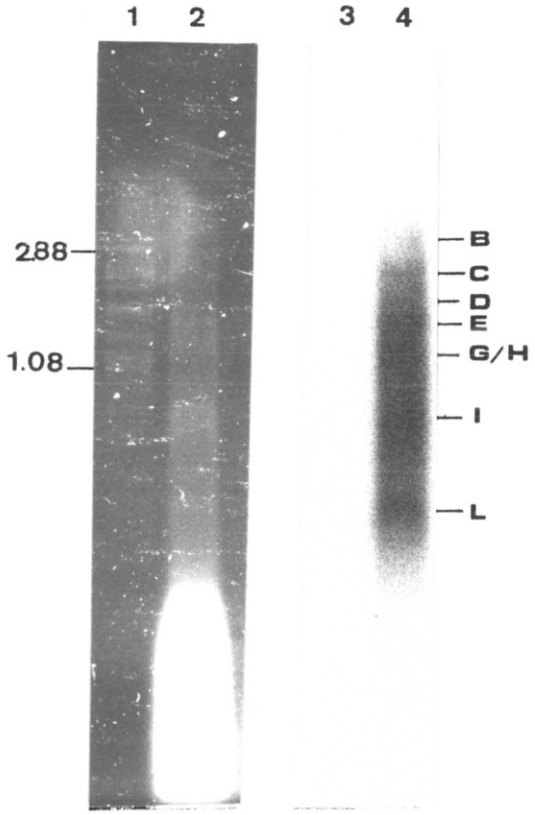


Fig.41 Methylmercury gel (Lanes 1,2) and Northern blot (Lanes 3,4) of BaYMV dsRNA from naturally infected plants. RNA was transferred to a GeneScreen *Plus*™ membrane and hybridised to cDNA synthesised to sucrose gradient-purified BaYMV RNA.

Lanes 1,3; MRDV dsRNA

Lanes 2,4; BaYMV dsRNA from 40g LC85

Denaturing gel electrophoresis again indicated that some dsRNAs were nicked as bands were barely visible following ethidium bromide staining (Fig.41). However, it appeared that dsRNA species were not fully denatured (to single strands) in this gel since a log plot derived from dsRNA standards did not match with one from ssRNA standards. In spite of this, transfer of RNA to hybridisation membranes was possible which was not the case for native dsRNA. DsRNA is notoriously difficult to denature and anomalous mobilities with respect to ssRNA were observed on other occasions (eg Figs.42,43). Since the pattern of BaYMV dsRNAs relative to MRDV is similar to that in Fig.40 ,in which the same dsRNA extract was used, both BaYMV and MRDV dsRNAs were assumed to be denatured to the same extent here. Band labelling and molecular weight assignment was thus with respect to MRDV.

Data in Fig.41 is summarised in Table 7 and it is apparent that most, if not all, dsRNA species present in the extract are BaYMV-specific and not derived from the host or coinfection of the plants in the field. At least seven dsRNA species are present but, with the exception of band C (possibly RNA-2 RF), it is not possible to ascribe a role in the genome expression of BaYMV to any of them without further information. Some may be RFs of subgenomic RNAs but since it not known whether BaYMV expresses any of its genes in this manner the suggestion must be tentative and the artefactual origin of at least some bands cannot be eliminated. Other possible derivations of these dsRNA species are RFs of hitherto undetected genomic RNAs and double-stranded forms of defective BaYMV RNAs containing the origin of replication.

3.6.5 Conclusions

Analysis of dsRNAs from BaYMV-infected plants revealed up to 14 different species of Mr $\geq 0.1 \times 10^6$ at least 7 of which hybridised to BaYMV sequences. Electrophoretic patterns produced were often similar but quantitative and qualitative differences were found. DsRNAs of the same size as ssRNA-1 and -2 were not always observed and when present were not necessarily the most abundant species. Results are summarised below.

Band	Mr ($\times 10^{-6}$)	Comment
A	>5	Probably RNA-1 RF. Only seen in naturally infected plants
B(b)	3-3.5	Often present. BaYMV-specific
C(c)	2.35-70	Probably RNA-2 RF. Often seen but not in inoculated plants. BaYMV-specific
D(β,d)	1.9-2.2	One or two bands, major species in some inoculated plants. At least one BaYMV-specific
E-H (e-k)	1.00-1.80	) Group of bands, variable in number) and size, including one found often) in inoculated plants (≈1.3) & at) least two BaYMV-specific (≈1.3,1.65)
I-K (k-n)	0.55-0.80	) Two-four bands, difficult to size) accurately. Often seen. At least) one BaYMV-specific
L	≈0.25	Seen in only one extract of naturally infected plants. BaYMV-specific. Size approximate

In 1.11 the potential of using dsRNA analysis as a means of detection and/or diagnosis of plant viral infection was discussed. From the results presented here on the levels of dsRNA found in infected plants it is clear that as far as BaYMV is concerned the technique is several orders of magnitude less sensitive than other detection methods based on serological or hybridisation assays. Antisera to several isolates of BaYMV exist and the techniques of ISEM, ELISA and Western blotting are now a routine exercise. Dot-blot hybridisation using cDNA probes is a more time consuming method but would be greatly facilitated by the existence of cloned fragments of BaYMV cDNA (see 3.7 below). Furthermore the electrophoretic patterns of dsRNAs observed in this study are probably too variable to be used in the diagnosis of BaYMV (or mixed) infection or footprinting as an aid to classification or strain comparison.

Purified dsRNAs have also been used as sources of viral RNA sequences. On account of the complexity and variability of dsRNA extracts it was felt that data from experiments using end-labelled dsRNAs as probes would be difficult to interpret. However, further experiments of the type illustrated in Fig.41 using cloned strand-specific or even gene-specific probes could be useful in the analysis of BaYMV replication strategy.

Table 8

Double-stranded molecular weights of commonly used standards ($\times 10^{-6}$)

Band	MRDV	3b1a	λ*Hind*III	*AfV*	*PsV*
1	2.88	4.1	15.0	4.1	1.11
2	2.50	3.5	6.12	2.7	0.99
3	2.35	1.6	4.26	2.5	0.94
4	2.35	1.54	2.84	1.87	0.89
5	2.12	1.45	1.5	1.70	0.46
6	1.75	1.43	1.32	1.44	
7	1.45	1.27	0.36	1.24	
8	1.25	1.19			
9	1.18	1.11			
10	1.08				

MRDV: Maize rough dwarf

3b1a: *G.g.t.* virus 3b1a

λ*Hind*III: *Hind*III digest of λ DNA

AfV: *Aspergillus foetidus* virus

PsV: *Penicillium stoloniferum* virus

Table 9

Single-stranded molecular weights of standards ($\times 10^{-6}$)

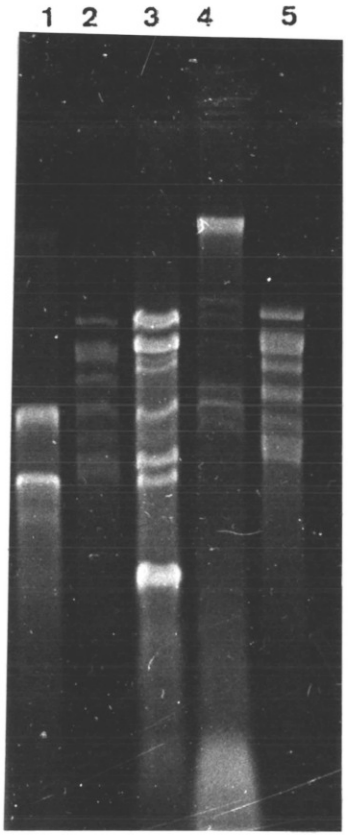
		Ribosomal RNA		
Band	CPMV	*E.c.*	Euk	TMV
1	2.02	1.05	1.35	2.0
2	1.37	0.56	0.70	

CPMV: Cowpea mosaic virus

E.c.: *E.coli* rRNA (23S & 16S)

Euk: Eukaryotic rRNA (25S & 18S)

TMV: Tobacco mosaic virus



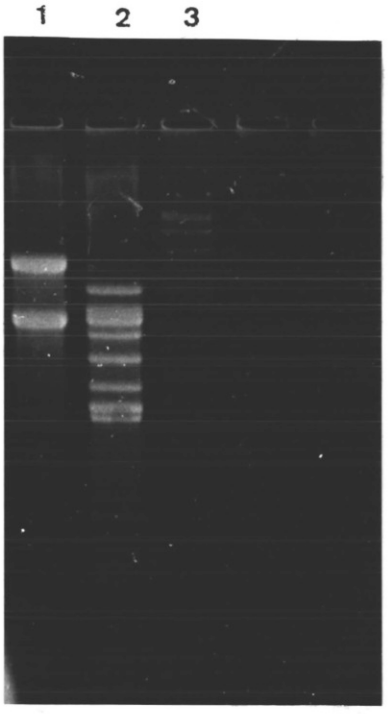


Fig.42

Fig.43

Fig.42 Methylmercury gel electrophoresis of molecular weight standards.

Lane 1; rRNA

Lane 2; MRDV

Lane 3; *P.s.*V + *A.f.*V

Lane 4; 3b1a

Lane 5; MRDV

Fig.43 Methylmercury gel electrophoresis of molecular weight standards.

Lane 1; CPMV

Lane 2; MRDV

Lane 3; λ *Hind* III

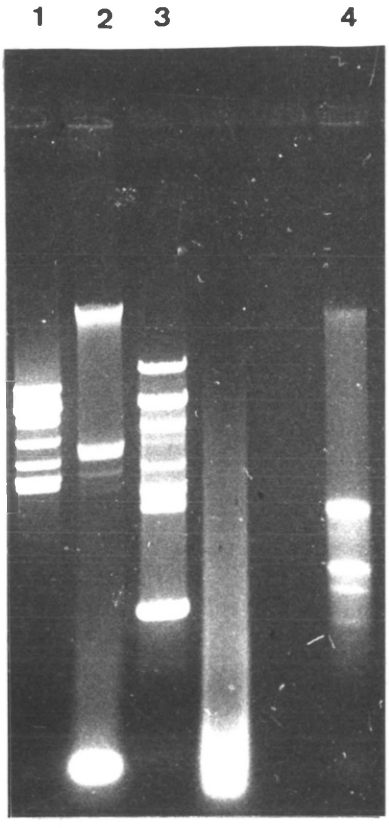


Fig.44 Agarose gel electrophoresis of molecular weight standards

Lane 1; MRDV

Lane 2; 3b1a

Lane 3; *P.s.*V + *A.f.*V

Lane 4; rRNA

3.7 cDNA Synthesis and M13 Cloning

3.7.1 cDNA Probes

All autoradiograms shown above were obtained by hybridisation of membranes to cDNA probes synthesised by random priming using as template RNA extracted from purified virus or purified by sucrose-gradient fractionation or elution from gels. Efficiencies of incorporation of radiolabel into cDNA were consistently low as compared with incorporation using TMV or PVX RNA as template under equivalent conditions. RNA extracted from agarose gels sometimes exhibited no template activity, probably as a result of residues of reverse transcriptase inhibitors derived from impurities in the agarose. In order to provide a source of high activity probes attempts were made to clone fragments of BaYMV RNA into bacteriophage M13. Initial attempts were made before the availability of purified RNA using RNA enriched in BaYMV sequences followed by differential screening of recombinant clones using cDNA probes derived from the same enriched preparations and RNA extracted in a similar manner from healthy plants. In later attempts recombinants were screened using probes made from purified BaYMV RNA.

3.7.2 cDNA cloning

The use of the "loop-back" method of ds cDNA synthesis in two experiments resulted in a total of 53 recombinant clones. Differential hybridisation of RF DNA dot-blots, and in some cases hybridisation of dot-blots of healthy and infected plant extracts with nick-translated clones, indicated a host origin for all clones, suggesting that a

larger population of clones was needed to find any specific for BaYMV.

The RNase H method of ds cDNA synthesis proved more efficient in terms of the number of recombinants produced. White plaques were obtained with all the combinations of restriction enzymes used (2.18.1) though at lower rates using the "blunt" end cutters. In all, 24 plates with between 20 and 100 recombinants each were obtained. These were screened by plaque-lift hybridisation with probes synthesised from sucrose-gradient purified BaYMV RNAs and autoradiography revealed 15 radioactive spots, all produced using "sticky"-end cutters. Shortage of time prevented any further characterisation of recombinants so positively hybridising plaques were picked and grown up then infected cell stocks were frozen at -70°C in 15% (v/v) glycerol. Clones will require further screening by dot-blot hybridisation of RF DNA and hybridisation of nick-translated RF probes to Northern blots of BaYMV RNA for two reasons: (1) the density of plaques did not allow absolute assignment of radioactive spots to individual plaques (all potential recombinants were picked separately) and (2) the possibility of background spots on autoradiograms cannot be eliminated.

Even if all these clones are BaYMV-specific 15 out of 1000s represents a very low proportion considering the RNA sample used (crude BaYMV RNA as in Fig.15) and the size selection procedures (see 2.18.1). This low efficiency of cDNA synthesis may be enhanced by using synthetic primers constructed after limited sequencing of RNA termini.

3.8 *In Vitro* Translation

BaYMV virion RNA-1 and RNA-2, together and separately, were used to programme cell-free *in vitro* translation systems (2.16). Whether RNA was extracted from virions,

eluted from gels or purified by sucrose-gradient centrifugation incorporation of radioactively labelled amino acids into translation products was very low compared to that with PVX or TMV RNA (<10%) and on many occasions no detectable incorporation occurred at all. Reasons for this are unclear but may be related to BaYMV RNA secondary structure. As a consequence of the inconsistency of results and the scarcity of RNA, translation systems were not optimised for K^{+} and Mg^{++} concentration. Conditions used were those described by suppliers as suitable for most mRNAs.

Translation of whole polysomes and polysomal RNA again resulted in low levels of incorporation. Additional reasons for this may be the presence of inhibitors in the relatively crude polysome preparations and the huge excess of ribosomal RNA over message in polysomal RNA preparations.

Fluorographs of MDL translation products of gel-eluted and sucrose-gradient purified RNA are presented in Figs.45 & 46. Fig.45 shows products of the sucrose-gradient fractions 5-9 shown in Fig.25b and products of two separate gel-extractions. Both the latter were extracted by the "freeze-thaw" method (2.11.7c) and contained equal amounts of RNA-1 and RNA-2. Fig.46 shows products of RNA-1 (from fractions 1 and 2 in Fig.25c) and RNA-2 (from fraction 5 in Fig.25c). The reason for the clouding of the fluorograph is not known. Clearly there is some cross-contamination of fractions but by comparison certain differences are apparent.

The major products are of molecular weights 23, 67, 83/4 (doublet) and 96K. Other discernible products are of 25 (doublet), 27, 44, 50, 58 and 110k.

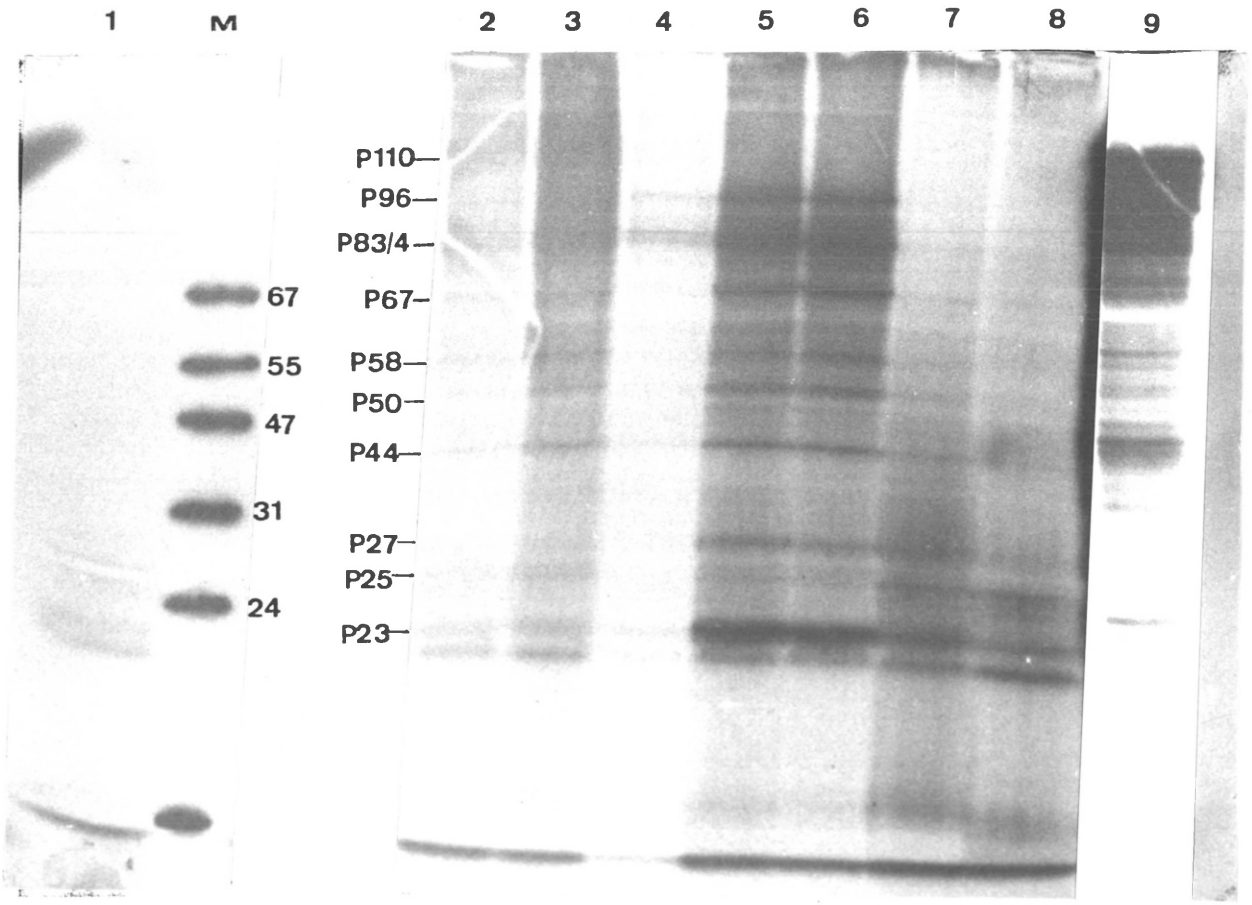


Fig.45 Fluorograph of BaYMV RNA translation products. RNAs were translated in MDL systems as described in 2.16 and fluorography carried out as in 2.17 after marking positions of molecular weight standards with radioactive ink.

Lane 1; H_2O control.

Lanes 2-6; Products of sucrose gradient fractions 5-9 illustrated in Fig.25b.

Lanes 7 & 8; Products of mixtures of BaYMV RNA-1 & -2 eluted from gels as described in 2.11.7c.

Lane 9; Products of PVX RNA translation

Lane M - Marker proteins (molecular weights are indicated)

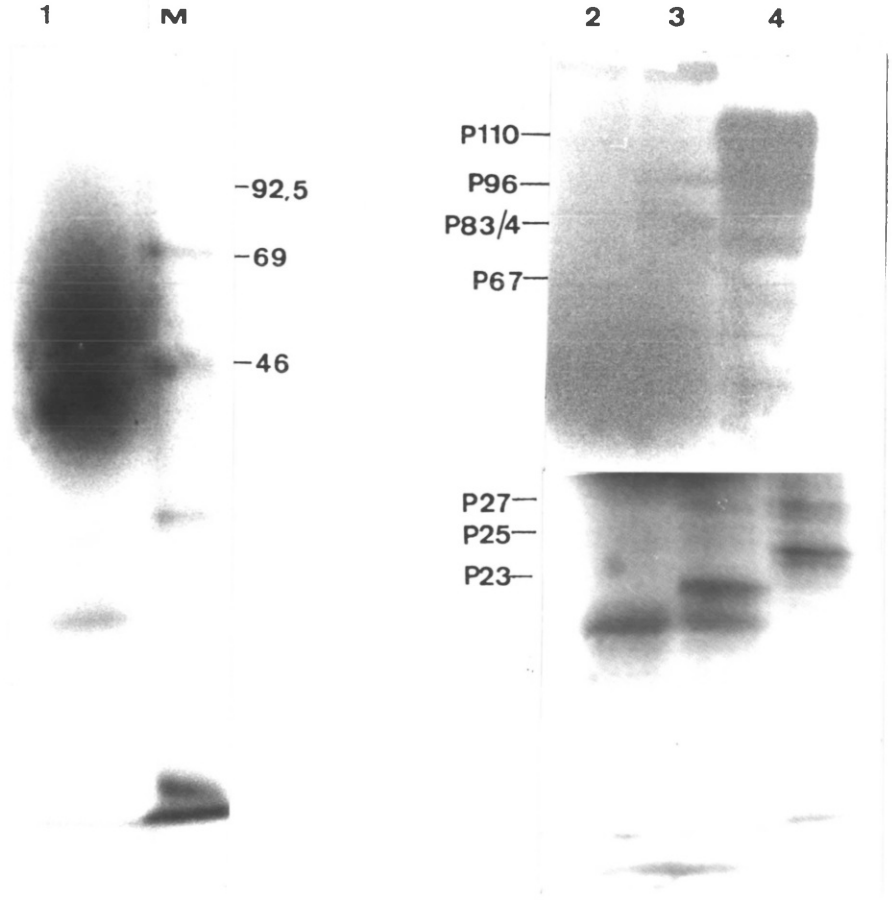


Fig.46 Fluorograph of BaYMV RNA translation products. RNAs were translated in MDL systems as described in 2.16 and fluorography carried out as in 2.17.

Lane 1; H_2O control.

Lane 2; Products of a mixture of sucrose gradient fractions 5 & 6 illustrated in Fig.25c (BaYMV RNA-1).

Lane 3; Products of sucrose gradient fraction 9 illustrated in Fig.25c (BaYMV RNA-2)

Lane 4; Products of PVX RNA translation.

Lane M - Marker proteins (molecular weights are indicated)

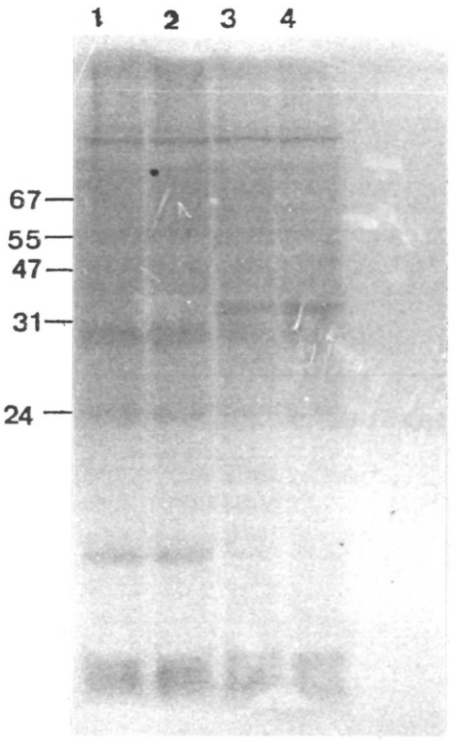


Fig.47 Fluorograph of translation products of whole polysomes and polysomal RNA from healthy (Lanes 1,3) and BaYMV-infected (Lanes 2,4) barley. Translation was carried out in wheatgerm extracts as described in 2.16.

Lanes 1 & 2; Products of whole polysome extracts prepared as in 2.11.6

Lanes 3 & 4; Products of total polysomal RNA

(Molecular weights of marker proteins are indicated)

P23 comigrates with the upper of two bands seen in the H_2O control track of Fig.45 but is stronger relative to the lower one in fractions 8 and 9. In Fig.46 P23 is found in the RNA-2 track but not in the control or RNA-1 tracks.

P67 can be seen in all BaYMV RNA tracks in Fig.5-9 but is relatively stronger in fractions 8 and 9. In Fig.46 P67 is only seen in the RNA-2 track.

P83/4 is present in all BaYMV RNA tracks but since RNA-1 fractions are inherently purer P83/84 is possibly an RNA-1 product.

P96 is much stronger in the RNA-2 track than the RNA-1 track in Fig.46 and relatively stronger in tracks 8 and 9 than in tracks 5, 6 and 7 in Fig.45.

P25 comigrates with a band in the control H_2O track of Fig.46 but in Fig.45 the lower band of the doublet is stronger in both eluted RNA tracks whereas both bands are of roughly equal intensity in gradient fractions.

P27 is stronger relatively in fractions 8 and 9 in Fig.45 and is only found in the RNA-2 track of Fig.46.

P110 is indistinct but is only found in fractions 5 and 6 in Fig.45 and the RNA-1 track of Fig.46.

P44, P50, P55 and P58 are of roughly equal intensity in all RNA tracks.

Though the evidence is not unequivocal some products can thus tentatively be assigned to particular RNAs:

RNA-1: P110, P83/84 and the lower band of the P25 doublet.

RNA-2: P23, P27, P67, and P96.

The similarity of the gel-electrophoretic patterns of the translation products of RNA isolated from two sucrose gradients and by two gel extractions is apparent and argues against artifactual origin of bands through contamination. Nevertheless the number of polypeptides produced is large

and not all are likely to be significant *in vivo*. Large numbers of proteins are also synthesised on *in vitro* translation of other plant viral RNAs, eg. PVX (see Figs.45,46 and S.E.ADAMS, 1985). Without further information it is not possible to ascribe a function to any of the BaYMV translation products. There are no products of 35K or 29/30K which would correspond, in size at least, to coat protein. Western blotting or immunoprecipitation experiments using antisera raised to purified virus or inclusion bodies might yield information of this sort.

Fig.47 shows the wheatgerm translation products of polysomes and polysomal RNA from healthy and infected tissue. There is no apparent difference between the translation products of RNA from either source. Thus viral and infection specific RNAs could at most constitute only a small fraction of RNA being translated in the infected tissue used.

3.9 Soluble Plant Proteins

Infection of many plant species with viruses induces the accumulation of so-called 'pathogenesis-related' proteins (PR-proteins) characterised by their selective extraction at low pH and intercellular location (reviewed VAN LOON, 1985). The existence of similar proteins in BaYMV-infected tissue was investigated as described. No such proteins have been reported for any virus-infected monocotyledonous plant. In view of the temperature-dependence of the infection process protein samples were extracted from infected plants grown under growth cabinet conditions, from plants grown in glass cloches on the roof in the winter and from plants transferred to the heated greenhouse (see 2.7.1a for growth conditions). In addition the effects of temperature on gene expression in a number of different cultivars of barley, varying in BaYMV susceptibility, were

investigated by growing virus-free plants in the greenhouse and growth cabinet and extracting proteins in a similar way. These plants were harvested as seedlings so were younger than plants used above. Finally intercellular fluids were extracted and monitored for the presence of PR-proteins.

Soluble plant protein samples, prepared as described (2.19) from equivalent amounts of tissue (≈0.2g), were analysed by non-denaturing disc-PAGE (2.6.3c). Yields of protein varied from sample to sample but these differences were not consistently cultivar- or infection-specific so were probably due to extraction efficiency. Thus only qualitative and relative quantitative comparisons can be made.

To summarise the results briefly; a broadly similar electrophoretic pattern of bands was produced in all cases but age-, cultivar- and temperature-specific quantitative differences and one possible infection-specific qualitative difference were found. Table 10 lists the major bands found and their approximate Rf's.

Table 10

Analysis of Figs. 48-50

Band	Rf	Comments
A	0.10	Doublet. Found in IF. Higher temperatures decrease in expression in some cultivars
B	0.18	Similar temperature effect as for A in the same cultivars.
C	0.27	
D	0.33	
E	0.36	
	-39	Low levels of expression in seedlings.
F	0.40	
	-44	Low levels of expression in seedlings.
G	0.45	
	-49	Most prominent band in cold grown plants.
H	0.56	Higher levels in Birgit & Igri. Higher temperatures
	-60	increase expression in healthy seedlings.
I	0.63	
	-67	Possible infection-specific protein.
J	0.70	
	-74	Stronger in Maris Otter than Tipper.
K	0.73	
	-78	

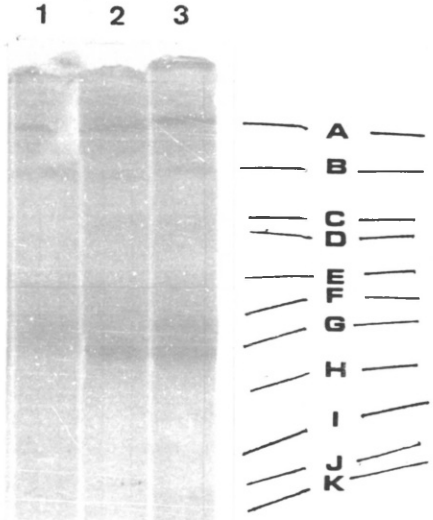


Fig.48

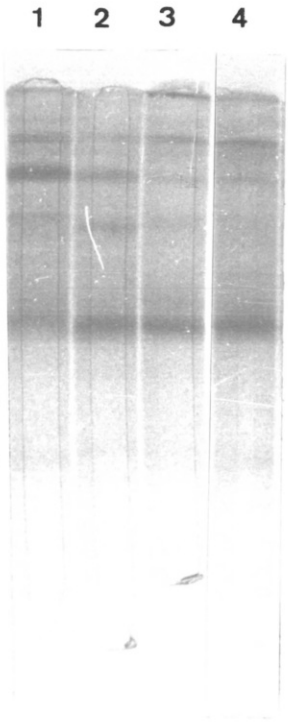


Fig.49

Fig.48 Tube gels of soluble proteins extracted as described in 2.19.1 from BaYMV-infected Maris Otter plants grown in:

Lane 1; Greenhouse

Lane 2; Glass cloches (outside)

Lane 3; Growth cabinet

Fig.49 Tube gels of soluble proteins extracted as described in 2.19.1 from healthy (Lane 1) and BaYMV-infected (Lanes 2-4) Tipper plants grown in:

Lane 1; Greenhouse

Lane 2; Greenhouse

Lane 3; Glass cloches (outside)

Lane 4; Growth cabinet

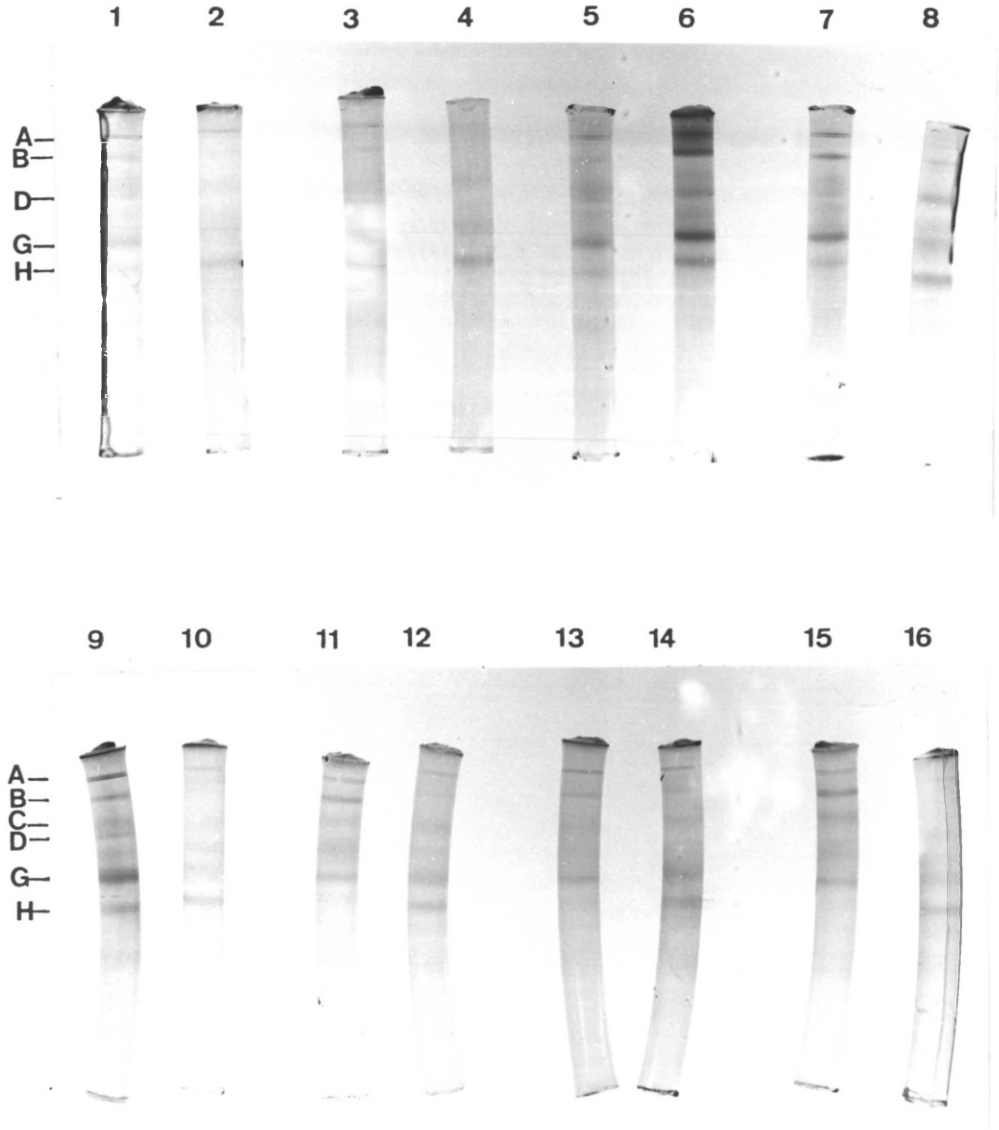


Fig.50 Tube gels of soluble proteins extracted as described in 2.19.1 from different barley cultivars grown in the growth cabinet (odd-numbered gels) and the greenhouse (even-numbered gels)

Gels 1 & 2; Birgit

Gels 3 & 4; Franka

Gels 5 & 6; Gerbel

Gels 7 & 8; Halcyon

Gels 9 & 10; Igri

Gels 11 &12; Maris Otter

Gels 13 & 14; Sonja

Gels 15 & 16; Tipper

Fig.48 shows the pattern of bands obtained from infected Maris Otter plants grown under the various conditions. Bands are labelled for reference to Table 10. Band G is decreased in greenhouse-grown plants relative to the cold-grown plants. Note the faint band I which is not present in extracts from healthy plants.

Fig.49 shows the pattern of bands obtained from healthy and infected Tipper plants grown under the various conditions. There are no major differences but there is a faint band (band I) present in extracts from infected but not healthy plants. Band J is barely visible.

Fig.50 shows the proteins extracted from seedlings of eight cultivars of barley grown in the refrigerated growth cabinet and the heated greenhouse. Yields are generally lower than for the older plants previously extracted and are not always equivalent for the same cultivar but certain trends are apparent. In Tipper, Maris Otter and Halcyon bands A and B are less strongly expressed in greenhouse grown plants relative to total protein. This effect in Igri and Birgit reflects the much lower yields of total protein in the respective greenhouse grown plants. Band H is more prominent in cold grown Gerbel and Igri seedlings than any other cultivars and levels are higher, relative to band G, in all greenhouse plants. In the latter plants band H represents the most abundant protein in nearly all cultivars, band G being equally abundant in the others.

Electrophoretic analysis of a dilution series of the intercellular fluids from healthy and infected Tipper plants showed that the only proteins detectable, even at zero dilution, were those constituting doublet A. The doublet was detectable in infected plants at approximately twice the dilution permissible for detection in healthy plants. However this may be due to extraction efficiency.

These results demonstrate that BaYMV infection of barley does not induce high levels of proteins with the characteristics of PR-proteins. One infection-specific

protein is detectable but at such low levels that this may be a quantitative rather than qualitative effect. This protein is not preferentially extracted in intercellular fluid extracts. Temperature-, age- and cultivar-specific differences in electrophoretic patterns are seen but without further information it is difficult to relate these to the infection process or resistance/susceptibility. However, in view of the temperature dependence of BaYMV infection, it is interesting to note that the decrease in expression with increased temperature of band A, which is also found in IF, is only apparent in the two-rowed, susceptible cultivars Halcyon, Maris Otter and Tipper. Furthermore band H, whose expression is increased in all healthy greenhouse grown cultivars, is also expressed in cold grown plants of the moderately resistant cultivars Igri and Gerbel.

3.10 Investigation of Infected Root Tissue

BaYMV transmission in the field is through the soil so roots are the natural sites of infection of barley plants. The roots of plants infected by mechanical inoculation must also contain virus since virus-free spores of *Polymyxa graminis* acquire BaYMV-infectivity on contact with such roots (KUSABA *et al.*, 1971). Roots are thus a potential site for virus replication from which transport would result in systemic infection. Root tissue from infected plants was therefore examined for the presence of virus particles and viral ss and dsRNA.

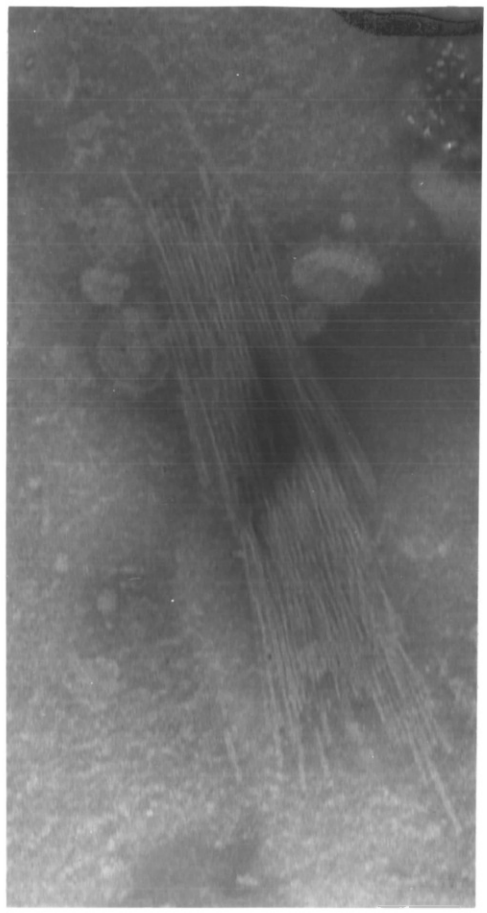
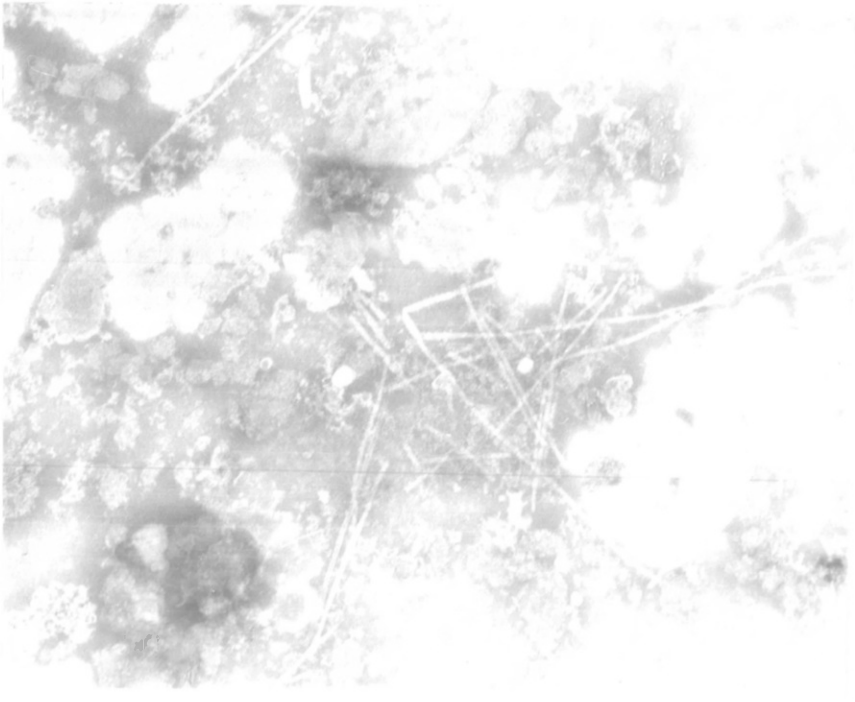


Fig.51 Electron micrograph of a 'root-dip' preparation from BaYMV-infected plants (×60,000).

(a)



(b)

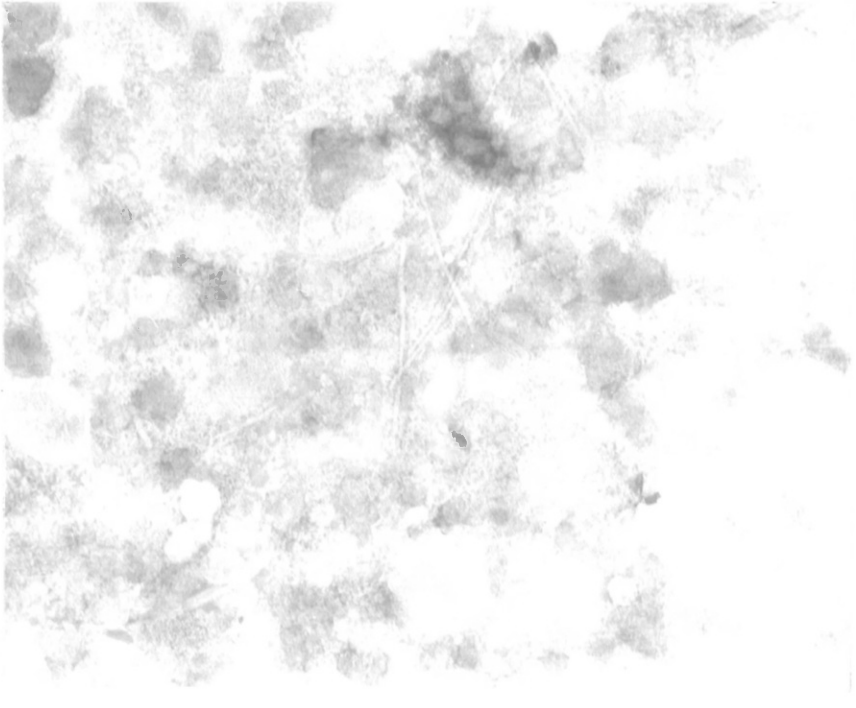


Fig.52 Electron micrographs of ultracentrifugation pellet preparations from roots of BaYMV-infected plants.
Magnifications; (a) ×56,000 (b) ×50,000

Small quantities of root tissue were used to prepare extracts for EM by the leaf dip method (2.9.2). 'Root dip' EM revealed the presence of thin, flexuous filaments at levels comparable to those of virus particles in infected leaves, sometimes aggregated as in Fig. 51. No such filaments were found in similar extracts from roots of uninfected plants. Larger quantities of root material (≈40g) were washed free of soil and treated as for a virus preparation up to the stage of the first ultracentrifuge pellet. Electron micrographs of these pellets (Fig.52) again revealed the presence of virus-like particles. A faint central canal can be seen in some filaments, in both root-dip and pellet preparations, and where this is visible it extends the entire filament length. For virus particles the visibility of the canal was suggested to depend on RNA depletion allowing more efficient stain penetration (3.3). RNA extraction of the ultracentrifugation pellets did not yield any intact BaYMV RNAs detectable by denaturing gel electrophoresis even at sensitivities 10× those required routinely to detect BaYMV RNAs in equivalent extracts from infected leaves. It was thus tempting to propose assembly of capsid proteins without RNA to explain the presence of virus-like filaments. However, in ISEM tests no particles were adsorbed from either the 'root dip' or pellet preparations while tests of leaves of the same plants were positive.

Three separate extractions of double-stranded RNA from roots, by the same procedure used for leaves, failed to produce detectable levels of dsRNA from 40g of material.

The presence of filaments antigenically distinct from virus particles in roots of BaYMV-infected plants is difficult to explain. The absence of virus particles in material from which *P.graminis* acquires infectivity is equally so. Speculation on the host or virus derivation of these filaments is hardly justifiable on the evidence but, if virus encoded, they may be inclusions or particles

modified in some fashion, possibly concerning their fungal transmission. In this context it is interesting to note that LANGENBERG & VAN DER WAL (1986) failed to detect BaYMV by immunogold labelling in thin sections of *P.graminis* in roots of BaYMV-infected plants. Particles were detected in host cells but it is unclear from the report if these were root or leaf cells. The lack of extractable ssRNA in filament preparations argues against a functional role for them in infection and the failure to find dsRNA is at variance with the suggestion of roots as a site of virus replication. While 40g of tissue does not greatly exceed the minimal amount of tissue required for routine dsRNA detection in leaves higher levels of dsRNA ought to be extractable from tissue active in virus replication.

4 CONCLUSIONS

Most plant viruses whose genome strategies have been analysed in detail replicate to high levels in host plants and are relatively easy to purify. Similar studies on viruses that do not have these experimental advantages may, however, reveal subtle variations on the strategies so far discovered. It is hoped that methods developed in this study in conjunction with the powerful technologies of molecular biology will help to determine if this is so for BaYMV. Furthermore, elucidation of the life cycle of BaYMV may suggest ways in which its pathological effects can be controlled until suitable resistant cultivars of barley are available.

The information obtained in this study represents the first steps in this direction. From the results presented here it appears that BaYMV is a multicomponent RNA virus encapsidating at least two RNA species which do not have extensive sequence homology. Since they were not found to be infectious it is possible that other genomic RNAs exist. Analysis of genome structure and organisation was hampered by difficulties in obtaining sufficient quantities of viral RNA but preliminary conclusions can be drawn.

The inability of viral RNAs to bind to oligo-dT-cellulose columns or support oligo-dT-primed cDNA synthesis argues against the presence of 3' poly(A) tails but a confirmation of this must await sequence analysis. No evidence was obtained on 5' terminal structures. *In vitro* translation of BaYMV RNAs produced a number of polypeptides, some of which could be tentatively ascribed to one particular RNA species, but none of which comigrated with BaYMV capsid protein whose gene is thus unlikely to be at the 5' end of either RNA. Plant virus coat protein cistrons are usually located at 3' ends as befits their expression late in the replication cycle. The total length of *in vitro* products exceeds the estimated coding capacity

of BaYMV RNAs (≈390K) so some polypeptides must be ascribed to readthrough, premature termination or the presence of discrete RNA breakdown products. Whether this occurs *in vivo* is not known. Shortage of RNA precluded time-course experiments which may have given evidence of polyprotein processing. Such a suggestion might be supported by the presence of genomic-length RNAs in polysome preparations except for the contamination of such extracts with virus particles. Furthermore, the large number of dsRNA species found are more compatible with a genome strategy involving subgenomic RNAs. Some of the dsRNAs may have artefactual origin but, for viruses such as CPMV which do employ protein processing, no other dsRNAs are found apart from those of genomic length. The two minor bands seen in Northern blots of polysomal RNA may be due to stacking artefacts involving ribosomal RNAs but if not could represent subgenomic RNAs of sizes ca. 0.38 & 0.7 $\times 10^6$. Putative replicative forms of these RNAs would have molecular weights of 0.76 & 1.4 $\times 10^6$ so could correspond to the dsRNA species of ca. 0.75 & 1.3 $\times 10^6$ found in extracts of nearly all infected plants investigated. These RNAs were often the major species in inoculated plants and both hybridised to BaYMV-specific cDNA. The smaller of the two would have a coding capacity of ca. 38K which would be sufficient for expression of capsid protein.

Many of the conclusions are speculative and further work is needed to determine the genome strategy of BaYMV but if it transpired that subgenomic RNAs are employed it would be difficult to justify inclusion of a virus with more than one non-polyadenylated RNA in the potyvirus group whose members have a single polyadenylated RNA component and appear to utilise polyprotein processing in their gene expression.

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