BIOCHEMICAL CHARACTERISATION OF VIRUSES ISOLATED FROM THE PHYTOPATHOGENIC FUNGUS, GAEUMANNOMYCES GRAMINIS VAR. TRITICI

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ABSTRACT

Characterisation of the isometric double-stranded (ds) RNA viruses of the fungus, Gaeumannomyces graminis var. tritici (Ggt), the causative agent of "take-all" disease of wheat and barley, is described. Total nucleic acid was extracted from homogenised mycelium of each of 20 field isolates differing widely in geographical origin. Extracts were treated with DNAase and RNAase under appropriate conditions to remove DNA and single-stranded RNA respectively; the remaining ds-RNA was then analysed by polyacrylamide gel electrophoresis. Isolates were found to contain different numbers of ds-RNA components varying from one up to twelve, with molecular weights from 1 x 10^6 to greater than 3 x 10^6. No two isolates contained the same distribution of ds-RNA components.

Virus preparations were obtained and purified from twelve of the isolates found to contain ds-RNA. Evidence was obtained for at least twelve different isometric variants infecting these isolates. Of the twelve variants, two were 40 nm in diameter and ten were 35 nm in diameter. The two 40 nm viruses, which were serologically different from each other and from the 35 nm viruses, had capsid polypeptides with molecular weights of 125,000 and 94,000 and ds-RNA components with molecular weights in the range 5.4 x 10^6 to 6.0 x 10^6. The ten 35 nm variants had capsid polypeptides with molecular weights ranging from 73,000 to 48,000 and ds-RNA components with molecular weights ranging from 1.64 x 10^6 to 1.00 x 10^6.

Inhibition of mycelial growth in certain isolates containing viruses was caused by a diffusible substance released by some ascospore isolates, not containing viruses, on potato dextrose agar buffered below pH 5.0 at 24°C. Isolates causing inhibition grew very little at or below this pH value but generally grew normally at values about pH 5.5.

The implications of these results in relation to the biology of Ggt and prospects for the practical control of "take-all" disease are discussed.

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INTRODUCTION

Mycoviruses (fungal viruses) have been recently reviewed by Lemke (1976), Buck (1977) and Hollings (1978).

Occurrence of viruses in the fungal kingdom

Virus particles have been discovered in just over 100 fungal species from more than 60 genera spread among all the main taxonomic groups of fungi. Bozarth (1972) estimated that 10 to 15% of randomly sampled fungus cultures contained virus particles, as detected by electron microscopy and therefore believed that a virus could be found in any species with diligent searching. Using a serological test for double-stranded RNA (ds-RNA), Moffitt and Lister (1975) found that of phenol extracts from 70 selected fungal isolates, 20 isolates reacted positively. Some, though not all, of the positive reactions were correlated with the presence of virus particles in clarified buffer extracts from mycelia and to ds-RNA detected in phenol extracts by polyacrylamide gel electrophoresis or sucrose density gradient centrifugation. This work suggested the widespread occurrence of ds-RNA viruses in fungi. At a conservative estimate, virus infection might therefore be expected in some 5000 fungal species (Hollings, 1978). The actual number of distinct fungal viruses will depend on how often a particular virus occurs in different fungal species and on the number of viruses which can infect a particular species.

Virus particles have been found in several plant pathogenic fungi (Table 1) (C.J. Rawlinson, personal communication).

Table 1 Plant pathogenic fungi infected with virus

Alternaria tenuis
Cochliobolus miyabeanus
Colletotrichum atramentarium
C. lindemuthianum
Corticium rolfsii
Cronartium fusiforme
Diplocarpon rosae
Erysiphe graminis f. sp. agropyri
E. graminis f. sp. hordei
E. graminis f. sp. tritici
Fusarium culmorum
F. oxysporum f. sp. lini
F. moniliforme
F. roseum
Gaeumannomyces graminis var. avenae
G. graminis var. graminis
G. graminis var. tritici
Glomerella cingulata var. orbiculare
Gonatobotrys sp.
Helminthosporium maydis
H. sacchari
H. victoriae
Microsphaera mougeotii
Periconia circinata
Phialophora radicicola var. graminicola
P. radicicola var. radicicola
Puccinia allii
P. coronata
P. graminis f. sp. tritici
P. horiana
P. malvacearum
P. recondita
P. striiformis
P. suaveolens
Pyricularia oryzae
Sclerotium cepivorum
Sphaerotheca fuliginea
Stemphylium botryosum
Thanatephorus cucumeris
Thielaviopsis basicola
Uromyces alopecuri
U. durus
Ustilago maydis
U. phaseoli
Verticillium dahliae
V. fungicola
V. malthousei

Some viruses in closely related fungal species have been found to be serologically related. An example is the serological relationship found between Penicillium cyaneo-fulvum virus and Penicillium chrysogenum virus (Buck and Girvan, 1977). In addition, the isometric particles were the same diameter, 36 nm and the amino acid compositions of their capsid polypeptides were very similar. There are also reports of serological relationships between viruses from different fungal genera, for example, between the isometric 34 nm virus from Diplocarpon rosae and Penicillium stoloniferum virus S (Bozarth et al., 1972). Mixed infections with two serologically unrelated viruses appear to be quite common in fungi, for example P. stoloniferum virus S and P. stoloniferum virus F (Buck and Kempson-Jones, 1970) and Aspergillus foetidus viruses S and F (Buck and Ratti, 1975a).

Properties of double-stranded RNA mycoviruses

Ds-RNA mycoviruses belong to class III of the Baltimore Classification (Baltimore, 1971). The other two families of ds-RNA viruses are the Reoviridae (Fenner, 1975 and 1976) and the Cystoviridae (Fenner, 1976). The Reoviridae have ten to twelve molecules of linear ds-RNA, total molecular weight 12 x 10^6 to 15 x 10^6, all enclosed within one virion, which also contains a transcriptase and other enzymes. The icosahedral particles diameter 50 to 80 nm have a characteristic double protein shell and multiply in the cytoplasm. The family contains several genera, for example reovirus (mammalian, avian and simian reoviruses), orbivirus (these have ring-like capsomers e.g. blue tongue virus of sheep), cytoplasmic polyhedrosis

viruses of insects, mammalian rotaviruses (these have a wheel-like appearance and are the major cause of infantile gastroenteritis) and plant viruses e.g. clover wound tumour virus. Bacteriophage ϕ6 from Pseudomonas phaseolytica, which is the only known member of the Cystoviridae family, has enveloped virions with icosahedral nucleocapsids. The linear ds-RNA is in three pieces, total molecular weight 13 x 10^6 which are all enclosed within one virion.

The known ds-RNA mycoviruses, which may ultimately be classified in more than one family, have one to six molecules of linear ds-RNA, total molecular weight 2 x 10^6 to 9 x 10^6 and each encapsidated in separate virions. The icosahedral particles of diameter 25 to 45 nm which contain an RNA polymerase, multiply and mature in the cytoplasm. Their molecular weights range from 6 x 10^6 to 21 x 10^6. The capsids are single-layered consisting of one major species of polypeptide with molecular weights ranging from 42,000 to 130,000. The molecular weights of the ds-RNA molecules encapsidated range from 0.3 x 10^6 to 6.3 x 10^6. The virus RNA polymerase molecules have been identified in only two cases: P. stoloniferum virus S particles contain one molecule of single-chain RNA polymerase of molecular weight 55,500 and 120 molecules of capsid polypeptide, molecular weight, 42,500 whereas P. stoloniferum virus F particles contain one or two molecules of RNA polymerase of molecular weight 59,000 (capsid polypeptide molecular weight, 47,000) (Buck and Kempson-Jones, 1974).

Unlike members of the Reoviridae which are virulent, many mycoviruses are avirulent, having no adverse effect on the host

cells and replicating in parallel with them. The viruses infecting the cultivated mushroom are the only well-documented example of virulent mycoviruses (Hollings and Stone, 1971). Studies of the polymerases of virus particles from P. stoloniferum and A. foetidus led Buck and Ratti (1975b) to propose that mycoviruses are replicated by doubling. This is in sharp contrast to other viruses which produce very many progeny from a single infecting particle in order to ensure that some survive the extracellular phase of their life cycle to infect other susceptible hosts. A replication cycle by doubling, however, may be particularly suited to the double-stranded RNA mycoviruses, which do not require an extracellular phase in their life cycle but always remain intracellular, being carried from one host generation to another. Mycoviruses have so far been found in conidiospores, basidiospores, urediniospores, zoospores and chlamydospores of some fungi, but not in ascospores. As might be expected from their life cycle, all attempts at transmission of mycoviruses by mechanical inoculation have failed, with the one exception of the mushroom viruses. Nearly all successful transmissions have been effected by hyphal anastomosis or heterokaryosis.

Since ds-RNA mycoviruses never need to infect from without, the separate encapsidation of genome segments is not a disadvantage, indeed it may be an advantage in that it allows considerable variation in the proportion of different RNA components, which could be important in the control of gene expression either by selective gene amplification or suppression. Multi-partite genomes are considered to promote rapid

recombinations in mixed infections with different strains of a virus, by selecting components from a pool, obviating the need for recombination enzymes and thus providing an evolutionary advantage for such a virus.

In addition to being avirulent, ds-RNA mycoviruses appear to have no direct effect on the production of secondary metabolites by their hosts, for example penicillin by P. chrysogenum (Lemke et al., 1973), or on the pathogenicity of phytopathogenic fungi, such as Gaeumannomyces graminis (Rawlinson et al., 1973) or Periconia circinata (Dunkle, 1974).

Some mycoviruses may, in fact, have a symbiotic relationship with their hosts. It has been suggested that virus particles in "killer" strains of the yeast Saccharomyces cerevisiae may confer a considerable selective advantage on the host under certain environmental conditions (Herring and Bevan, 1974). The killer of Saccharomyces cerevisiae has been recently reviewed by Wickner (1976). The killer reaction in yeast, Saccharomyces cerevisiae, was discovered by Bevan and Makower (1963), who described how certain strains could be classified into one of three phenotypes which they termed killer, sensitive and neutral. When killer and sensitive cells are grown together in the same culture medium, a high proportion of the latter are killed. Neutral cells neither kill sensitive cells nor are they killed by killer cells. Killing can occur without cell contact between killer and sensitive cells and is caused by the release of killer factor by the killer cells. The killer factor is an unstable glycoprotein which is inactivated by papain. It has a highly specific action spectrum; the optimal pH for the

production of the killer factor in liquid medium lies within the narrow range pH 4.6 to 4.8 and it is inactivated by temperatures above 25°C (Woods and Bevan, 1968). These properties bear a striking resemblance to those of the bacteriocins, which are plasmid-coded.

Both killer and neutral phenotypes are inherited cytoplasmically but a number of nuclear genes affects the maintenance and expression of killer and neutral determinants. Normal killer strains always have two types of viruses, V1 and V2. V1 has a ds-RNA molecule P1 of molecular weight 2.92×10^6 and a capsid polypeptide of molecular weight 75,000. V2 has a ds-RNA molecule P2 of molecular weight 1.39×10^6 and also has a capsid polypeptide of molecular weight 75,000. Sensitive cells either possess only V1 or completely lack virus (Bevan and Mitchell, 1978). The maintenance or replication of V2 requires nine chromosomal genes (mak), whereas expression of killing and resistance requires three other chromosomal genes (kex and rex). Under a variety of conditions where sensitive clones have been segregated from killer parents, it was found that the mutation to the stable sensitive phenotype was accompanied by the loss of the smaller ds-RNA (Mitchell et al., 1973). This suggests that the smaller ds-RNA may code for both killer factor and an immunity factor. Since V1 can occur alone in sensitive strains, it is reasonable to assume that the P1 ds-RNA is dicistronic, coding for both the capsid polypeptide and RNA polymerase.

A survey of laboratory killer strains revealed that although the P1 ds-RNA species had the same estimated molecular weights, the P2 species differed in molecular weight, although,

with one exception, these strains produced the same type of toxin, TOX1.

Killer strains are not confined to laboratory stocks of Saccharomyces cerevisiae. They are found amongst both pathogenic yeasts and those used in wine and beer fermentations (Rogers and Bevan, 1978). The strains tested fell into four groups designated TOX1 to TOX4 according to their killing/immunity reaction with one another and their ability to kill mutants resistant to one or more of the toxins. In all such strains with the exception of one, an isolate of Candida albicans, ds-RNA has been shown to be present wherever it has been looked for. Associated viruses have so far only been demonstrated in a few cases.

Killer strains of Ustilago maydis have also been discovered and the ability to produce killer and immunity factors, which are inherited cytoplasmically, is associated with the presence of ds-RNA virus particles (Koltin and Day, 1976). In this case, however, resistance to killer toxin can also be conferred by nuclear genes. Three different killer specificities have been found in U. maydis; all three killers can kill each other as well as sensitive strains. Killer strains carry virus particles about 41 nm in diameter. Each killer possesses distinct ds-RNA components that range in molecular weight from 0.46×10^6 to 2.9×10^6. Two components are shared by all three killers whereas immune strains possess some different components. Crosses and heterokaryons between different killers revealed unilateral or mutual restrictions that prevent inclusion of two killer specificities in the same cell.

Yeast mycoviral ds-RNA has been found to hybridize to host cell DNA regardless of the strains from which the respective nucleic acids were prepared (Vodkin, 1977). One possible explanation of the sequence homology between virion nucleic acids and cell DNA is that DNA copies of the virus RNA may become integrated into the host DNA as a provirus, which is then replicated as part of the host genome (Buck, 1978). This would then constitute an explanation of a way in which mycoviruses are transmitted from generation to generation. There is some circumstancial evidence in support of this suggestion from studies on the introduction of Gaeumannomyces graminis var. tritici (Ggt), and its viruses, into crops of barley or wheat, grown in a field which had carried no cereal crop and hence no Ggt for more than 100 years (Rawlinson, 1976). For three years, no virus-infected isolates were found but within a further three months, 17 of 38 isolates examined from widely separated plants contained the smaller virus only of the two (27 nm and 35 nm diameter) commonly found in the fungus. One possible explanation of these observations is the induction of provirus from the host genome. This seems to be the most plausible of the possible explanations which include the postulation of either a very efficient vector in soil, a new virus-infected source of take-all inoculum, or that free RNA (unencapsulated), or undetected low amounts of virus were suddenly stimulated to multiply.

The viruses of Gaeumannomyces graminis var. tritici and their biological implications

Take-all disease of cereals is caused by Gaeumannomyces

graminis (Sacc.) Arx and Olivier (syn. Ophiobolus graminis (Sacc.) Sacc.). This serious disease is primarily a root rot which in an advanced stage can also damage the stem base severely (Nilsson, 1969). Severely infected plants have extensively blackened roots, stunted aerial growth, chlorotic foliage and frequently occur in patches in the crop. Those that survive until ripening die prematurely and become very conspicuous as blanched plants, which in wet weather are blackened by mould growth. These plants called "whiteheads" are easily pulled and contain little or no grain. Less severe infections adversely affect grain size and appearance, but even without aboveground symptoms yield is frequently reduced. The symptoms are caused by mycelial invasion of the roots leading to disintegration and blockage of tissues both in the cortex and vascular cylinder, thereby impairing plant nutrition and water supply. No resistant varieties or practical and economical fungicides are known for the field control of this disease. Crop rotation and adequate use of fertilizers (especially nitrogen) are the only methods currently available which offer some measure of control. Economic considerations dictate cereal monoculture in many areas which may restrict the use of even these practices. The absence of economic, conventional control measures for this disease has encouraged research into possible methods of biological control.

Gaeumannomyces graminis var. tritici (Ggt) affects wheat and barley and, when these crops are grown consecutively, disease may increase to a maximum (often in the third crop) and then subside in subsequent crops, exhibiting the phenomenon of take-all

decline (Slope and Cox, 1964; Gerlagh, 1968). Attempts to explain take-all decline have included ideas such as microbial antagonism, effects of nutritional changes in the rhizosphere and hypovirulent or avirulent fungi antagonistic to the pathogen. In a recent review, Hornby (1978) concluded that take-all decline was a theorist's paradise.

In 1970, Lapierre et al. found virus particles, diameter 29 nm, in two of the seven Ggt isolates examined. These were isolated after six and twelve years of continuous culture of wheat (during the period of take-all decline). The virus infected isolates in pure culture were little different in growth to healthy isolates; they were also able to infect the host plant but only produced weak symptoms, a weak attack of the roots without necrosis on the stem base. These authors concluded that a relationship existed between the presence of virus particles and the reduction of pathogenicity. The next year, Lemaire et al. (1971) described two methods of biologically controlling take-all disease. The first method utilised the antagonistic properties of soil microorganisms which were enhanced by the incorporation of nutrient proteins in the soil. The second method involved the mixing of virus-infected weakly pathogenic isolates, taken from take-all decline soils, with uninfected pathogenic isolates, taken from soils in which the take-all disease had not then reached its maximum. This constituted the first report of biological control of a plant pathogen with a virus.

At Rothamsted Experimental Station, Rawlinson et al. (1973) found that isometric viruses measuring 35 nm and 27 nm occurred

in cultured Ggt mycelium. The 35 nm virus was absent from eleven Ggt isolates from first cereal crops after fallow or non-susceptible break crops; two of these contained the 27 nm particles. More than half of 145 isolates, from cereals after two to twelve consecutive susceptible crops, contained either 35 nm or 27 nm viruses. Viruses were not confined to Ggt isolates from soils exhibiting take-all decline nor consistently associated with weak pathogenicity or with isolates of unusual growth, morphology, pigmentation, lysis or readiness to form perithecia. Isolates with one kind of particle were mostly more pathogenic and those with both kinds less pathogenic than isolates without virus. The proportion of isolates with 27 nm and 35 nm particles increased progressively in samples from different consecutive crops during the first nine years of cropping, then decreased. This work was therefore in accord with the discovery that many mycovirus infections are latent. Table 2 lists the sizes of virus particles found in a number of Ggt isolates.[1] A full description of the isolates is to be found in Table 3.

Table 2 Diameters of isometric virus particles in the Ggt field isolates examined

(Determined at Rothamsted)

Isolate	Diameter(s) of virus particles (nm)	Isolate	Diameter(s) of virus particles (nm)
45/10	35	F6	35
45/9	35 and 27	F7	40 and 35
45/8	27	F10	40 and 35
021/5	27	F11	40 and 35
235-1	27	F17	40, 35 and 27
254-2	27	F20	40 and 35
38-4	35 and 27	F22	40 and 35
PP2a	35	African 1	35
374-2	35	African 3	35
019/6	35	U.S.A. 3	35
3b1a	35 and 27	01-1-3	40 and 30
0gA	35 and 27	01-1-4	30
F3	40, 35 and 27	Aust. T1	35

Three types of vegetative incompatibility of fungi have been distinguished:

(i) Heterokaryon incompatibility. Hyphae from opposing mycelia anastamose and interaction of the two protoplasms leads to destruction of the affected region in each hypha. (Garnjobst and Wilson, 1956).

(ii) Fusion incompatibility. Inability of hyphae to anastamose caused by the production of diffusible sterility - inducing substances by sterility mutants (Carr and Olive, 1959).

(iii) Killer reactions. Killing at a distance of sensitive strains by a diffusible killer toxin produced by killer strains (Bevan and Makower, 1963).

Vegetative incompatibility reactions between paired Ggt isolates were observed (C.J. Rawlinson, unpublished work) which resembled the killing reactions of Saccharomyces cerevisiae (Bevan and Makower, 1963) and of Ustilago maydis (Koltin and Day, 1976). This suggested that some isolates of Ggt were capable of producing a killer factor. The observation by Lemaire et al. (1975) that wheat roots preinoculated with virus-infected weakly pathogenic Ggt isolates produced a substance, diffusible in agar, which inhibited the growth of pathogenic isolates also indicated that some Ggt isolates could produce a killer factor, which was associated with virus infection.

Scope of the thesis

Although Rawlinson et al. (1973) found no obvious correlation between phytopathogenicity and presence or absence of the two sizes of virus particles commonly found in Ggt, possible

correlations with specific ds-RNA molecules were not investigated. In addition, Herring and Bevan (1974) found that killer strains of yeast were infected with viruses which contained two specific ds-RNA molecules whereas other strains possessed only one. It was therefore decided to prepare virus from a range of Ggt isolates of differing pathogenicity and to compare the RNA and polypeptide components of each type of virus particle. This work was seen as a prerequisite for the examination of possible relationships between specific ds-RNA components and fungal pathogenicity. It was also decided to investigate further the vegetative incompatibility reactions between certain paired Ggt isolates in order to determine whether they were a killer reaction and, if so, whether there was a relationship between specific virus ds-RNA components and the production of the killer factor.

This fundamental study of the rôle of virus in Ggt was considered to be of value in furthering work on the biological control of the fungus, either by using virus infection to lower fungal pathogenicity or by the use of strains producing killer factors.

It was decided to begin the study by isolating, purifying and characterising the 35 nm virus particles from Ggt 45/10, the 27 nm virus particles from Ggt 235-1 and a mixture of the two virus particle sizes from Ggt 45/9.

ABBREVIATIONS

These are as given in the instructions to authors in the Biochemical Journal (1976).

Buffers are abbreviated:

P buffer:	0.03M-sodium phosphate, pH 7.6.
PK buffer:	0.03M-sodium phosphate + 0.15M-potassium chloride, pH 7.6.
PNaCl buffer:	0.03M-sodium phosphate + 0.15M-sodium chloride, pH 7.6.
TAE buffer:	0.04M-tris + 0.02M-sodium acetate + 0.0013M-EDTA, pH 8.0.
SSC:	0.15M-sodium chloride + 0.015M-sodium citrate, pH 7.0.
TSE buffer:	0.1M-tris-HCl + 0.1M-sodium chloride + 0.01M-EDTA, pH 7.0.
STM buffer:	0.01M-tris-HCl + 0.1M-sodium chloride + 0.01M-magnesium chloride, pH 7.3.
CP buffer:	0.04M-disodium hydrogen phosphate titrated with 0.1M citric acid to the required pH.

Other abbreviations used are:

p.a.g.e.:	polyacrylamide gel electrophoresis.
PEG:	polyethylene glycol.
SDS:	sodium dodecyl sulphate.
Ggt:	*Gaeumannomyces graminis* var. *tritici*.
DNAase:	deoxyribonuclease.
RNAase:	ribonuclease.
PDA:	potato dextrose agar.
ds-RNA:	double-stranded RNA.

MATERIALS

Glass distilled water was used throughout.

Reagents: all reagents, unless otherwise stated, were of the highest available grade.

Coomassie Brilliant Blue G was obtained from the Sigma Chemical Co. (St. Louis, Missouri, U.S.A.) and Toluidine Blue was from Hopkin and Williams (Chadwell Heath, Essex, England).

DEAE cellulose (Whatman DE52) was supplied by Whatman Ltd. (Maidstone, Kent, England).

Visking tubing (Scientific Instrument Centre, London, England) was used for all dialyses.

Standard buffer solution, pH 7.0 (phosphate) (Fisons Ltd., Loughborough, Leicestershire, England) was used for the standardisation of the direct reading pH meter.

E. coli RNA polymerase, phosphorylase a, aldolase, pyruvate kinase, glutamate dehydrogenase and lactate dehydrogenase were obtained from Boehringer GmbH (Mannheim, Germany). Bovine serum albumin was from Koch-Light Laboratories Ltd. (Colnbrook, Buckinghamshire, England). DNAase 1 (RNAase free) and RNAase A were obtained from the Worthington Biochemical Corp. (Freehold, New Jersey, U.S.A.).

PDA (dehydrated) was from Oxoid Ltd. (London S.E.1., England) or Difco Laboratories (Detroit, Michigan, U.S.A.).

GENERAL METHODS

G.M.1. Organisms and microbiological methods

The following Ggt field isolates were used:

Table 3 Description of the Ggt field isolates

Isolate	Host	Source and Site/Field	Date isolated
45/10	12th consecutive wheat	C.J. Rawlinson, Rothamsted Little Knott field, England	1972
45/9	12th consecutive wheat	C.J. Rawlinson, Rothamsted Little Knott field, England	1972
45/8	12th consecutive wheat	C.J. Rawlinson, Rothamsted Little Knott field, England	1972
021/5	2nd barley after wheat	C.J. Rawlinson, Rothamsted Highfield, England	1972
235-1	2nd wheat. No previous cereals for more than 100 years	D.B. Slope, Rothamsted Barnfield, England	1975
254-2	2nd wheat. No previous cereals for more than 100 years	D.B. Slope, Rothamsted Barnfield, England	1975
38-4	barley	D.B. Slope, Rothamsted Hoosfield, England	1973
PP2a	2nd wheat	C.J. Rawlinson, Rothamsted Pennells Piece, England	1971
374-2	3rd wheat	D.B. Slope, Rothamsted Summerdells, England	1974
019/6	barley after wheat	C.J. Rawlinson, Rothamsted Highfield, England	1972
3b1a	3rd wheat	M. Chu Chou, Rothamsted Little Knott field, England	1970
OgA	2nd wheat	J.E.E. Jenkins, Selby, England	1972
F3	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974

Table 3 Description of the Ggt field isolates (continued)

Isolate	Host	Source and Site/Field	Date isolated
F6	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F7	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F10	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F11	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F17	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F20	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F22	2nd wheat	C.J. Rawlinson, Lerheu near Rennes, France	1974
F911-1	12th wheat	J-M. Lemaire, Rennes, France (single ascospore isolate)	1969
African 1	wheat	K. Bock, Kenya	1974
African 3	wheat	K. Bock, Kenya	1974
U.S.A. 3	wheat	R.M. Lister, Vincennes, Indiana, U.S.A.	1973
01-1-3	wheat	S. Yamashita, Gunma, Tokyo, Japan	1950
01-1-4	wheat	S. Yamashita, Gunma, Tokyo, Japan	1950
Aust. T1	wheat	P.T.W. Wong, Carnarvah, Australia.	1974

Cultures were maintained at 4° C in aerobic conditions on unbuffered PDA slopes either in loosely capped McCartney or Bijou bottles and were subcultured at 6 monthly intervals. The Oxoid PDA was prepared by suspending 39 g in 1000 ml cold water and

heating to boiling to dissolve completely. The medium was sterilised by autoclaving for 15 min at 15 lb/in^2 (121° C).

The liquid culture medium used, unless otherwise stated, was basal/corn steep liquor (percentage w/v):

glucose monohydrate	1.00
potassium dihydrogen phosphate	0.10
magnesium sulphate (hydrated)	0.05
ferrous sulphate (hydrated)	0.001
corn steep liquor	3.00

The pH was adjusted to 6.0 with sodium hydroxide solution and the medium sterilised by autoclaving for 20 min at 15 lb/in^2 (121° C).

Occasionally, Weste and Thrower's liquid medium was used (percentage w/v):

glucose monohydrate	1.00
L-asparagine	0.20
potassium dihydrogen phosphate	0.10
magnesium sulphate (hydrated)	0.05
ferrous sulphate (hydrated)	0.0001
manganese chloride (hydrated)	0.0004
zinc sulphate (hydrated)	0.0001
biotin	5.0×10^{-7}
thiamine	1.0×10^{-5}

The pH was adjusted to 6.0 with sodium hydroxide solution and the medium sterilised by autoclaving for 20 min at 15 lb/in^2 (121° C).

Cultures in liquid media (100 ml medium in 500 ml conical flasks) were grown from 1 ml aliquots of mycelium from "master" cultures, suspended in sterile saline and incubated at 24° C for 7 to 14 days on a rotary shaker (200 rev./min, 4.5 cm throw). For small scale preparations of virus, these primary cultures were harvested by filtration. For large scale preparations, they were used to inoculate secondary cultures (1 litre medium in 3 litre conical flasks), which were grown by shaking for 3 to 5 days. A fully grown secondary culture was used as the inoculum for a 60 litre fermenter.

Pilot plant fermentations were carried out in two jacketed stainless steel, sparger aerated fermenters of a total capacity of 100 litres and working capacity of 60 litres. The agitator shaft speeds (183 to 367 rev./min) and air flow rates (30 to 60 litres/min) facilitated heat transfer and culture aeration. Fermenters were maintained at an air pressure of 15 lb/in^2 and the fermentations terminated after 3 days by filtration through a filter press.

G.M.2 Large scale preparation and purification of virus

The isolates were grown on a pilot plant scale as described in G.M.1. A typical wet weight of mycelium was 7.5 kg. The mycelium was suspended in 20 litres of P buffer at 4^{o} C and homogenised by passage through an A.P.V. Manton-Gaulin homogeniser (The A.P.V. Co., Crawley, Sussex, England) at 7,500 lb/in^2. The homogenate was mixed with a further 30 litres of P buffer which was chilled in order to lower the temperature of the homogenate. The degree of breakage of the mycelium was checked by microscopic examination. If the breakage was sufficient, the insoluble debris was removed by passage through a Sharples Super centrifuge (Sharples Centrifuges Ltd., Camberley, Surrey, England), developing a centrifugal force of 13,200 $g_{av.}$ at a flow rate of about 4 litres/min. The volume of the supernatant was adjusted to 70 litres and 6 kg of sodium chloride were added, stirring to dissolve. 30 litres of 30% (w/v) PEG 6000 in P buffer was added dropwise with stirring over 2 to 3 h. Stirring was continued for 1 h and then the suspension was left to precipitate overnight. The precipitate was collected

by passage through the Sharples Super centrifuge and resuspended in 4 litres of P buffer. The final volume was made up to 5 litres. The suspension was left to stir for several hours at 4^{o} C, then the insoluble debris was removed by centrifugation at 23,000 g for 1 h. Virus was pelleted from the supernatant by centrifugation overnight at 23,000 to 30,000 g. The pellets were taken up in a total of 100 ml of P buffer and the solution was clarified by low speed centrifugation. A number of final purification methods, described in the experimental and results section, were tried.

G.M.3 Small scale preparation and purification of virus

The mycelium was grown as described in G.M.1 and was collected by filtration. The mycelium was then suspended in 4 ml/g wet weight of chilled PK buffer and homogenised as in G.M.2. After the degree of breakage was checked to be sufficient by microscopic examination, insoluble debris was removed by centrifugation at 23,000 g for 30 min. Sodium chloride was dissolved in the supernatant and 30% (w/v) polyethylene glycol 6000 in PK buffer was added dropwise so that the final concentrations were 1M and 10% (w/v) respectively. The precipitate formed after standing overnight at 4^{o} C was collected by centrifugation at 23,000 g for 20 min, then resuspended in 35 ml of PK buffer by stirring for 2 h. The insoluble material was removed by low speed centrifugation (38,000 g for 30 min) and virus was pelleted from the supernatant by centrifugation overnight at 65,000 g. The virus pellet was resuspended in 0.5 ml of PK buffer and debris was removed from the suspension by centrifugation at 38,000 g for 20 min.

The final purification was performed by centrifugation of the crude virus preparation (0.25 ml) in a 5 ml 10% to 45% (w/w) sucrose gradient in PK buffer at 45,000 rev./min for 90 min in a Spinco SW50.1 rotor. After centrifugation, the centrifuge tube was placed in a Büchler gradient fractionator (Büchler Instrument Inc., Fort Lee, New Jersey, U.S.A.) and surrounded by ice. The bottom of the tube was pierced by a hollow needle and 0.1 ml fractions were collected. Virus particles containing ds-RNA were located in the fractions by spotting 2 µl of each onto a 0.5% (w/v) agarose gel plate containing 2×10^{-3} mg/ml ethidium bromide, then irradiating the gel with short wavelength ultraviolet light. Those fractions containing "full" virus particles fluoresced under these conditions. Fractionation of the "full" virus particles across the velocity sucrose gradient was studied by p.a.g.e. of the ds-RNA and polypeptides using a semi-micro gel system (G.M.7), before appropriate fractions were combined for dialysis against PK buffer (PNaCl buffer if required for antiserum production).

G.M.4 Preparation of dialysis tubing

Suitable lengths of 1-8/32" Visking tubing were boiled in six changes of 0.1% (w/v) sodium bicarbonate. The tubing was rinsed between each change with water. The tubing was then rinsed once with ethanol and once with water. The tubing was boiled twice in 1 mM EDTA, rinsing between each change with water. After boiling for a third time in 1 mM EDTA, the tubing was left in the EDTA solution and stored at 4^{o} C. Each length of the tubing was rinsed with water before use.

G.M.5 Preparation of DEAE cellulose

DEAE cellulose (Whatman DE 52) was prepared in the phosphate form as follows: DEAE cellulose was suspended in dilute phosphoric acid, pH 2.0, evacuated to remove carbon dioxide and neutralised with sodium hydroxide solution to pH 7.6. The DEAE cellulose was suspended in P buffer (0.3 M) and washed until the pH was constant. It was then suspended in P buffer (0.03 M), washed five times and stored at 4^{o} C in P buffer.

G.M.6 Spectrophotometry

U.v. spectra were measured with either a Cary "15" recording spectrophotometer (Applied Physics Corp., Moravia, California, U.S.A.) or a Unicam SP 800 spectrophotometer (Pye-Unicam, Cambridge, England) using silica cells of 1 cm path length. Point measurements were also made using a Gilford spectrophotometer (Gilford Instrument Laboratory Inc., Oberlin, Ohio, U.S.A.). An extinction unit ($E_{260}U$) is defined as the extinction at 260 nm in a 1 cm cell, multiplied by the sample volume. An approximate correction for light scattering was made by subtraction of the reading at 350 nm.

G.M.7 Analytical gel electrophoresis

(a) Polyacrylamide gels for whole virus. 4% gels were prepared essentially as described by Loening (1967). 10 ml of gel solution contained: 1.0 ml of 39.6% (w/v) acrylamide containing 0.4% (w/v) methylene bis-acrylamide, 1.0 ml of 1% (v/v) N, N, N', N'-tetramethylethylenediamine, 1.0 ml of 1.5%

(w/v) ammonium persulphate, 0.4 ml of 25 x TAE buffer and 6.6 ml of deaerated water. The mixture was cooled to 4° C, deaerated for about 2 min and pipetted into vertical glass tubes 7.5 cm long and with internal diameter of 0.45 cm (standard size) sealed with parafilm over the lower ends. When the gels had almost set, water was layered onto the tops of the gels with a finely drawn Pasteur pipette to give a flat surface to the gels. The gels were pre-electrophoresed at 6 ma/tube for 1 h with a running buffer of TAE. Virus samples were normally 50 µl (E_{260} 0.5 to 2.0) and contained 20% (w/v) sucrose. About 3 µl of a saturated bromophenol blue solution was added to each sample to act as a tracker dye. Electrophoresis was carried out at 6 ma/tube for 2 h with running buffer. The capsid polypeptides of the intact virus were visualised by staining with 0.1% (w/v) Coomassie Brilliant Blue G + 10% (w/v) 5-sulphosalicylic acid + 10% (w/v) trichloroacetic acid in water overnight, followed by destaining in 10% (v/v) acetic acid. The RNA of the intact virus was visualised by either staining with 2% (w/v) acridine orange in 10% (v/v) acetic acid overnight, followed by destaining in 10% (v/v) acetic acid or staining with 0.01% (w/v) aqueous toluidine blue overnight, followed by destaining in water.

(b) Polyacrylamide gels for analysis and molecular weight determination of virus RNA. 4% gels were prepared in glass tubes as described above. 10 ml of gel solution contained: 1.0 ml of 39.6% (w/v) acrylamide containing 0.4% (w/v) methylene bis-acrylamide, 1.0 ml of 1% (v/v) N, N, N' N'-tetramethylethylenediamine, 1.0 ml of 1.5% (w/v) ammonium persulphate, 0.8 ml of 25 x TAE

buffer and 6.2 ml of deaerated water. The glass tubes used were 7.5 cm long and with internal diameters of either 0.45 cm (standard size) or 0.25 cm (semi-micro size). The gels were pre-electrophoresed at 6 ma/tube for either 1 h (standard size gels) or 30 min (semi-micro size gels) with a running buffer of 2 x TAE. Prior to electrophoresis, virus preparations were dissociated by addition of SDS (1% (w/v)final concentration) and incubation at 60^{0} C for 20 min. The maximum volume of sample loaded onto standard size gels was 100 µl and onto semi-micro size gels, 10 µl. Samples contained 20% (w/v) RNAase free sucrose and bromophenol blue solution. Electrophoresis was carried out at 6 ma/tube for 2 to 5 h using standard size gels and 1 to 2 h using semi-micro size gels with running buffer. RNA was located by staining with 0.01% (w/v) aqueous toluidine blue overnight, followed by destaining in water.

Molecular weights less than 2.20×10^{6} were determined by co-electrophoresis with suitable ds-RNA markers, fitting a least squares regression line to the two variables, x= mobility in cm and $y = \log_{10}$ (mol. wt.), hence determining the virus RNA molecular weights. Unless otherwise stated, three determinations were performed and the molecular weights for each band averaged. The following ds-RNA molecular weight markers were used: Penicillium chrysogenum virus (PcV): 2.18×10^{6}, 1.99×10^{6} and 1.89×10^{6} (Wood and Bozarth, 1972), Penicillium stoloniferum virus F (PsV-F): 0.99×10^{6} and 0.89×10^{6} (Bozarth, et al., 1971) and Penicillium stoloniferum virus S (PsV-S): 1.11×10^{6} and 0.94×10^{6} (Buck and Kempson-Jones, 1973). Molecular weights greater than 2.20×10^{6} were determined by

coelectrophoresis with suitable ds-RNA markers and a graph drawn of $y = \log_{10}$ (mol. wt.) against x = mobility in cm, hence determining the virus RNA molecular weights (Bozarth and Harley, 1976). Three determinations were performed and the molecular weights for each band averaged. The following ds-RNA molecular weight markers were used: phage Ø6 : 4.87×10^6, 2.87×10^6 and 2.00×10^6 (Semancik et al., 1973) and Aspergillus foetidus virus S (AfV-S): 4.10×10^6, 2.60×10^6 and 0.27×10^6 (Buck and Ratti, 1977).

(c) Polyacrylamide gels for analysis and molecular weight determinations of virus polypeptides. 8% gels of the following composition were prepared in glass tubes as described above. 10 ml of gel solution contained: 2.0 ml of 39.6% (w/v) acrylamide containing 0.4% (w/v) methylene bis acrylamide, 1.0 ml of 1% (v/v) N, N, N', N'-tetramethylethylenediamine, 1.0 ml of 1.5% (w/v) ammonium persulphate, 1.0 ml of 1% (w/v) SDS and 5.0 ml of 0.2M-sodium phosphate buffer containing 0.02M-sodium EDTA, pH 7.2. Gels were pre-electrophoresed as described above with running buffer (0.1 M-sodium phosphate + 0.01 M-sodium EDTA + 0.1% (w/v) SDS, pH 7.2). Prior to electrophoresis, virus preparations were dissociated by addition of SDS (1% (w/v) final concentration) and 2-mercaptoethanol (1% (v/v) final concentration) and heating to 100^0 C for 3 min. The maximum volume of sample loaded onto standard size gels was 100 µl and onto semi-micro size gels, 10 µl. Samples contained 20% (w/v) sucrose and bromophenol blue solution. Electrophoresis was carried out at 6 ma/tube for 2 to 5 h using standard size gels and 1 to $1\frac{1}{2}$ h using semi-micro size gels with running buffer. Polypeptides were located as described in G.M.7(a).

Molecular weights were calculated as for the virus RNA components with molecular weights less than 2.20×10^6, as described above, using as standards: phosphorylase a (rabbit muscle) subunit mol. wt. 100,000, bovine serum albumin mol. wt. 68,000, lactate dehydrogenase (hog muscle) subunit mol. wt. 36,000 (Weber et al., 1972) and glutamate dehydrogenase (bovine liver) subunit mol. wt. 55,390 (Smith et al., 1975).

(d) Agarose gels for whole virus. 0.5% (w/v) agarose was made up in buffer, boiled for 15 min and dispensed into vertical glass tubes (7.50 x 0.45 cm) sealed with parafilm over the lower ends. When the gels had set, a small square of nylon netting was placed over the top of each tube and secured in position with an elastic band. The tubes were then inverted and the gels slipped down the tubes, giving a flat surface on the top of each gel. The netting prevented the gels from slipping out of the tubes during electrophoresis. Virus samples were normally 50 µl (E_{260} 0.5 to 2.0) and contained 20% (w/v) sucrose. 3 µl of a saturated bromophenol blue solution was added to each sample. Electrophoresis was performed at 6 ma/tube for $\frac{1}{2}$ to 2 h using the buffer which the gels contained. The capsid polypeptides and RNA of the intact virus were visualised as described in G.M.7 (a).

(e) Cellulose acetate paper electrophoresis for whole virus. The strip of cellulose acetate paper was placed horizontally between two filter paper wicks dipping into electrode reservoirs containing buffer. 5 µl of virus preparation was applied to the centre of the strip and was electrophoresed at 20 V/cm for 5 to 10 min. The capsid polypeptides of the intact virus were visualised as described in G.M.7 (a), but staining for only 5 min.

G.M.8 Analytical ultracentrifugation

Analytical ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge, equipped with a monochromator and a double beam u.v. absorption optical system with a photoelectric scanner (Hanlon et al., 1962).

(a) Sedimentation coefficients. These were determined by centrifuging preparations of virus in P buffer containing 0.1 M KCl in cells with 2.5° double-sector aluminium-filled Epon centrepieces, at 16,000 rev./min in the AN-F rotor. Detection of the moving boundary was made with the u.v. scanner at 265 nm. The cell was scanned at 4 min intervals. Figure 1 shows a typical photoelectric scanner trace. For each scan, the distance of the boundary from the centre of rotation, $\overline{x}$ cm is:

$$\overline{x} = \frac{\text{distance from the front(inside) reference hole to the middle of the boundary}}{\text{magnification factor}} + 5.70$$

where the magnification factor is the product of the magnification of the optical system and that due to the trace. The gradient of the graph of $\log_e \overline{x}$ against t and the standard deviation were found by the method of least squares. The sedimentation coefficient s was determined from the formula:

$$s = \frac{\text{gradient}}{w^2}$$

where w = angular velocity in radians/sec. At the low concentrations used (E_{260} 0.3 to 0.4), the sedimentation coefficients determined were effectively s^o (Schumaker and Schachman, 1957). s^o_{20} was found using the approximation:

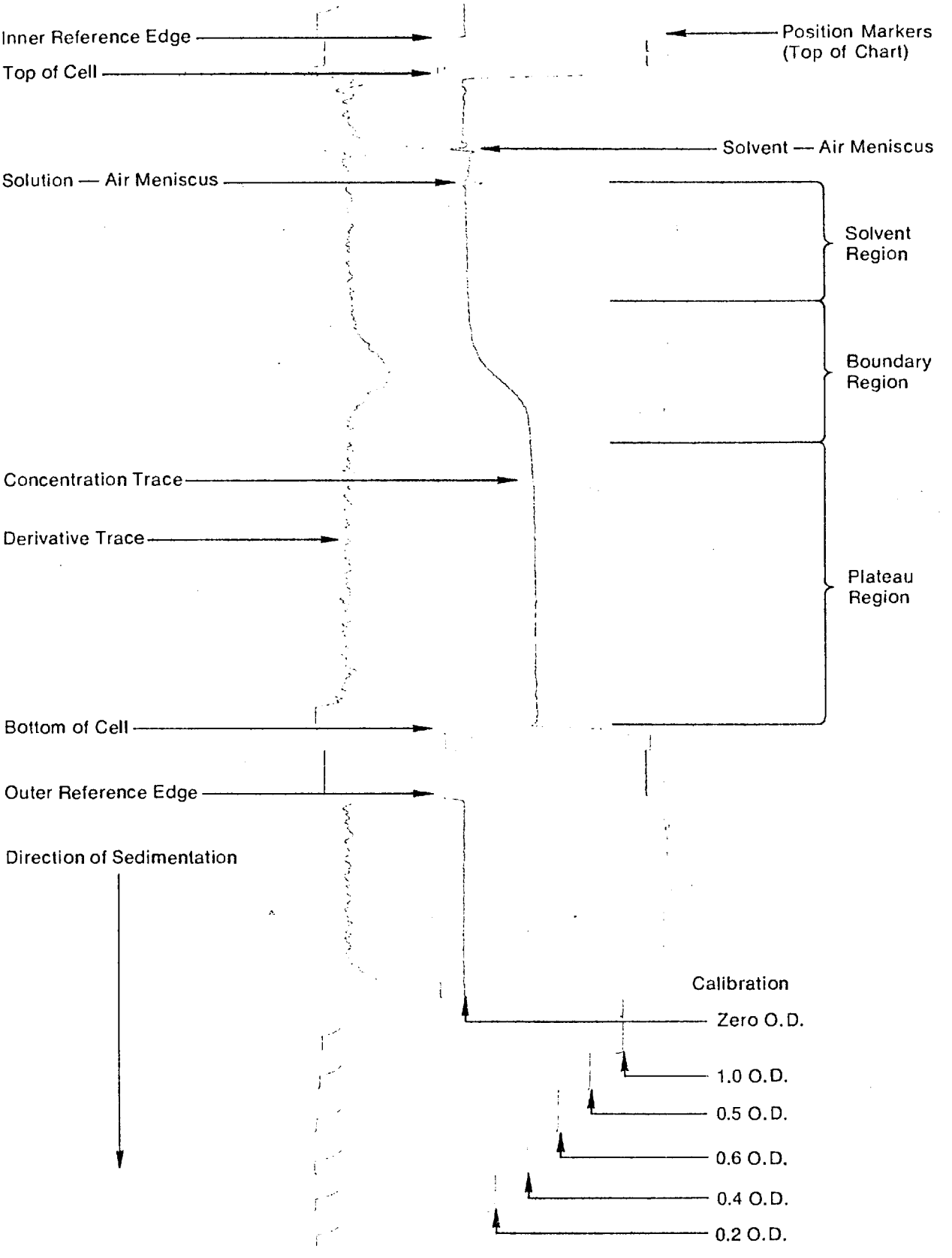


Figure 1 Typical photoelectric scanner trace for the determination of sedimentation coefficient

$$s^{o}_{20} = s^{o}\left(\frac{\eta t,\ w}{\eta 20,\ w}\right)$$

where $\eta t,\ w$ = viscosity of water at the temperature of the determination

$\eta 20,\ w$ = viscosity of water at 20^{o} C.

The value s^{o}_{20} was expressed in Svedbergs (S), where $1S = 10^{-13}$ sec^{-1}.

(b) Determination of buoyant densities of virus. Virus preparations in P buffer were centrifuged to equilibrium (18 to 24 h) in gradients of caesium chloride at 34,000 rev./min at 22 to 24^{o} C. Centrifugation was performed using cells with double-sector charcoal-filled Epon centrepieces in the AN-F rotor and profiles were obtained with the u.v. scanner at 265 nm in conjunction with the Multiplexer accessory (Figure 2). Average densities of caesium chloride solutions were calculated from the refractive indices as described in G.M.9 and G.M.10 For the determination of the buoyant densities of a mixture of viruses, Aspergillus foetidus virus F (ρ = 1.351, 1.362, 1.367 and 1.380 g/ml) (Ratti and Buck, 1972) was used as an external standard. For the determination of the buoyant density of one virus, Aspergillus foetidus virus S components S1a and S1b (ρ = 1.396 and 1.403 g/ml respectively) were used as internal standards. The conversion coefficient a of the distances (cm) in the cell to density increments (g/cm^{3}) is calculated using the formulae:

$$a = \frac{\rho_1 - \rho_2}{Do}$$

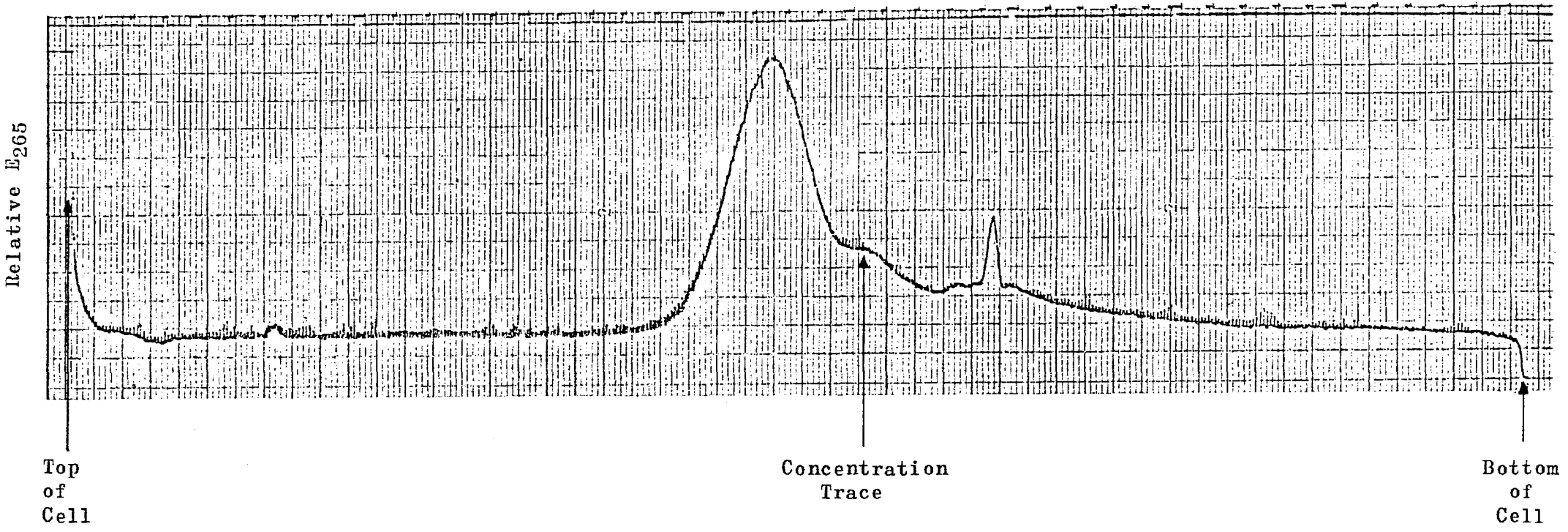


Figure 2 Typical photoelectric scanner trace for the determination of buoyant density

where ρ_1 = density of the S1b component
ρ_2 = density of the S1a component
Do = corrected distance between the bands in the cell

and $Do = D\ (1 + K)$

where $D = \frac{\text{measured distance between the bands on the trace}}{\text{magnification factor}}$

K = a function of the distance E_1, which the centre A between the bands is shifted from the cell centre, K (Figure 2)

Having calculated a, the density of the unknown virus is calculated using appropriate substitutions in the above formulae.

G.M.9 Measurement of refractive index

Refractive index measurements were made at 25° C using an Abbé High Accuracy "60" refractometer (Bellingham and Stanley Ltd., London N.5., England) with a sodium lamp (Gallenkamp and Co. Ltd., London E.C.2., England). The instrument was calibrated with a quartz block of known refractive index (1.54422 for the ordinary ray).

G.M.10 Determination of density of caesium chloride

For caesium chloride solutions, the refractive index was converted to density using the relation (Ifft et al., 1961):

$$\rho^{25.0} = 10.8601\ n_D^{25.0} - 13.4974$$

where $\rho^{25.0}$ = density at 25°C (g/ml)

$n_D^{25.0}$ = refractive index at 25°C at the wavelength of the sodium D_1 line (589.6 nm)

Analytical ultracentrifugation for density determinations in caesium chloride was performed between 22° C and 25° C. It was unnecessary to correct densities quoted to 3 decimal places for temperature since the variation in density per degree was 0.00008 g/ml.

G.M.11 Nucleic acid extraction

(a) Preparation of total nucleic acid extracts from mycelia. Mycelium (30 to 60 g wet weight) grown as described in G.M.1 and suspended in 150 ml of chilled TSE buffer was homogenised as described in G.M.2. SDS was added to the homogenate to bring the final concentration to 1% (w/v). The homogenate was then extracted at room temperature with a 100 ml aliquot of 90% (v/v) phenol + 9.9% (v/v) TSE buffer + 0.1% (w/v) 8-hydroxyquinoline. Insoluble debris was removed by centrifugation at 23,000 g for 20 min. The aqueous layer of the supernatant was extracted again with 100 ml of the phenol solution then centrifuged again to effect separation of the aqueous and phenolic layers. The aqueous layer was made 0.2 M with respect to sodium acetate, then $1\frac{1}{2}$ volumes of absolute ethanol, precooled to -20° C, was added. This solution was stored in a centrifuge bottle overnight at -20° C to allow the nucleic acid to precipitate. The nucleic acid precipitate was collected by centrifugation at 23,000 g for 15 min then taken up in 2 ml of STM buffer and dialysed against this buffer. Any precipitate remaining after dialysis was removed by centrifugation at 38,000 g for 15 min.

(b) Preparation of virus RNA. The method used was of Marcus et al. (1974). EDTA (0.1 M) and SDS (10% (w/v)) were added to a virus preparation so that the final concentrations were 5.0 mM and 0.5% (w/v) respectively. The preparation was then extracted with an equal volume of EDTA washed phenol. The aqueous and phenolic layers were separated by low speed

centrifugation. The extraction was performed three times in total. The aqueous layer containing the RNA was dialysed against six changes of water then stored at -20° C.

G.M.12 Action of DNAase 1 on total nucleic acid extracts

Total nucleic acid extracts in STM buffer were incubated for 2 h at 37° C with a final concentration of 10 µg/ml of DNAase 1 (RNAase free). The solutions were then examined by p.a.g.e. (G.M.7).

G.M.13 Action of RNAase A on total nucleic acid extracts and on virus RNA

Total nucleic acid extracts in STM buffer, 2 x SSC and 0.1 x SSC were incubated for 2 h at 24° C with final concentrations of RNAase A of 0.2 µg/ml, 1.0 µg/ml and 1.0 µg/ml respectively before being examined by p.a.g.e. (G.M.7).

Virus RNA in 2 x SSC and 0.1 x SSC was incubated for 2 h at 24° C with final concentrations of RNAase A of 5 µg/ml or 0.5 µg/ml, depending on the RNA concentration, before being examined by p.a.g.e.

G.M.14 Electron microscopy

A few drops of the virus sample were mixed with an equal volume of negative stain (2% (w/v) potassium phosphotungstate, pH 7.0). A drop of albumin solution was added to help spread the sample. The mixture was poured into a high velocity spray-gun (Aerograph Super 63) and droplets sprayed onto 200 mesh copper grids coated with collodion stabilised with carbon from a distance of about 30 cm. The preparations were examined in

a Siemens Elmiskop 1A electron microscope. The minimum beam intensity, consistent with photography, was used to minimise damage to the viruses. Photographs were taken on Ilford EM plates. A magnification of x 40,000 was used, followed by a x 2.5 magnification for the prints giving an overall magnification of x 100,000.

G.M.15 Preparation of antisera

Antisera were raised in mice to virus preparations using the following procedure. For the first injections, 0.3 ml of virus E_{260} = 0.1 was emulsified with 0.3 ml of Freund's complete adjuvant; 0.1 ml of the emulsion was injected intramuscularly into each of six mice immediately after the emulsion had been made. The second and third injections were given two and four weeks after the first injections respectively. For these, 0.3 ml of virus E_{260} = 0.1 was emulsified with 0.3 ml of Freund's incomplete adjuvant; 0.1 ml of the emulsion being injected into each of the six mice as described above. The mice were bled one week after the last injection and the whole blood was stored in a centrifuge tube at 37° C for one hour then overnight at 4° C. The next day, the clots were removed by low speed centrifugation and an equal volume of glycerol was added to the serum supernatant to act as a bacteriostat. The antisera were stored at 4° C.

G.M.16 Gel immunodiffusion

Solutions of 1.0% (w/v) purified agar (Oxoid Ltd., London S.E.1, England) in PNaCl buffer were prepared by steaming to dissolve the agar then autoclaving for 15 min at 10 lb/in^2.

When the solution had cooled to 60° C, sodium azide was added to give a final concentration of 0.01% (w/v). It was found convenient to prepare 200 ml of the purified agar solution, dispensing 15 ml aliquots into clean McCartney bottles. The aliquots were then steamed to melt as required. 1.5 ml aliquots of the molten agar solution were poured quickly onto each clean 3" x 1" microscope slide using a Pasteur pipette. The slides were allowed to set for 15 min and were then each placed in a 9 cm diameter plastic Petri dish containing moist filter paper. Appropriate patterns of wells, 7.5 mm centre to centre and 2.5 mm apart, were cut using a template. The agar discs were removed using a Pasteur pipette connected to a water pump. Either 5 µl or 10 µl of antigen or antiserum was loaded into each well. Dilutions of antigen were made with the appropriate buffer and of antiserum with 0.15 M-sodium chloride. The slides were allowed to develop in the Petri dish for one to two days at room temperature. At the end of this time, the slides were washed with PNaCl buffer then soaked in a change of the buffer overnight. The next day, the slides were washed with water then soaked in a change of water for one to two hours. The gels were then floated onto clean microscope slides. Wet, hardened Whatman 50 filter papers (Whatman Ltd., Maidstone, Kent, England) were laid on the slides which were allowed to air dry overnight. The next day, the filter papers were wetted again then peeled off some minutes later, before allowing the slides to air dry for a few hours. Precipitin lines were located in the same way as the virus polypeptides in G.M.7 (a).

G.M.17 Testing cultures for incompatibility

The buffered PDA plates were prepared as follows: 100 ml of CP buffer of a required pH and 100 ml of distilled water + 7.8 g of Difco PDA (Difco laboratories, Detroit, Michigan, U.S.A.) were autoclaved separately at 15 lb/in^2 for 15 min. Both the PDA solution and buffer solution were allowed to cool to about 40^o C before they were mixed aseptically and 25 ml aliquots were poured into 9 cm diameter plastic Petri dishes. The PDA was allowed to cool and set.

The Ggt isolates to be tested were subcultured from the "master" cultures described in G.M.1 onto unbuffered PDA plates. Discs were cut in these plates to act as inocula. One disc of each of the two isolates to be tested against each other were placed about 2.0 to 3.0 cm apart in the centre of the plate, with the mycelium in contact with the buffered PDA. The plates were incubated in the dark at 24^o C and examined at suitable time intervals.

EXPERIMENTAL AND RESULTS

Section 1 - Isolation, purification and characterisation of virus from Ggt 45/10

(A) Preliminary small scale isolation and purification of virus

Ggt 45/10 was grown on Weste and Thrower's medium as described in G.M.1 but in two 4 litre conical flasks each containing 1 litre of medium. The isolate grew little after 5 days and was harvested after 10 days by filtration. The wet weight of mycelium was 2 g/100 ml of culture medium. The mycelium was suspended in chilled P buffer, so that the suspension was three times the wet weight of the mycelium and homogenised as in G.M.2. The homogenate was made up to 1 litre by addition of chilled P buffer and insoluble debris was removed by centrifugation at 25,000 g for one hour. The virus was pelleted from 500 ml of the supernatant by centrifugation overnight at 35,000 g. The pellets were taken up in 5 ml of P buffer and the suspension clarified by centrifugation at 25,000 g for 20 min. 2 ml of the crude virus preparation was layered onto each of two 10% to 50% (w/w) sucrose gradients in P buffer and centrifuged at 25,000 rev./min for $2\frac{1}{2}$ hours in a Spinco SW 25.2 rotor. The gradient was scanned at 254 nm and fractionated with the ISCO Model D density gradient fractionator (Instrument Specialities Inc., Lincoln, Nebraska, U.S.A.). A pump rate of 2.0 ml/min and a flow cell path length of 0.5 cm were used and 5 ml fractions collected. The whole virus and RNA

components in the fractions were analysed by p.a.g.e. as in G.M.7 (a) and (b).

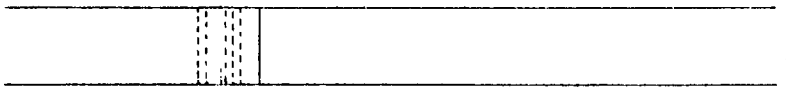
The RNA gels showed that RNA components 5 and 11 peaked in fraction A3 and RNA component 1 peaked in fraction A4 (Figure 3 and Table 4). This fractionation showed that the seven RNA components of twelve found in a later preparation, visible in the gel run of fraction A3, could not be contained by the same capsid. If they were, the relative intensities of the RNA components would be unaltered in all the gels. The whole virus gels showed that the virus had not migrated more than 1 mm. The few protein bands visible indicated that a reasonable degree of purification had been achieved by rate centrifugation in a sucrose density gradient.

Appropriate sucrose gradient fractions were combined, dialysed against P buffer and their E_{260} values measured as in G.M.6. Assuming that 5 E_{260} units is equivalent to 1 mg of virus, the yield of virus was found to be 90 µg/g wet weight of mycelium.

(B) Large scale isolation and purification of virus

(a) Preparation of crude virus

The isolate was grown on basal/corn steep liquor for three days in two 60 litre fermenters (Weste and Thrower's medium was too expensive to use on this scale). One fermenter was run at one atmosphere pressure and the other at two atmospheres pressure. Since there was no difference in the growth of the isolate in the two fermenters, the two were bulked together on harvest and the mycelium collected by



Fraction A3

RNA components 1, 3, 5, 6, 7, 9 and 11



Fraction A4

RNA components 1, 5 and 11



Fraction A5

RNA component 1

Figure 3 Polyacrylamide gel electrophoresis of Ggt 45/10 virus RNA from sucrose density gradient fractions following rate centrifugation: the isolate was grown on Weste and Thrower's medium. The direction of migration was from left to right



(a)

RNA components 1, 5, 8 and 9



(b)

RNA components 1, 2, 3, 4, 5, 7 or 8, 9, 10 and 11

Figure 5 Polyacrylamide gel electrophoresis of the RNA from Ggt 45/10 virus prepared by (a) direct pelleting and (b) PEG precipitation followed by pelleting: the isolate was grown on basal/corn steep liquor. The direction of migration was from left to right

filtration through a filter press. The wet weight was 7.5 kg. The mycelium was suspended in 20 litres of chilled P buffer and homogenised as in G.M.2. The homogenate was mixed with a further 30 litres of P buffer which was chilled in order to lower the temperature of the homogenate. Three litres of the suspension were taken, in order to investigate direct pelleting and DEAE cellulose adsorption followed by pelleting as methods of virus isolation (Sub-sections (i) and (ii)). Insoluble debris was removed from the remainder of the suspension by passage through a Sharples Super centrifuge as in G.M.2. The volume of the supernatant was adjusted to 70 litres then used to investigate PEG precipitation and pelleting as a method of virus isolation (Sub-section (iii)). The division of the preparation for the investigation of the three methods of virus isolation is summarised by a flow diagram (Figure 4).

(i) Direct pelleting. The three litres of the suspension were clarified by centrifugation at 23,000 g for one hour. Virus was pelleted from 1.5 litres of the clarified supernatant by centrifugation overnight in a Spinco Type 19 rotor at 17,000 rev./min. The pellets were resuspended in 50 ml of P buffer and clarified by centrifugation at 38,000 g for 30 min. The virus RNA was analysed by p.a.g.e. (G.M.7 (b)). Only RNA components 1, 5, 8 and 9 were found to be present (Figure 5 and Table 4), three components less than when the isolate was grown on Weste and Thrower's medium and the virus prepared by direct pelleting (Figure 3).

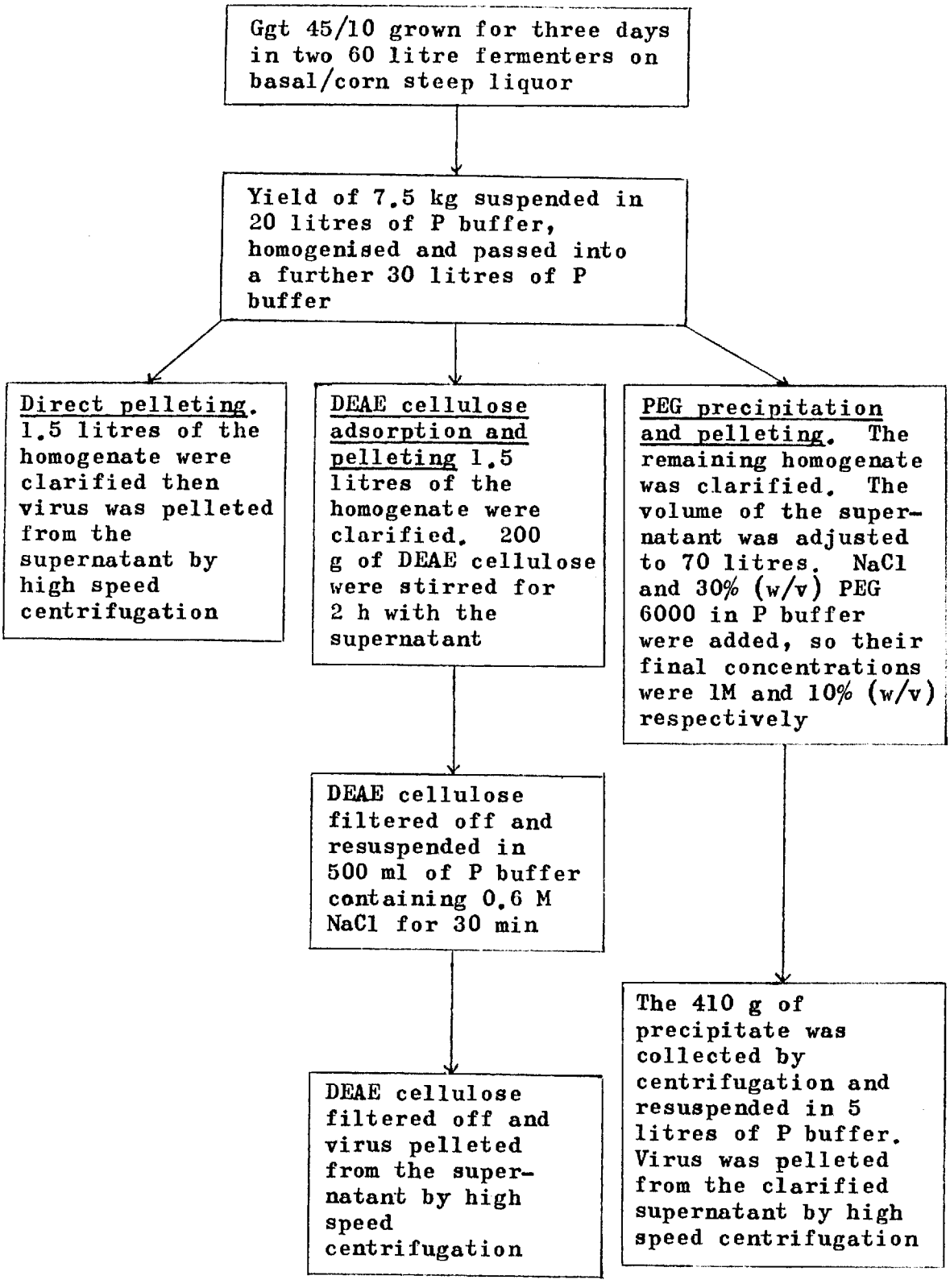


Figure 4 Investigation of three methods of virus isolation from Ggt 45/10 grown on a large scale

(ii) DEAE cellulose adsorption and pelleting. DEAE cellulose is an anion exchanger which can be used to separate substances with differing charges. It has been widely used in the purification of proteins and enzymes and was used here, in the hope that the virus would be bound in preference to the impurities, thus effecting an initial concentration.

The other half of the clarified supernatant (Sub-section (i)) was stirred for two hours with 200 g of Whatman DE52 prepared as in G.M.5. The suspension was filtered and the DE52 cake was resuspended in 500 ml of P buffer containing 0.6 M NaCl in order to remove any virus which had been adsorbed. After stirring for 30 min, the DE52 was filtered again under reduced pressure and virus pelleted from 250 ml of the supernatant by centrifugation overnight in a Spinco Type 19 rotor at 17,000 rev./min. The pellet was resuspended in 15 ml of P buffer and then clarified by centrifugation at 38,000 g for 30 min. No virus RNA was detected on analysis by p.a.g.e. (G.M.7 (b)) showing that the virus was not adsorbed by the DEAE cellulose.

(iii) PEG precipitation and pelleting. Six kg of sodium chloride was added to the 70 litres of supernatant (see above), stirring to dissolve. 30 litres of 30% (w/v) PEG 6000 in P buffer were added dropwise with stirring over 2 to 3 h. Stirring was continued for 1 h then the suspension was left to precipitate overnight. The precipitate (410 g) was collected by passage through the Sharples Super centrifuge and resuspended in 4 litres of P buffer. The final volume

was made up to 5 litres. The suspension was left to stir for several hours at 4^{0} C, then the insoluble debris was removed by centrifugation at 23,000 g for 1 h. Virus was pelleted from the supernatant by centrifugation overnight at 23,000 to 30,000 g. The pellets were taken up in a total of 100 ml of P buffer and the solution was clarified by low speed centrifugation. The virus RNA was analysed by p.a.g.e. (G.M.7 (b)). RNA components 1, 2, 3, 4, 5, 7, or 8, 9, 10 and 11 (Figure 5 and Table 4) were found to be present.

PEG precipitation and pelleting had therefore isolated a virus preparation containing five more RNA components than that isolated by direct pelleting. This observation was further evidence that the RNA components were contained in more than one capsid and that the virus isolated from Ggt 45/10 was not one virus but a mixture of different viruses. Since PEG precipitation and pelleting had produced a virus preparation containing more RNA components than contained by the other virus preparations, it was adopted as a standard method of virus isolation.

(b) Purification and fractionation of crude virus

(i) Column chromatography on DEAE cellulose. Since the virus was not found to adsorb to DEAE cellulose (Section 1 (B) (a) (iii)), it was thought that a considerable degree of purification of the crude virus preparation could be effected by column chromatography on DEAE cellulose, if most of the protein impurities were bound to the column.

A 20 ml column of Whatman DE52 prepared as in G.M.5, was equilibrated with P buffer. 7 ml of crude virus prepared as in Section 1 (B) (a) (iii) were loaded onto the column and eluted with 40 ml of P buffer then with a continuous gradient of 0 to 0.6 M NaCl in P buffer. 2 ml fractions were collected, their E_{260} values measured as in G.M.6 and the RNA of selected fractions analysed by p.a.g.e. (G.M.7 (b)). The virus was found to be eluted immediately after the void volume.

The experiment was repeated, eluting only with P buffer and collecting 1 ml fractions. Again the virus was found to be eluted immediately after the void volume. Whole virus gels run of the crude virus and the virus eluted from the column (G.M.7 (a)) showed that a considerable degree of purification was effected. The fractions containing virus were combined and concentrated by PEG precipitation and pelleting as in G.M.3. The PEG pellet was resuspended in 2 ml of P buffer.

(ii) Isopycnic sucrose density gradient centrifugation. The virus suspension (1.8 ml of the 2 ml) was layered onto a 36 ml 25% to 60% (w/w) sucrose gradient in P buffer and was centrifuged overnight at 25,000 rev./min in a Spinco SW 27 rotor. The gradient was scanned at 254 nm and fractionated with the ISCO Model D density gradient fractionator (Figure 6). A pump rate of 1.0 ml/min and a flow cell length of 0.5 cm were used. 0.5 ml fractions were collected and their E_{260} values were measured as in G.M.6. The virus RNA and polypeptides of selected fractions were analysed by p.a.g.e.

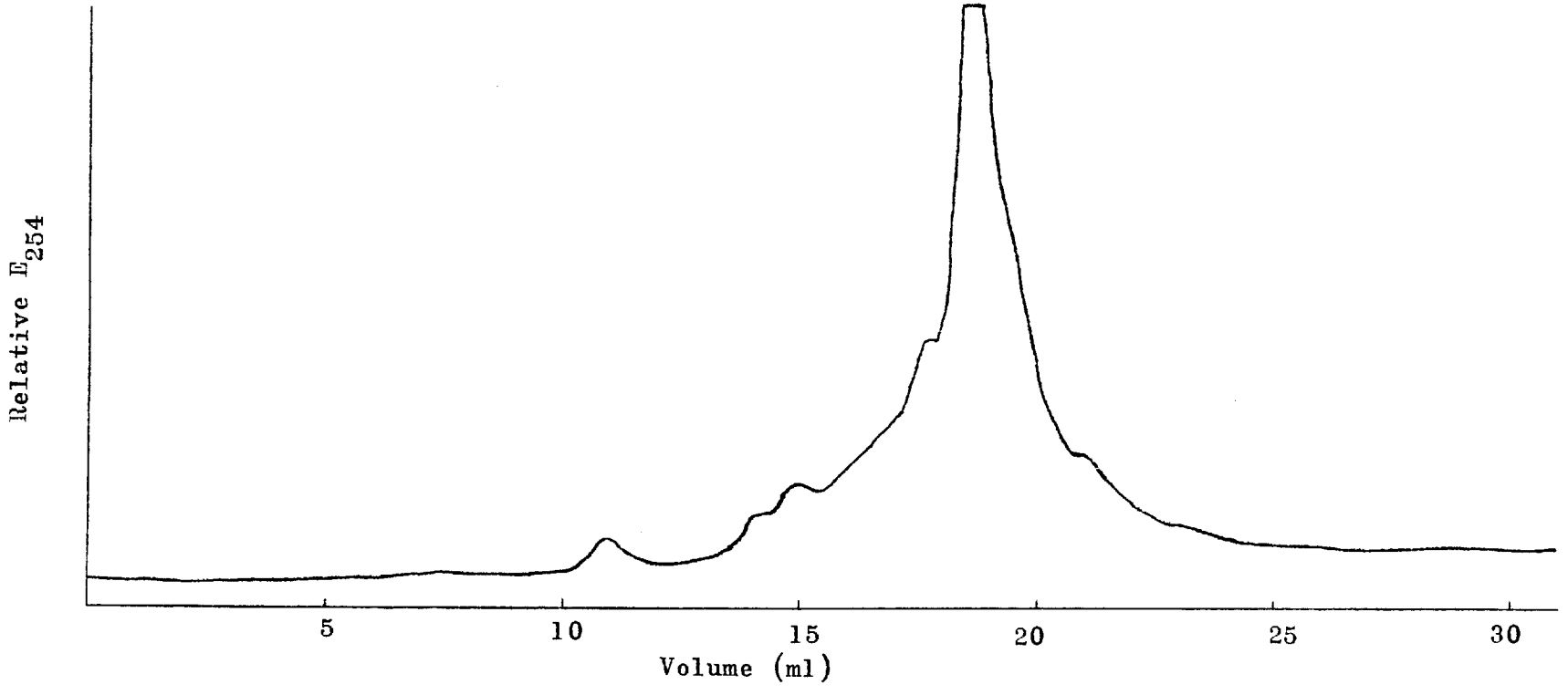


Figure 6 ISCO u.v. trace of a sucrose density gradient following isopycnic centrifugation of Ggt 45/10 virus

(G.M.7). RNA component 11 peaked in fraction 35, RNA components 5, 8, 9 and 10 peaked in fraction 37 and RNA components 1, 2, 3 and 7 peaked in fraction 39 (Figure 7 and Table 4). Significantly, RNA 7 peaked in the densest fraction 39 together with the heaviest RNA components and RNA 5 peaked in fraction 37 rather than the other way round. This proved that RNA 7 was separately encapsidated and in a different capsid to RNA 5. The polypeptide analysis showed that there were four capsid polypeptides. P1 peaked in fractions 39 and 40, P2 and P3 in fractions 39, 40, 41 and 42 and P5 in fraction 37 (Figure 8 and Table 4). This fractionation of the polypeptides showed that the virus capsids each contained one major polypeptide. The results taken as a whole were good evidence that Ggt 45/10 was infected by four different viruses.

(iii) Isopycnic caesium chloride density gradient centrifugation. 85 ml of crude virus prepared by PEG precipitation and pelleting as in Section 1 (B) (a) (iii) were partially purified by DEAE cellulose chromatography as in Section 1 (B) (b) (i). The virus was concentrated by PEG precipitation and pelleting as in G.M.3. The PEG precipitate was resuspended in 5 ml of P buffer and clarified by centrifugation at 38,000 g for 20 min. The resultant 29.7 E_{260} units of virus were layered onto two 54 ml 10% to 45% (w/w) caesium chloride gradients in P buffer, which were centrifuged overnight at 22,000 rev./min in a Spinco SW 25.2 rotor. Gradients were scanned at 254 nm and fractionated with the ISCO Model D density gradient fractionator (Figure 9).



Fraction 35

RNA components 1 and 11



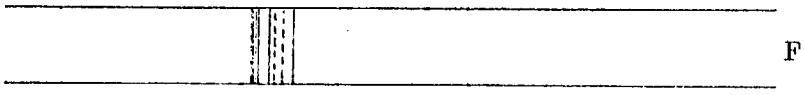
Fraction 37

RNA components 1, 2, 3, 5, 7, 8, 9, 10 and 11



Fraction 39

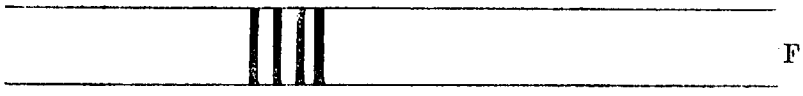
RNA components 1, 2, 2a, 3, 4, 5 and 7



Fraction 41

RNA components 1, 2, 2a, 3, 4, 5 and 7

Figure 7 Polyacrylamide gel electrophoresis of Ggt 45/10 virus RNA from sucrose density gradient fractions following isopycnic centrifugation. The direction of migration was from left to right



Fraction 35

Polypeptides 1, 2, 4 and 5



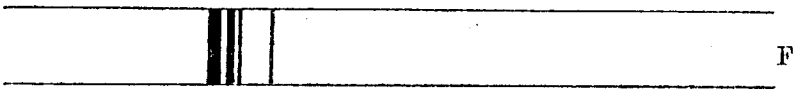
Fraction 37

Polypeptides 1, 2 and 5



Fractions 39 and 40

Polypeptides 1, 2, 3 and 5



Fraction 42

Polypeptides 1, 2, 3 and 5

Figure 8 Polyacrylamide gel electrophoresis of Ggt 45/10 virus polypeptides from sucrose density gradient fractions following isopycnic centrifugation. The direction of migration was from left to right

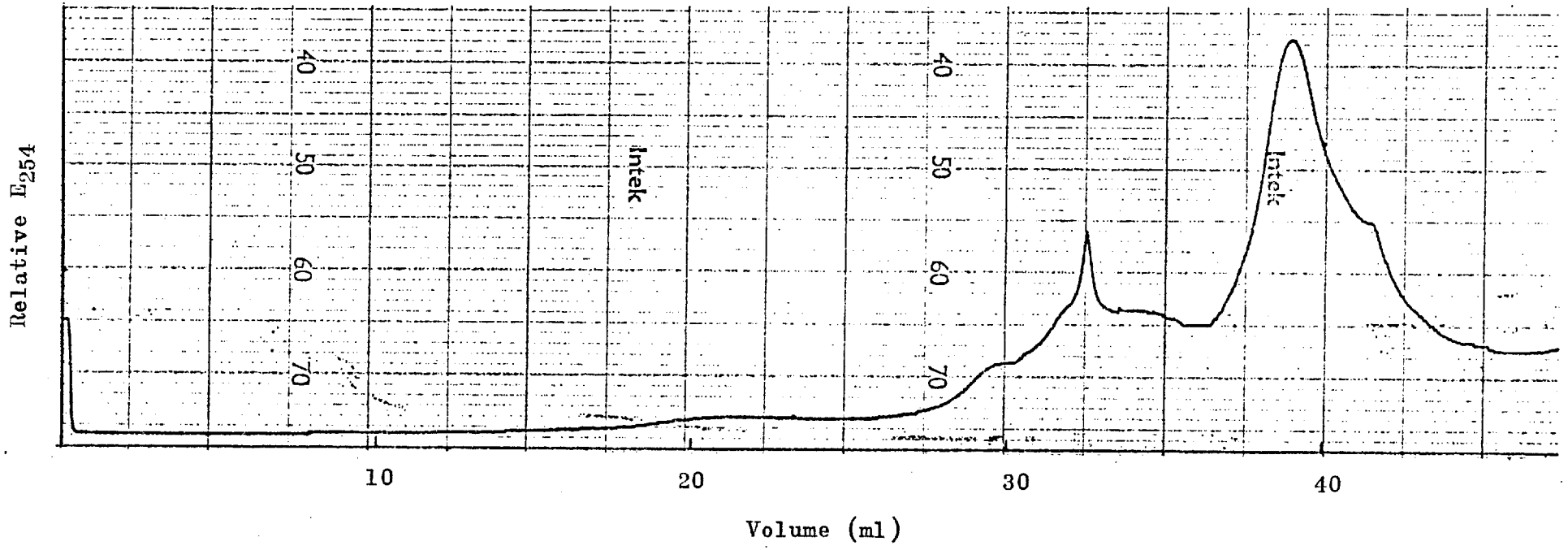


Figure 9 ISCO u.v. trace of a caesium chloride density gradient following isopycnic centrifugation of Ggt 45/10 virus

A pump rate of 1.0 ml/min and a flow cell path length of 0.5 cm was used. 1.0 ml fractions were collected and their E_{260} values measured as in G.M.6.

The buoyant densities of the virus particles in selected fractions were determined by analytical centrifugation as in G.M.8 (b) using Aspergillus foetidus virus F as an external standard. The results showed that the virus preparation was heterogeneous with a main broad peak of buoyant density 1.362. Scans of fractions A37 and A40 are to be found in Figure 10.

These fractions were dialysed against P buffer and their RNA and polypeptide components were analysed on standard size polyacrylamide gels (G.M.7). RNA components 1 to 12 with the exception of RNA 7 peaked in fraction A37, the latter peaked in fraction A41 (Figure 11 and Table 4). Polypeptide components 1 to 5 with the exception of P3 peaked in fraction A37, the latter peaked in fraction A41 (Table 4). It was therefore concluded that P3 encapsidated RNA 7. The RNA molecular weights were determined using PcV and PsV RNA as standards. The polypeptide molecular weights were determined using E.coli RNA polymerase subunits α and σ with molecular weights of 41,000 and 86,000 respectively (Travers, 1971), phosphorylase a (rabbit muscle) molecular weight 100,000 and pyruvate kinase (rabbit muscle) subunit molecular weight 57,000 (Weber et al., 1972).

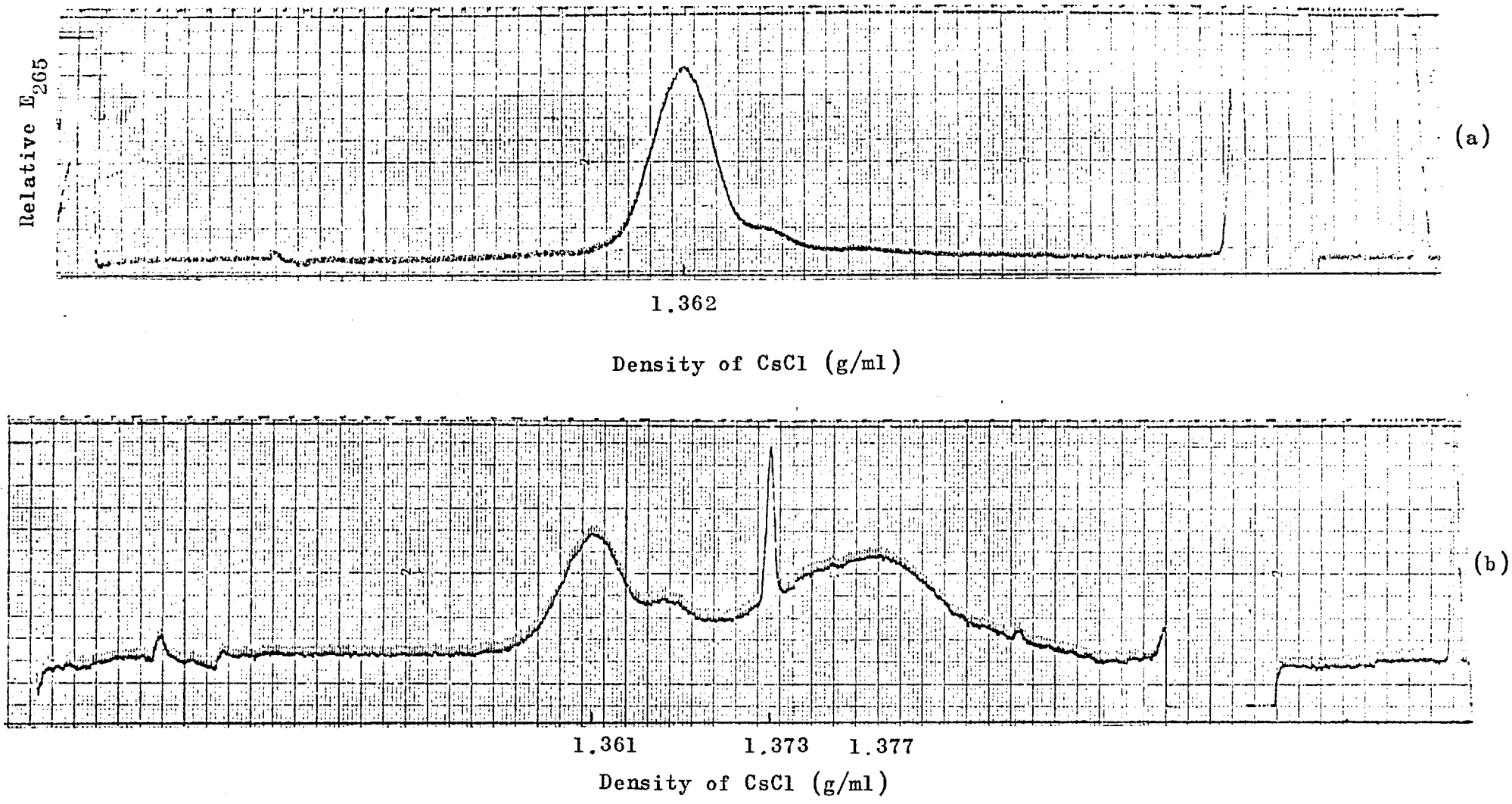
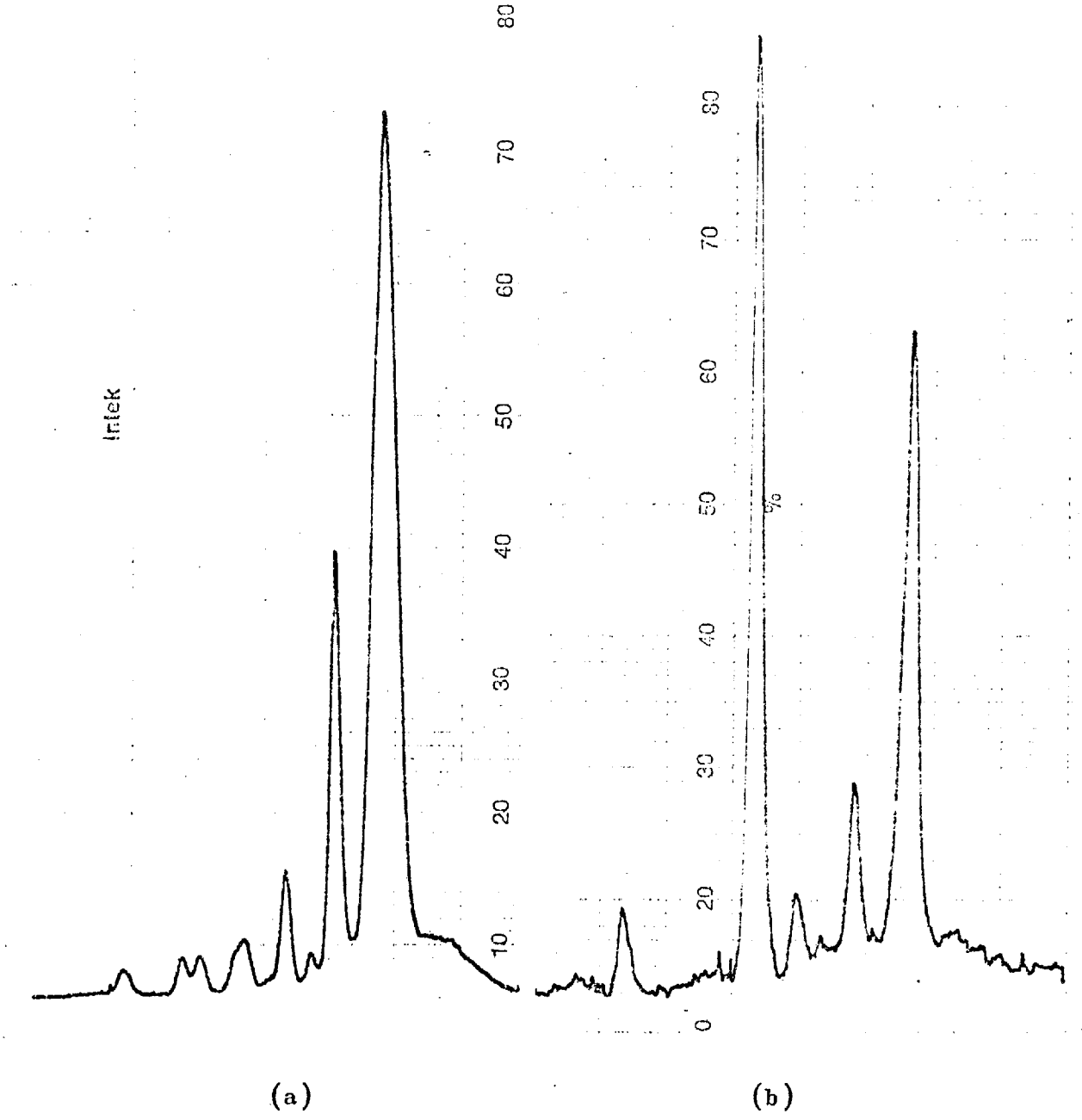


Figure 10 Photoelectric scanner traces for the determination of the buoyant densities of Ggt 45/10 virus in caesium chloride density gradient fractions (a): A37 and (b): A40



(a) (b)

RNA components 1, 2, 2a, 3, 4, 5, 6, 7, 8, 9, 10 and 11

RNA components 1, 2, 2a, 3, 4, 5, 7 and 11

Figure 11 Densitometer scans of polyacrylamide gels of Ggt 45/10 virus RNA from caesium chloride density gradient fractions (a): A37 and (b): A41 following isopycnic centrifugation. The direction of migration was from right to left

Table 4 RNA and polypeptide component molecular weights of viruses of Ggt 45/10

RNA molecular weight x 10^{-6}	Description	Polypeptide molecular weight	Description
1.57	RNA 1	73,000	P1
1.54	RNA 2	69,000	P2
1.53	RNA 2a	65,000	P3
1.43	RNA 3	60,000*	P4
1.39	RNA 4	56,000	P5
1.34	RNA 5		
1.30	RNA 6		
1.25	RNA 7		
1.23	RNA 8		
1.15	RNA 9		
1.12	RNA 10		
1.01	RNA 11		

*P4 had the same electrophoretic mobility as pyruvate kinase. The latter was found to have a molecular weight of 60,000 using the standards described in G.M.7 (c).

(iv) Attempted electrophoretic separation of the viruses. Polyacrylamide gel electrophoresis using standard size gels, essentially as described in G.M.7, was used under the following conditions to attempt a separation of the viruses; in each case, aliquots of virus were dialysed against the appropriate running buffer: 4% (w/v) polyacrylamide gels with running buffers of TAE, pH 8.0, tris-HCl (I = 0.05), pH 8.5 and tris-HCl (I = 0.05), pH 9.0. Under these conditions, the viruses only entered the

first millimetre of the gels. The following systems also failed to separate the viruses: 0.5% (w/v) agarose gels with running buffers of NaAc/HAc (I = 0.05), pH 5.0, TAE, pH 8.0, tris-HCl (I = 0.05), pH 8.5 and tris-HCl (I = 0.05), pH 9.0 also cellulose acetate paper electrophoresis with a running buffer of NaAc/HAc (I = 0.05), pH 5.0. In all cases, the viruses did not migrate from the origin.

These results showed that either the viruses were all individually weakly charged in the pH range 5.0 to 9.0 or more likely that the viruses had either formed a non-covalently bonded complex which was weakly charged in this pH range or that some of the viruses had precipitated out of solution when dialysed against the appropriate running buffer.

(v) Precipitation studies. A virus sample which had been purified by caesium chloride density gradient centrifugation as described in Section 1 (B)(b)(iii) was found to contain RNA components 1, 2, 2a, 3, 5, 7 or 8 (Table 4). When dialysed against 0.01 M-tris-HCl, pH 8.0, a running buffer to be used in an attempted electrophoretic separation of the viruses, precipitation occurred. The RNA and polypeptide components of the precipitate and supernatant were analysed by p.a.g.e. (G.M.7). The precipitate was found to contain RNA components 3 and 7 or 8 in equal proportion and a small amount of RNA 1 and RNA 2a. The supernatant contained RNA 1 (major), RNA 2 and RNA 5 (minor). Polypeptide analysis showed that the precipitate contained polypeptides 2 and 3 whereas the supernatant contained polypeptides 1 (major) and 5 (minor). From the analysis of the supernatant, it was deduced that

polypeptide 1 encapsidated RNA 1 and that polypeptide 5 encapsidated RNA 2 or 5. Since polypeptide 3 was known to encapsidate RNA 7 (Section 1 (B)(b)(iii)), it was deduced from the analysis of the precipitate that polypeptide 2 encapsidated RNA 3 or RNA 8.

Since some of the viruses had been found to precipitate in certain buffers, it was decided to investigate the insoluble material removed from the virus sample before purification on a caesium chloride density gradient by centrifugation at 38,000 g for 20 min (Section 1 (B)(b)(iii)). This insoluble material was re-suspended by stirring overnight in P buffer (10 ml) then clarified by centrifugation at 38,000 g for 20 min. The supernatant was found to contain RNA components 1, 2, 3, 5 (major), 2a, 4, 8, 9, 11 (minor); 6 (trace) and 7 absent and polypeptides 1, 2 and 5 (major, in equal proportions) and 4 (minor). Consideration of the relative proportions of the RNA and polypeptide components showed that polypeptide 2 encapsidated RNA 3 rather than RNA 8. When this supernatant was dialysed against 0.01 M-tris-HCl pH 8.0, a precipitate formed which was removed by low speed centrifugation and washed with 11 ml of the tris HCl buffer. The viruses soluble in 0.01 M-tris HCl pH 8.0 were found to contain RNA components 1 and 5 (major), 2, 8, 9, 10 and 11 (minor) and polypeptides 1 and 5 (major) and 2 (trace). The precipitate contained RNA 3 (major), RNA 1, 2a, 4 (minor) and RNA 7 (trace) and polypeptides 2 (major), 1 (minor) and 3 (trace). Consideration of the relative proportions of the RNA and polypeptide components showed that polypeptide 5 encapsidated RNA 5 rather than RNA 2.

Summarising, the deductions made were:

polypeptide 1 encapsidated RNA 1
polypeptide 2 encapsidated RNA 3
polypeptide 3 encapsidated RNA 7
polypeptide 5 encapsidated RNA 5

(vi) Electron microscopy. Electron micrographs (x 100,000 magnification) of dialysed caesium chloride fraction A39 (Section 1 (B)(b)(iii)) were taken as described in G.M.14. 73% of the virus particles measured were 35 nm in diameter (Figure 12 and Table 22).

(C) Variability and problems in the isolation and purification of virus

It was decided to isolate and purify more virus from Ggt 45/10 both for the production of an antiserum and to act as secondary standards during the characterisation of viruses infecting other isolates.

The virus was prepared on a large scale as described in G.M.2. This preparation was found to contain only RNA components 1, 5, 7 or 8, 9, 10 and 11 (G.M.7), although the crude virus yield (5 µg/g wet weight of mycelium) was about four times that produced in the first large scale preparation by PEG precipitation and pelleting (Section 1 (B)(a)(iii)). A subsequent small scale preparation (G.M.3) was found to contain only RNA components 1, 5, 9, 10 and 11. Since the virus prepared from the isolate grown on Weste and Thrower's medium contained seven RNA components (Section 1 (A)), the isolate was grown for 14 days on that medium. The virus prepared contained only four RNA components and the yield of pure virus was only 0.7 µg/g wet weight of mycelium which was

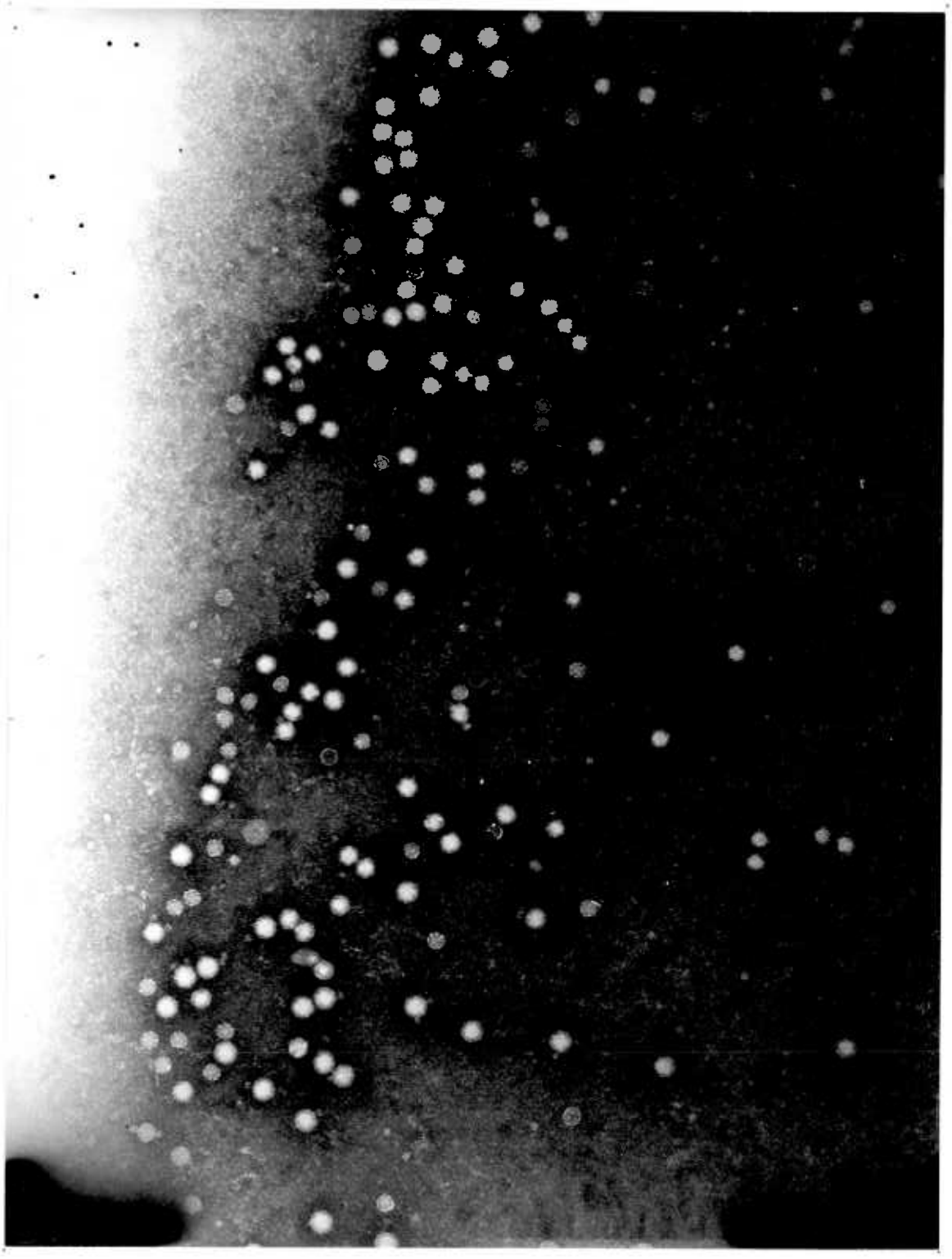


Figure 12 Electron micrograph of Ggt 45/10 virus (x 100,000 magnification)

less than a hundredth of the yield originally obtained using Weste and Thrower's medium (Section 1 (A)).

There seemed to be two likely reasons for the failure to isolate some of the viruses and for the variable yield:

(i) The isolate had been subcultured at two monthly intervals (G.M.1). Since the field isolate is probably a mixture of individuals, certain individuals could overgrow the rest of the population on agar to agar transfer so that, after several transfers, the culture could contain a different population to that previously present. Perlman (1971) discussed this phenomenon as a limitation of the maintenance of streptomycete cultures on agar slants.

(ii) The P buffer used in the harvesting and purification of the virus was not the optimal buffer. It is probable that some of the viruses from Ggt 45/10 precipitated unpredictably out of solution. The early success using this buffer had probably been fortuitous.

Using a master culture of Ggt 45/10, which had only been sub-cultured at six monthly intervals, as inoculum, virus was prepared on a small scale using the following buffers: 0.03 M-sodium phosphate buffer + 0.15 M-KCl, pH 7.6, 0.03 M-sodium phosphate buffer, pH 7.6, 0.05 M-borate buffer, pH 8.6 and 0.05 M-veronal, pH 8.6. Of these, the PK buffer was found to solubilise the virus best, the yield being about 10 fold that in P buffer (R. McGinty, personal communication). However, even using PK buffer, more than 90% of the virus was still adsorbed to cell debris, as judged by SDS disruption and RNA analysis (G.M.7 (b)).

Using the same master culture of Ggt 45/10, virus was obtained, purified and characterised as described in G.M.3. The yield of pure virus was 7 µg/g wet weight of mycelium. The semi-micro size gel system was used as a method of analysis since it used less sample and was found to be faster than the standard size gel system. Figure 13 shows the fluorescence of the sucrose fractions spotted onto an ethidium bromide agarose gel plate in order to locate the virus. Figure 14 shows the RNA and polypeptide gels run of the sucrose fractions found to contain virus.

The RNA molecular weights were determined as in G.M.7 (b), using PcV and PsV-S as standards and semi-micro size gels. The polypeptide molecular weights were determined as in G.M.7 (c) using semi-micro size gels. The RNA was extracted from a crude virus preparation as in G.M.11 (b) and proven to be double-stranded by resistance to RNAase A in 2 x SSC and degradation by the enzyme in 0.1 x SSC (G.M.13).

Table 5 Ds-RNA and polypeptide molecular weights of viruses of Ggt 45/10

Ds-RNA		Polypeptides	
Molecular weight x 10^{-6}	Intensity of band	Molecular weight	Intensity of band
1.64	strong	72,000	strong
1.61	medium	68,000	strong
1.59	medium	66,000	medium
1.48	strong	55,000	medium
1.42	faint	51,000	faint
1.26	strong		
1.22	faint		
1.18	strong		
1.05	strong		
9 ds-RNA components		5 polypeptides	

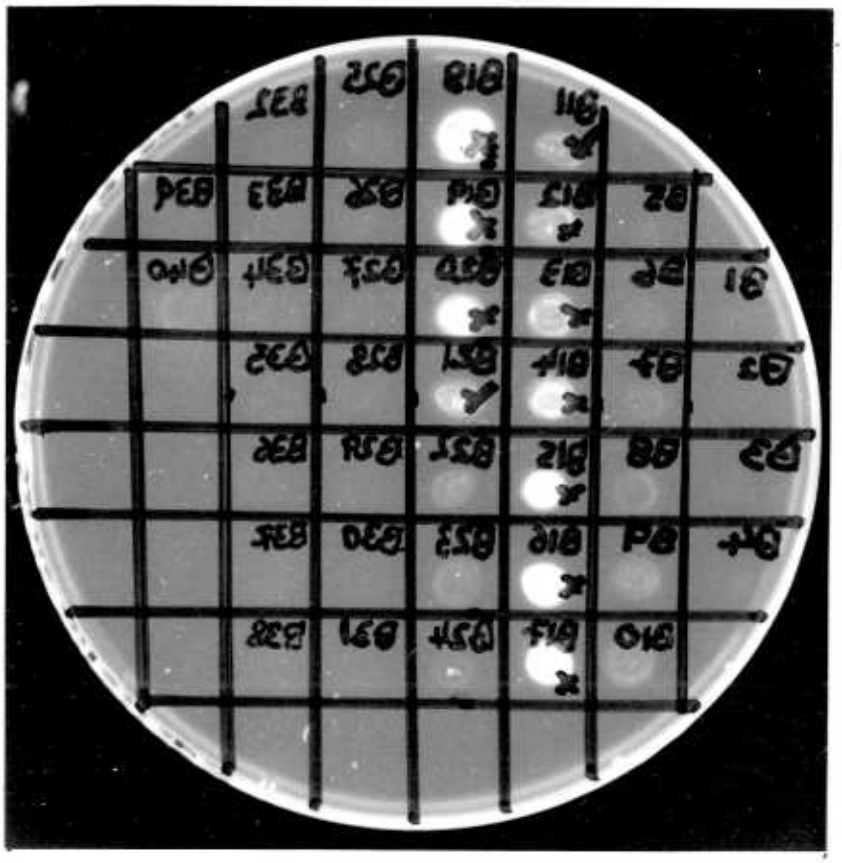
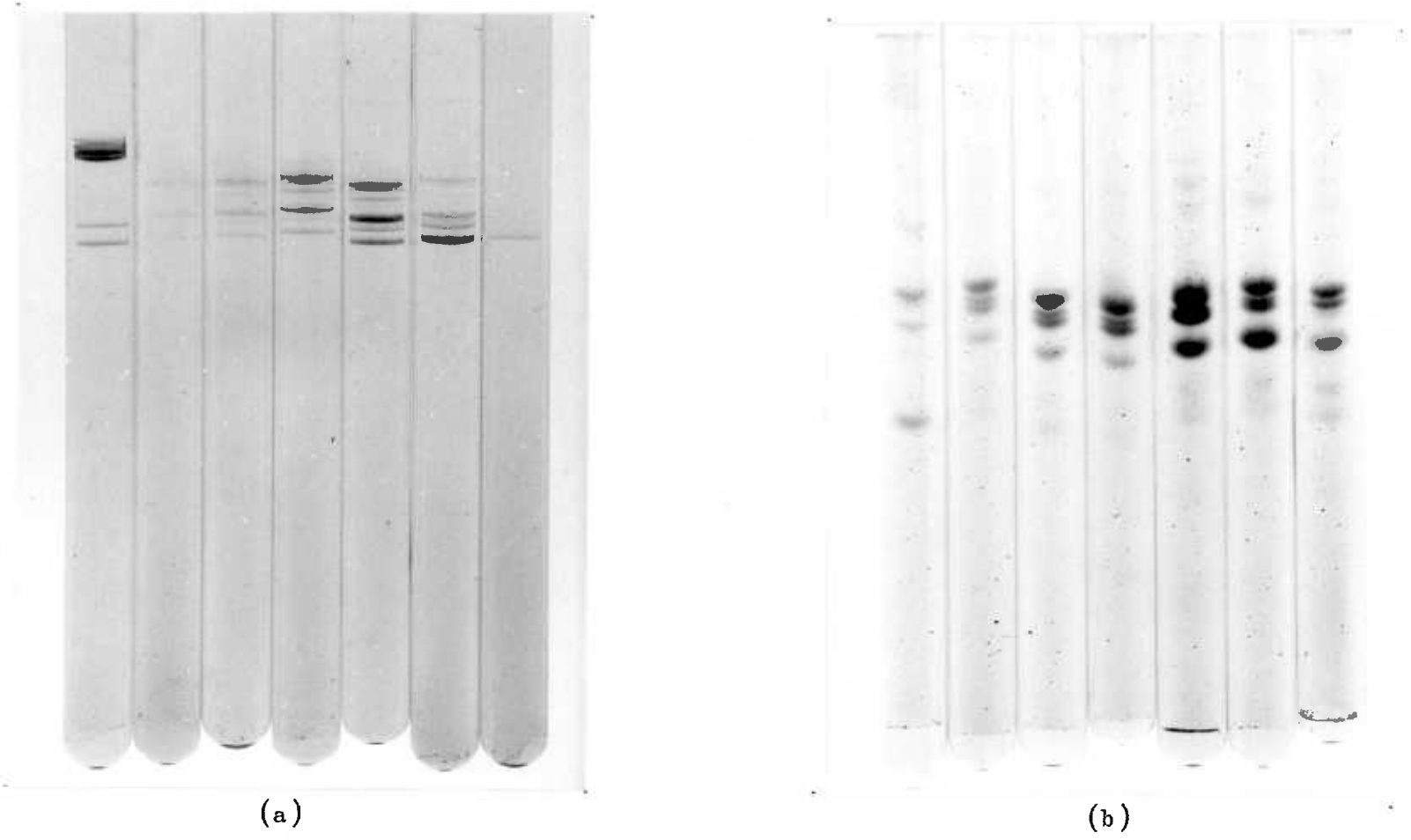


Figure 13 Sucrose density gradient fractions of Ggt 45/10 virus spotted onto an ethidium bromide agarose plate following rate centrifugation



(a) (b)

Figure 14 Polyacrylamide gel electrophoresis of Ggt 45/10 virus RNA (a) and polypeptides (b) from sucrose density gradient fractions following rate centrifugation. From left to right: standards; fraction B11; fraction B13; fraction B15; fraction B17; fraction B19; fraction B21. The direction of migration was from top to bottom.

The RNA molecular weights determined were higher than those determined using the same standards but using the standard size gel system (Table 4) in the higher molecular weight range. The RNA molecular weights were therefore redetermined more accurately using standards nearer in molecular weight to those of the unknown. They were PsV-S RNA and AfV-F RNA. The molecular weights of the latter are 1.87×10^6, 1.70×10^6 and 1.44×10^6 (Ratti and Buck, 1972). The redetermined molecular weights are given in Table 6 (K.W. Buck, personal communication).

Table 6 Redetermined ds-RNA molecular weights of viruses of Ggt 45/10

Molecular weight $\times 10^{-6}$	Description
1.60	RNA 1
1.56	RNA 2
1.54	RNA 2a
1.43	RNA 3
1.37	RNA 4
1.22	RNA 8
1.20	RNA 8a
1.16	RNA 9
1.05	RNA 10a

RNA components 1, 2, 2a, 3 and 4 were correlated with the RNA components of the original preparation (Table 4), based on the similarity of molecular weights and the pattern of the bands on gels. The correlation of RNA 8 and RNA 9 was established

when it was found that they had the same electrophoretic mobility as those components of the virus from Ggt 45/9 Section 2 (B)(a). Having correlated the RNA components of the new preparation with those of the original, it is obvious that the increased molecular weights in the higher molecular weight range as determined on the semi-micro size gel system are attributable to an increased curvature of the graph of $\log_{10}$ (mol. wt.) against mobility over that given by the standard size gel system.

It was also noted that, although some of the RNA components of the original preparation were missing from the new, two new components 8a and 10a had been gained. The new preparation also contained a new polypeptide of molecular weight 51,000 in addition to P1, P2, P3 and P5. This variation may have been caused by a change in the population of the individuals within the isolate during maintenance on PDA slopes.

Section 2 - Isolation, purification and characterisation of virus from Ggt 45/9

(A) Large scale isolation and purification of virus

(a) Column chromatography on DEAE cellulose

DEAE cellulose chromatography was used for the reasons described in Section 1 (B)(a)(ii). Virus was prepared on a large scale as described in G.M.2. 8 ml of the crude virus (49.6 E_{260} units) was eluted from a 20 ml DEAE cellulose column, preequilibrated with P buffer, using the same buffer. Measurement of the E_{260} values of the fractions (G.M.6) and analysis of their RNA components by p.a.g.e. (G.M.7 (b)) showed that the virus was eluted immediately after the void volume as was the virus from Ggt 45/10 under similar conditions (Section 1 (B) (b)(i)). Since most of the host impurities were known to bind to the column under these conditions, a considerable degree of purification had been effected. However, when a 250 ml DEAE cellulose column was used to purify the rest of the crude virus preparation, no virus was eluted from the column, even after elution with P buffer containing 0.5 M-NaCl. It was concluded that the virus had precipitated out of solution while on the column. DEAE cellulose column chromatography as a general method of purification was abandoned thereafter.

(b) Isopycnic caesium chloride density gradient centrifugation

Virus was prepared again on a large scale as in G.M.2 except that the virus pellets were taken up in 430 ml of 0.1 M-sodium phosphate buffer, pH 7.6. The higher ionic strength buffer was used in an attempt to prevent the precipitation which

was described in Section 2 (A)(a). The clarified supernatant was centrifuged at 78,000 g for 3 h to repellet the virus which was then taken up in 20 ml of 0.1 M-sodium phosphate buffer, pH 7.6 and clarified by low speed centrifugation. The crude virus was layered onto three 54 ml 15% to 50% (w/w) caesium chloride gradients in 0.1 M-sodium phosphate buffer, pH 7.6 and centrifuged overnight at 22,000 rev./min in a Spinco SW 25.2 rotor. Centrifuge tubes were placed in a Büchler gradient fractionator and surrounded by ice. The tubes were then pierced by a needle and fractions were collected as the gradient dripped out. The first 16 ml from the bottom of each gradient was collected followed by 20 ml of 0.5 ml fractions and the remainder as 1 ml fractions. The E_{260} of each fraction was measured on the Cary "15" recording spectrophotometer. Fractions with the highest E_{260} values from the centres of the gradients were combined and centrifuged at 35,000 rev./min for 21 h in a Spinco SW 50.1 rotor. The gradients were fractionated using the Büchler fractionator. For each gradient, the first 1 ml was collected in 0.2 ml fractions, then the next 3 ml as 0.1 ml fractions, finally the last 1 ml as 0.2 ml fractions. The volume of each fraction was made up to 0.3 ml with 0.1 M-sodium phosphate buffer, pH 7.6 and the E_{260} values measured on the Cary "15" recording spectrophotometer. Fractions with the highest E_{260} values were combined and dialysed against P buffer. The yield of virus was 0.2 µg/g wet weight of mycelium.

(B) Characterisation of the virus

(a) Molecular weights of the RNA and polypeptide components

The RNA and polypeptide components of the virus from Ggt 45/9 were analysed by p.a.g.e. (G.M.7). The RNA molecular weights were determined using PcV and PsV RNA as standards and the polypeptide molecular weights determined using the following standards: phosphorylase a (rabbit muscle) mol. wt. 100,000 and aldolase (rabbit muscle) subunit mol. wt. 40,000 (Weber et al., 1972). The RNA molecular weights were 1.32×10^6, 1.21×10^6, 1.15×10^6 and 1.11×10^6. The polypeptide molecular weight was found to be 54,000.

(b) Buoyant density in caesium chloride

The buoyant densities of the virus components in a preparation purified as described in Section 2 (A)(b) followed by stirring with DEAE cellulose, were determined by analytical centrifugation in a Beckman Model E analytical ultracentrifuge as in G.M.8 (b) using Aspergillus foetidus virus S as an internal standard. The buoyant densities of the virus particles were found to be 1.366 and 1.372. Since all the RNA components were encapsidated by the same polypeptide, it was probable that the denser peak consisted of those particles containing the RNA of molecular weight 1.32×10^6.

(c) Sedimentation coefficient

Using a virus preparation purified as described in Section 2 (A)(b), the sedimentation coefficient was determined as in G.M.8 (a). The average value of s_{20} was 117.3 ± 3.9.

(d) Electron microscopy

Electron micrographs (x 100,000 magnification) of a purified virus preparation were taken as described in G.M.14. 98% of the virus particles measured were 35 nm in diameter (Table 22).

(e) Comparison of the virus from Ggt 45/9 and Ggt 45/10

The RNA components of the virus from Ggt 45/9 had the same electrophoretic mobility as RNA components 5, 8, 9 and 10 of Ggt 45/10 virus (Table 4), while the polypeptide of the virus from Ggt 45/9 had the same electrophoretic mobility as polypeptide 5, as shown by co-electrophoresis in each case. Although identity of molecular weights is far from a proof of identity, which can only be unequivocally shown by sequencing, the fact that both RNA components and the polypeptide component of the virus from Ggt 45/9 had the same molecular weights as those of a virus of Ggt 45/10 was taken as good evidence that one virus was common to both isolates.

(C) Variability and problems in the isolation and purification of virus

It was decided to isolate and purify more virus from Ggt 45/9 both for the production of an antiserum and to act as a secondary standard during the characterisation of viruses infecting other isolates.

Since Weste and Thrower's medium had been found to give a good yield of virus from Ggt 45/10 (90 µg/g wet weight of mycelium) (Section 1 (A)), Ggt 45/9 was grown on the medium for 24 days. It was hoped to increase the yield by increasing the

time before harvest. The virus RNA in a crude preparation produced by PEG precipitation and pelleting was analysed by p.a.g.e. (G.M.7 (b)). These gels showed that the yield of crude virus was about 1 µg/g wet weight of mycelium so that the overall yield was inadequate for the purpose intended.

In order to investigate the conditions required for optimal yields of virus, it was decided to grow Ggt 45/9 on a small scale in basal/corn steep liquor medium as in G.M.1 and to compare the yields of virus obtained by:

(i) Suspension of the isolate mycelium in 4 ml/g wet weight of chilled P buffer followed by homogenisation and PEG precipitation and pelleting (G.M.3).

(ii) Suspension of the isolate mycelium in 4 ml/g wet weight of chilled P buffer followed by homogenisation in which the homogenate was directly passed into a further 6 ml/g wet weight of chilled P buffer followed by PEG precipitation and pelleting (G.M.3).

(iii) Suspension of the isolate mycelium in 4 ml/g wet weight of chilled P buffer followed by homogenisation in which the homogenate was directly passed into a further 6 ml/g wet weight of chilled P buffer. After removal of insoluble debris by low speed centrifugation, the virus was pelleted by high speed centrifugation.

An equal wet weight of mycelium was used in each of the three procedures. Each virus pellet was taken up in 3 ml of PK buffer and clarified by low speed centrifugation. Analysis of the virus RNA in the crude preparations by p.a.g.e. (G.M.7 (b)) gave the following results:

(i) Using the intensity of staining of the four RNA bands as an indication of yield, there was no increase of yield when the volume of buffer in which the homogenate was suspended finally was increased from 4 ml/g wet weight to 10 ml/g wet weight.

(ii) Direct pelleting gave a slightly better yield than the PEG precipitation and pelleting method. However, the directly pelleted preparation contained far more impurities and as such would be much harder to purify.

An attempt to purify the crude virus preparation by using self forming caesium chloride gradients led to disruption of the virus, releasing its RNA, as judged by the fluorescence pattern of the fractions spotted onto ethidium bromide agarose gel plates and irradiated by short wavelength u.v. (G.M.3). This was entirely unexpected since the virus had seemed stable in caesium chloride gradients (Section 2 (A)(b)).

The data accumulated on the viruses from Ggt 45/10 and Ggt 45/9 (Section 1 and this section) led to the adoption of the following procedures in the small scale preparation and purification of all subsequent isolate viruses (G.M.3):

(i) Use of PK buffer throughout.

(ii) Homogenisation of the mycelium in 4 ml/g wet weight of buffer with no further dilution.

(iii) Final purification by rate sedimentation in sucrose gradients using similar conditions to that used in the purification of the yeast virus (Buck et al., 1973).

(iv) Location of the virus in the sucrose gradient fractions using agarose gel plates containing ethidium bromide. The plates were shown to be as sensitive as the semi-micro polyacrylamide gel system (G.M.7 (b)) for the detection of virus RNA (Limit of detection is 0.1 µg of RNA).

Section 3 - Isolation, attempted purification and characterisation of virus from Ggt 235-1

(a) Electron microscopy

Virus from Ggt 235-1 was prepared on a large scale as in G.M.2. Electron micrographs (x 100,000 magnification) of the crude virus preparation were taken as described in G.M.14. 54% of the virus particles measured were 27 nm in diameter (Figure 15 and Table 22). Many of the virus particles had been penetrated by the stain.

(b) RNA and polypeptide analysis

The crude virus preparation was analysed for RNA and polypeptides by p.a.g.e. as in G.M.7. Although no virus RNA could be detected, the capsid polypeptide was found to have the same molecular weight as polypeptide 3 of the viruses isolated from Ggt 45/10 (65,000).

(c) Isopycnic caesium chloride density gradient centrifugation

It was possible that no virus RNA was detected in the crude preparation simply because it was too dilute. It was therefore decided to attempt to concentrate the virus using a self-forming caesium chloride gradient.

Solid caesium chloride was added to 5 ml of the crude virus preparation until the density was equal to that of the estimated buoyant density of the virus particles (G.M.9 and G.M.10). This solution was centrifuged overnight at 40,000 rev./min in a Spinco SW 50.1 rotor. Using a Büchler fractionator, ten 0.3 ml fractions were dripped out from the centre of the

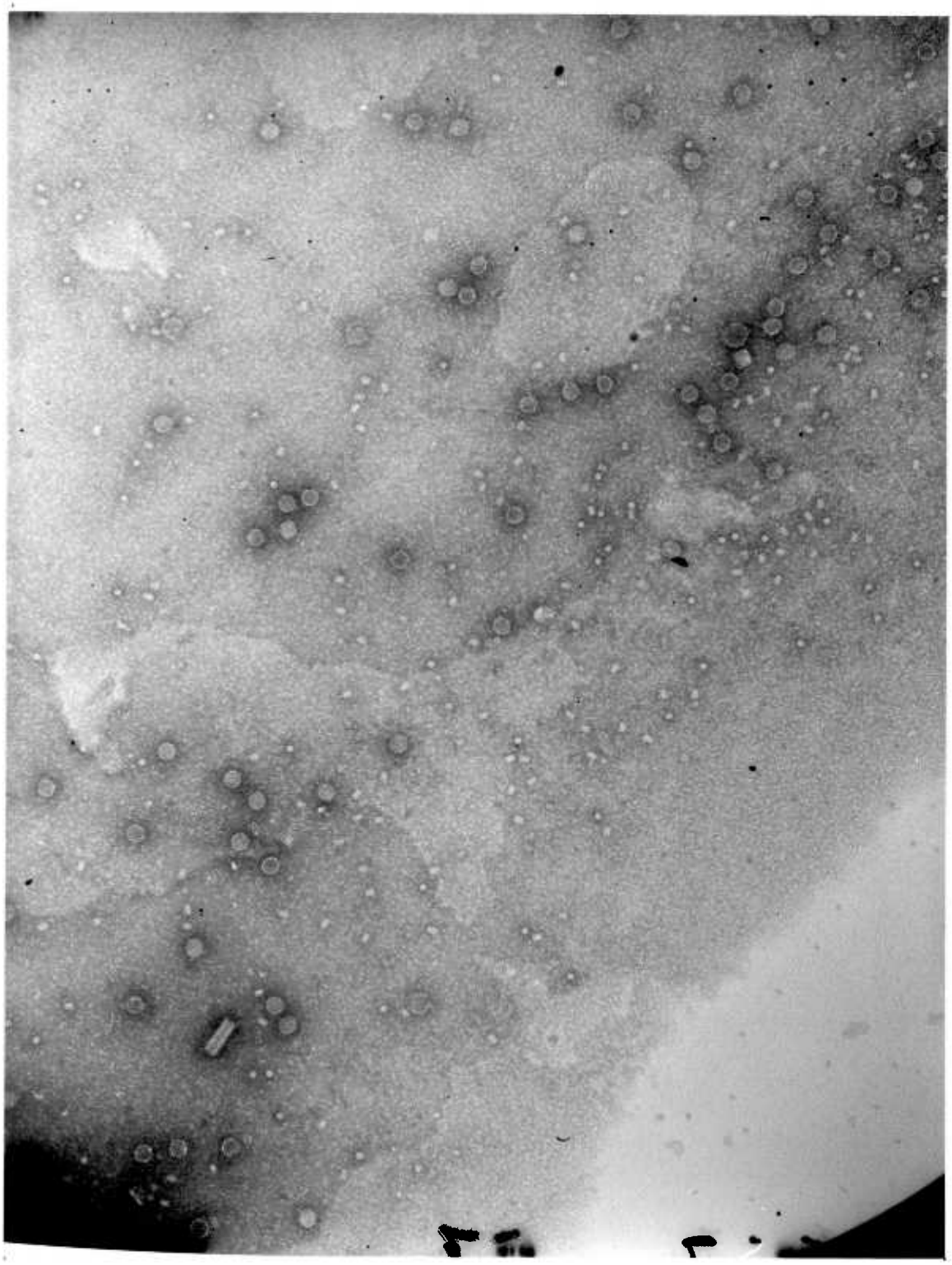


Figure 15 Electron micrograph of Ggt 235-1 virus (x 100,000 magnification)

gradient, where two faint bands could be seen by light scattering. RNA analysis by p.a.g.e. (G.M.7 (b)) of these fractions after dialysis against P buffer detected no RNA.

(d) Attempted preparation of virus RNA

RNA was extracted from 40 ml of the crude virus as described in G.M.11 (a). The precipitate was taken up in 1 ml of STM buffer and dialysed once against this buffer. Analysis of the preparation by p.a.g.e. (G.M.7 (b)) using standard size gels detected no virus RNA.

The limit of detection of ds-RNA on a standard size gel is approximately 100 µl of a preparation whose E_{260} value is 0.05 i.e. 2.5 µg/ml. Assuming that the ds-RNA is 20% of the mass of the virus (Rawlinson et al., 1973), this corresponds to a virus concentration of 12.5 µg/ml or 1.25×10^{-2} mg/ml. The limit of detection of virus particles by electron microscopy is 10^8 particles/ml (Anderson et al., 1966). Assuming that the molecular weight of the virus was 9×10^6, the minimum concentration that could be detected by electron microscopy was 1.5×10^{-6} mg/ml. It was therefore concluded that no virus RNA was detected in the crude preparation either because the virus concentration was between 1.5×10^{-6} mg/ml and 1.25×10^{-2} mg/ml or because the virus consisted of empty capsids.

Section 4 - Screening of selected Ggt isolates for ds-RNA

The preliminary investigations described in Sections 1, 2 and 3 indicated that the viruses infecting Ggt were far more numerous than the two suggested by preliminary investigations (Rawlinson et al., 1973). It was decided to screen a number of isolates, in which virus had been detected by electron microscopy (see Introduction), for ds-RNA for the following reasons:

(i) It was thought that the viruses of Ggt 45/10 might occur singly in some isolates. Such "biological separation" would permit the completion of their characterisation. It was therefore necessary to identify those isolates containing a minimum of ds-RNA components and therefore a simple virus system.

(ii) The isolation and purification of the virus from these isolates would be facilitated by the identification of those isolates which contained no detectable ds-RNA.

All the Ggt isolates were grown on basal/corn steep liquor as described in G.M.1 and harvested at the primary stage. Total nucleic acid extractions were performed as described in G.M.11 (a) and the nucleic acid preparations analysed by p.a.g.e. (G.M.7 (b)). RNA was identified as double-stranded by the characteristic sharpness of the bands on the gels, by its property of staining pink with toluidine blue (single-stranded nucleic acids stain blue) and by its resistance to DNAase 1 treatment (G.M.12). It was not possible to prove that all these RNA components were double-stranded by resistance to

RNAase A in 2 x SSC and degradation by RNAase A in 0.1 x SSC (G.M.13). This was probably due to a combination of inappropriate [RNA] to [RNAase A] ratios for the demonstration of the differential action and competition from the host single-stranded RNA as a substrate. PcV and PsV ds-RNA were used as standards in an approximate determination of the molecular weights of the ds-RNA components of each extract. In some cases, distortion of the RNA bands on the gel increased the possible error in the molecular weight determinations. The limit of detection of ds-RNA by the method used was estimated to be 0.1 µg/g wet weight of mycelium.

No ds-RNA was detected in the following isolates: 45/8, 235-1, 254-2, F7, F911-1 and 01-1-3. The remaining isolates were found to contain different numbers of ds-RNA components varying from one up to eleven. The results are summarised in Figure 16. A striking observation was that no two isolates contained the same distribution of ds-RNA components.

n + = n ds-RNA components within the molecular weight range given

MOLECULAR WEIGHT x 10^{-6}	021/5	38-4	PP2a	374-2	019/6	3b1a	OgA	F3	F6	F10	F11	F17	F20	F22	African 1	African 3	U.S.A.3	01-1-4	Aust. T1	Ggt isolates
>2.20						2+		2+	2+		1+	1+	2+	1+		3+			1+	
2.10-2.19																				
2.00-2.09																				
1.90-1.99			2+																	
1.80-1.89																				
1.70-1.79										1+										
1.60-1.69						1+				1+						1+			1+	
1.50-1.59		1+				1+	1+	1+	1+		1+	1+	1+			1+				
1.40-1.49						2+		1+	1+				2+		2+	2+	1+			
1.30-1.39																				
1.20-1.29	1+	1+		1+		1+				1+			2+		1+	3+	3+		1+	
1.10-1.19	2+	2+		2+	1+	2+		2+	1+	1+			1+		2+	1+	2+	1+		
1.00-1.09		1+		1+				1+										1+		
0.90-0.99								1+			1+	1+								
	ENGLISH										FRENCH				AFRICAN		AMERICAN	JAPANESE	AUSTRA-LIAN	

Figure 16 Distribution of ds-RNA components in Ggt isolates

Section 5 - Isolation, purification and characterisation of virus from selected Ggt isolates

Virus preparations were obtained and purified from ten of the isolates found to contain ds-RNA, representing a range of complexity. Isolates containing a minimum of ds-RNA components were chosen in the hope that the viruses of Ggt 45/10 occurred in them singly and the others were chosen because their ds-RNA molecular weights indicated that they contained some completely different viruses. The following isolates were chosen: 38-4, PP2a, 019/6, 3bla, OgA, F3, F6, F10, 01-1-4 and Aust. T1.

Every isolate was grown on basal/corn steep liquor as described in G.M.1 and harvested at the primary stage. Virus was obtained and purified as in G.M.3 and characterised as follows:

(i) Size. A histogram was constructed of the sizes of 200 particles in a purified virus sample (or less in the case of a dilute sample) using electron micrographs of x 100,000 magnification, in order to determine the predominant size(s) (G.M.14)(Table 22). P. 119

(ii) U.v. spectrum. This was determined as in G.M.6.

(iii) RNA molecular weights. These were the average of three determinations by p.a.g.e. (G.M.7 (b)) using PcV and PsV-S ds-RNA markers as internal standards and the semi-micro size gel system. The virus RNA used was extracted from the crude preparation (G.M.11 (b)).

(iv) Proof of double-strandedness of the RNA. Double-strandedness of the virus RNA was proven by resistance to RNAase A in 2 x SSC and degradation by RNAase A in 0.1 x SSC (G.M.13).

(v) Polypeptide molecular weights. These were the average of three determinations by p.a.g.e. using pure virus, internal standards and the semi-micro size gel system (G.M.7 (c)).

Sedimentation coefficients of virions were not determined, since, in most cases, the virus preparations characterised were mixtures of viruses.

The results for the viruses of each isolate are tabulated below. The intensity of the staining of each RNA and polypeptide band on the polyacrylamide gels was estimated visually in order to give an estimate of their relative proportions. It should be noted, however, that since the RNA was obtained from crude virus preparations, which could have contained some free RNA, that the proportions of specific RNA and polypeptide components are not comparable.

(A) The viruses of isolates PP2a, 019/6, OgA and Aust.T1.

Table 7 Properties of viruses of isolates PP2a, 019/6, OgA and Aust.T1

Host isolate	Particle size (nm)	E_{260}: E_{280} value	Molecular weight* ds-RNA (x 10^{-6})	polypeptide
PP2a	35	1.64	1.59m 1.55m	(2 aggregated)
019/6	35	1.63	1.28s 1.20f	60,000s
OgA(I)+	35	1.65	1.22m	48,000m
OgA(II)+	35	1.44	1.53m 1.42m 1.23s	68,000f 55,000f
Aust. T1	35	1.45	1.54s	74,000s 68,000f

*Intensity of bands on polyacrylamide gels signified as s = strong, m = medium and f = faint

+Two separate preparations of virus from the same isolate.

(B) The virus of isolate 38-4

Size of virus particles: 35 nm.

E_{260}: E_{280} = 1.52.

Table 8 Ds-RNA and polypeptide molecular weights of virus of isolate 38-4

Ds-RNA		Polypeptides	
Molecular weight x 10^{-6}	Intensity of band	Molecular weight	Intensity of band
1.59	faint	55,000	strong
1.55	faint		
1.29	strong		
1.21	medium		
1.09	medium		
5 ds-RNA components		1 major polypeptide	

Encapsidation of ds-RNA components

From the partial fractionation of the virus components across a rate sucrose gradient, it was deduced that the major polypeptide of molecular weight 55,000 encapsidated the RNA components of molecular weights 1.29×10^6, 1.21×10^6 and 1.09×10^6 (Figure 17)

(C) The viruses of isolate 3bla

Sizes of virus particles: 35 nm (89%) and 40 nm (10%)

E_{260}: E_{280} = 1.50.

Table 9 Ds-RNA and polypeptide molecular weights of viruses of isolate 3bla

Ds-RNA		Polypeptides	
Molecular weight x 10^{-6}	Intensity of band	Molecular weight	Intensity of band
4.10	strong	127,000	faint
3.13	medium	120,000	faint
1.62	strong	87,000	strong
1.56	strong	83,000	medium
1.49	medium	78,000	strong
1.46	medium	68,000	strong
1.29	medium	66,000	strong
1.23	medium	55,000	medium
1.20	medium	54,000	medium
1.11	faint		
10 ds-RNA components		9 unaggregated polypeptides	

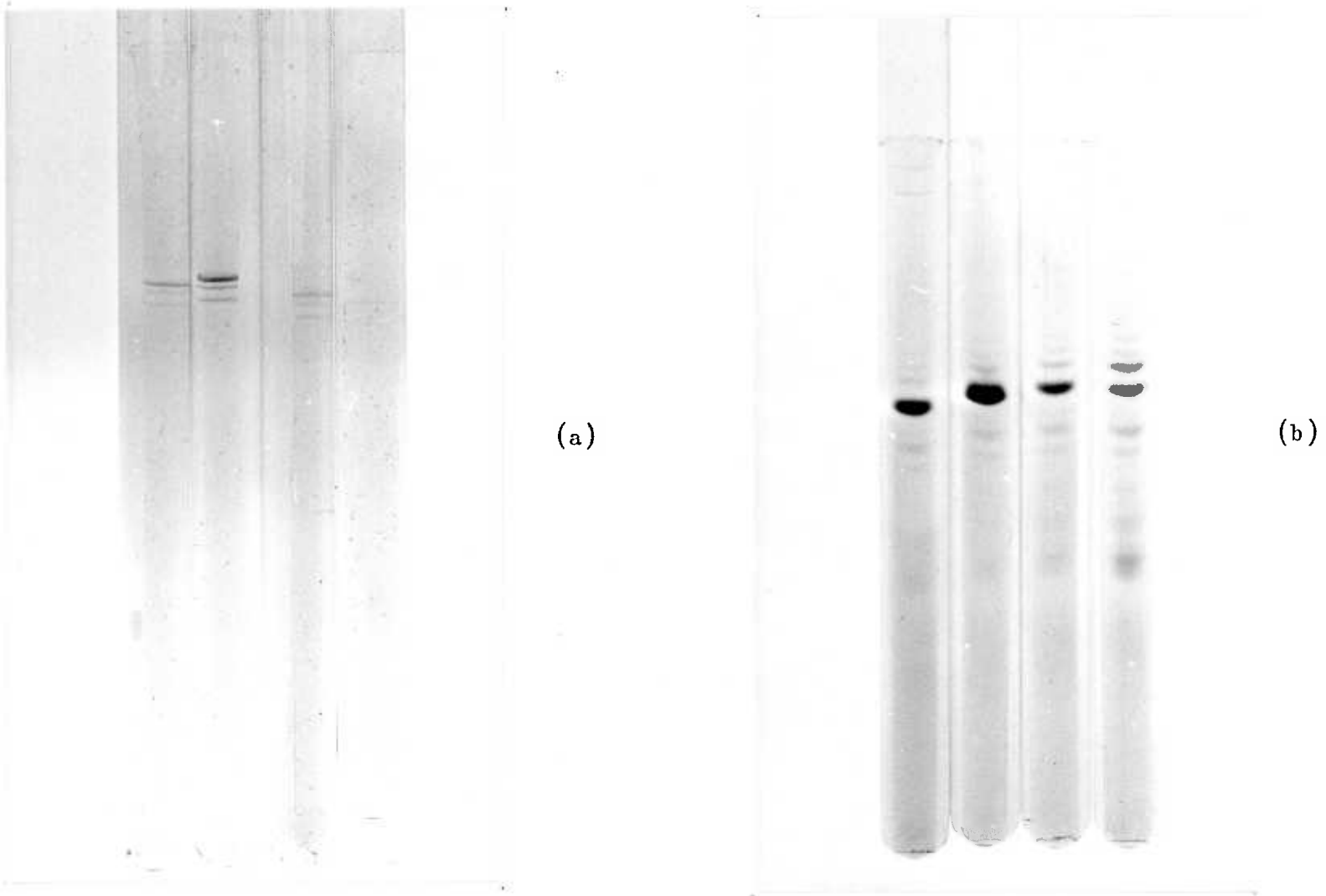


Figure 17 Polyacrylamide gel electrophoresis of Ggt 38-4 virus RNA (a) and polypeptides (b) from sucrose density gradient fractions following rate centrifugation. From left to right: fraction B20; fraction B22; fraction B24; fraction B26. The direction of migration was from top to bottom

Encapsidation of ds-RNA components

From the partial fractionation of virus components across a rate sucrose gradient, it was deduced that the polypeptide of molecular weight 68,000 encapsidated the RNA components of molecular weights 1.62×10^6 and 1.56×10^6 (Figure 18).

Sizes of virus particles and their capsid polypeptides

Comparison of the relative abundance of the 40 nm and 35 nm particles and the relative intensities of the polypeptide bands on polyacrylamide gels showed that the polypeptide of molecular weight 68,000 was the capsid polypeptide of one of the 35 nm particles.

(D) The viruses of isolate F3

Sizes of virus particles: 40 nm (52%) and 35 nm (41%) (Figure 19)

E_{260}: E_{280} = 1.27.

Table 10 Ds-RNA and polypeptide molecular weights of viruses of isolate F3

Ds-RNA		Polypeptides	
Molecular weight x 10^{-6}	Intensity of band	Molecular weight	Intensity of band
5.8 to 6.0	medium	125,000	strong
5.4 to 5.5	medium	55,000	faint
1.60	strong		
1.54	strong		
1.26	medium		
1.20	faint		
1.00	faint		
7 ds-RNA components		2 unaggregated polypeptides	

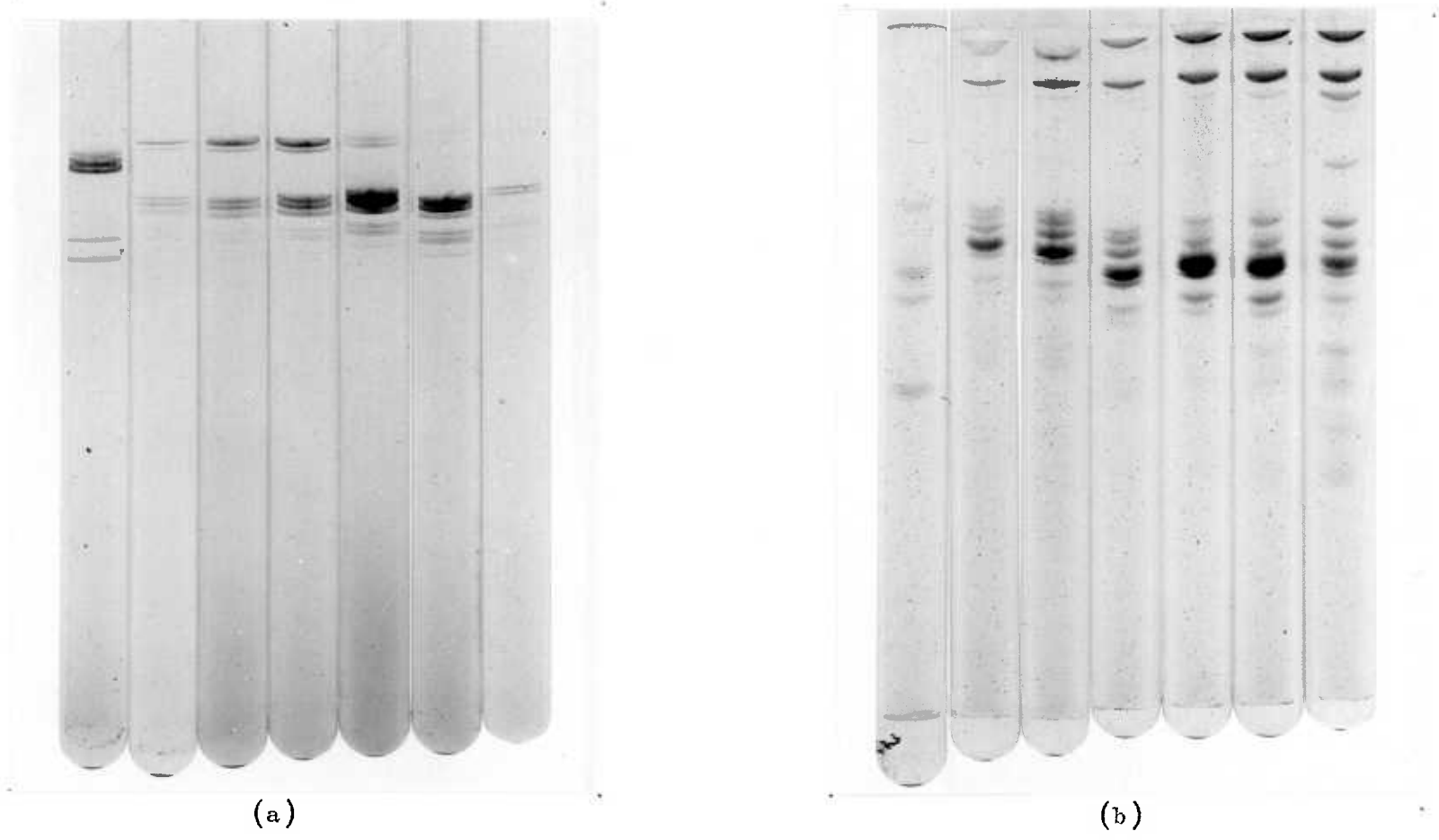


Figure 18 Polyacrylamide gel electrophoresis of Ggt 3bla virus RNA (a) and polypeptides (b) from sucrose density gradient fractions following rate centrifugation. From left to right: standards; fraction A6; fraction A9; fraction A12; fraction A16; fraction A20; fraction A23. The direction of migration was from top to bottom



Figure 19 Electron micrograph of Ggt F3 virus (x 100,000 magnification)

Encapsidation of ds-RNA components

From the partial fractionation of virus components across a rate sucrose gradient, it was probable that the polypeptide of molecular weight 125,000 encapsidated the RNA components of molecular weights 5.8 x 10^6 to 6.0 x 10^6 and 5.4 x 10^6 to 5.5 x 10^6. However, the aggregation of the polypeptides observed on polyacrylamide gels confused the interpretation.

Sizes of virus particles and their capsid polypeptides

Comparison of the relative abundance of the 40 nm and 35 nm particles and the relative intensities of the polypeptide bands on polyacrylamide gels showed that the polypeptide of molecular weight 125,000 was the capsid polypeptide of the 40 nm particles and that the polypeptide of molecular weight 55,000 was the capsid polypeptide of the 35 nm particles.

(E) The viruses of isolate F6

Sizes of virus particles: 35 nm (93%) and 40 nm (6%) (Figure 20)

E_{260}: E_{280} = 1.45.

Table 11 Ds-RNA and polypeptide molecular weights of viruses of isolate F6

Ds-RNA		Polypeptides	
Molecular weight x 10^{-6}	Intensity of band	Molecular weight	Intensity of band
5.9 to 6.4	medium	90,000	faint
4.10	medium	86,000	faint
2.78	medium	79,000	medium
1.70	faint	55,000	strong
1.64	strong		
1.59	strong		
1.56	strong		
1.53	medium		
1.27	strong		
1.18	medium		
10 ds-RNA components		4 unaggregated polypeptides	

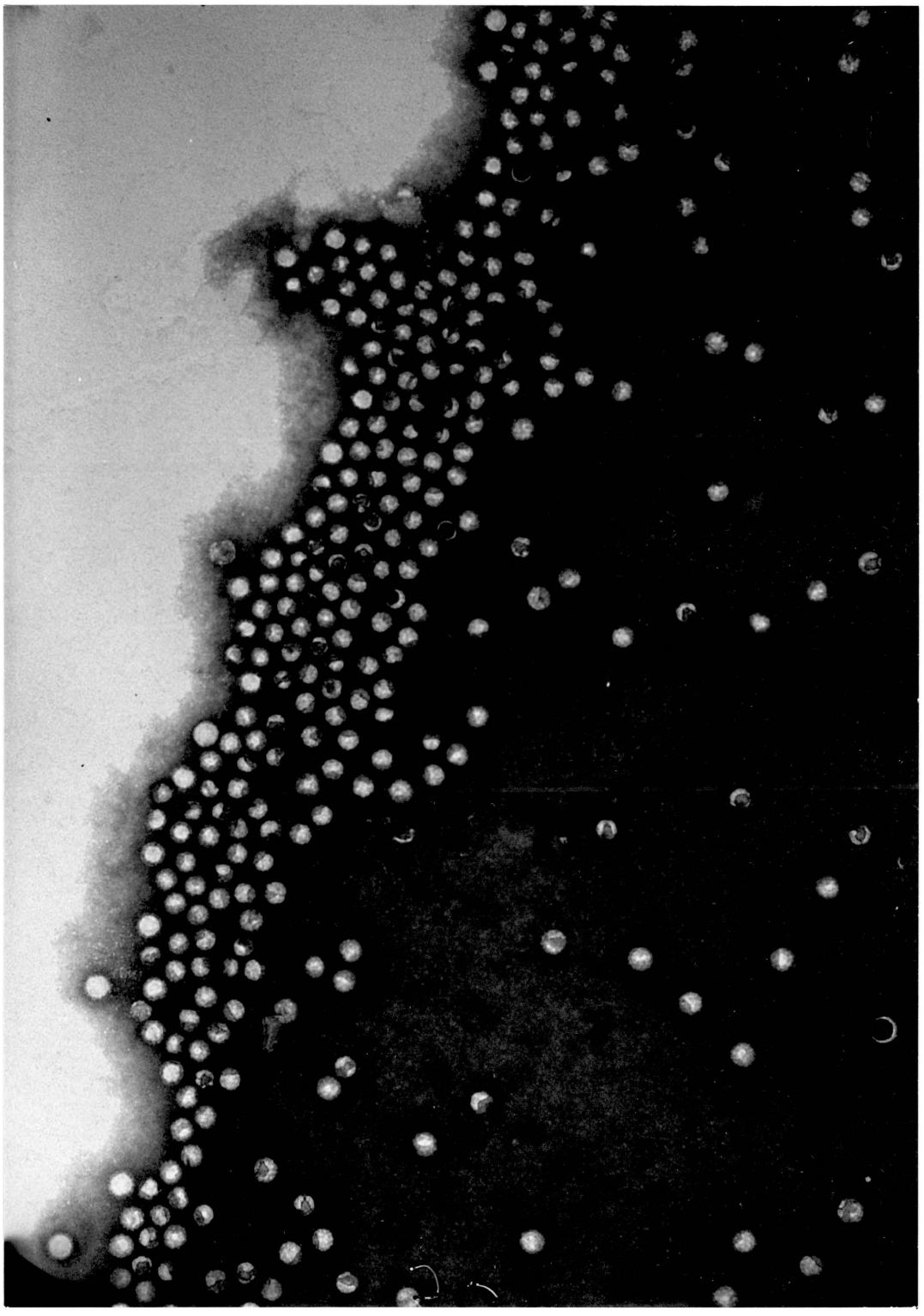


Figure 20 Electron micrograph of Ggt F6 virus (x 100,000 magnification)

Encapsidation of ds-RNA components

From the partial fractionation of virus components across the rate sucrose gradient, it was probable that the polypeptide of molecular weight 55,000 encapsidated the RNA components of molecular weights 1.64×10^6, 1.59×10^6, 1.56×10^6, 1.53×10^6, 1.27×10^6 and 1.18×10^6. This interpretation assumed that if the aggregated polypeptides were unaggregated that the relative intensities of the unaggregated polypeptide bands on polyacrylamide gels would remain unaltered (Figure 21).

Sizes of virus particles and their capsid polypeptides

The capsid polypeptide of the 35 nm particles had a molecular weight of 55,000.

(F) The virus of isolate F10

Size of virus particles: 40 nm

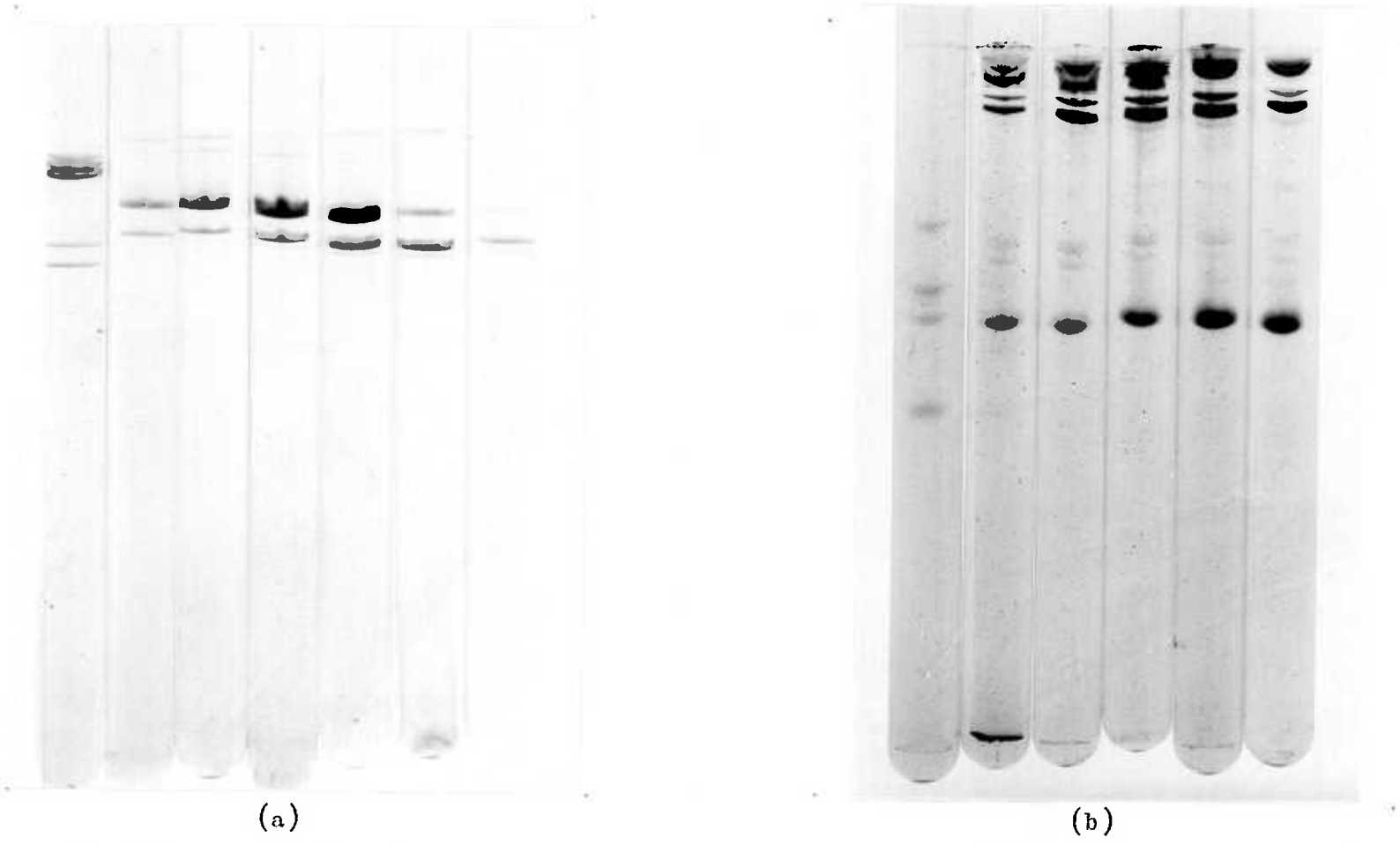
E_{260}: E_{280} = 1.33.

Table 12 Ds-RNA and polypeptide molecular weights of virus of isolate F10

Ds-RNA		Polypeptides	
Molecular weight $\times 10^{-6}$	Intensity of band	Molecular weight	Intensity of band
5.4 to 5.8	medium	94,000	medium
1 ds-RNA component		1 polypeptide	

Encapsidation of ds-RNA components

RNA components of molecular weight 1.85×10^6 and 1.71×10^6 were also detected in the crude virus preparation.



(a) (b)

Figure 21 Polyacrylamide gel electrophoresis of Ggt F6 virus RNA (a) and polypeptides (b) from sucrose density gradient fractions following rate centrifugation. From left to right: standards; fraction A9; fraction A11; fraction A13; fraction A15; fraction A17; fraction A19. The direction of migration was from top to bottom

Only the virus with a polypeptide molecular weight of 94,000 encapsidating the RNA component of molecular weight 5.4 x 10^6 to 5.8 x 10^6 remained intact following rate centrifugation in a sucrose density gradient.

(G) The viruses of isolate 01-1-4

Sizes of virus particles: 35 nm (63%) and 30 nm (26%) (Figure 22)

E_{260}: E_{280} = 1.53.

Table 13 Ds-RNA and polypeptide molecular weights of viruses of isolate 01-1-4

Ds-RNA		Polypeptides	
Molecular weight x 10^{-6}	Intensity of band	Molecular weight	Intensity of band
1.25	medium	55,000	medium
1.16	medium		
2 ds-RNA components		1 polypeptide	

Encapsidation of ds-RNA components

The polypeptides of molecular weight 55,000 encapsidated the two RNA components of molecular weights 1.25 x 10^6 and 1.16 x 10^6.

Sizes of virus particles and their capsid polypeptides

The polypeptide of molecular weight 55,000 was the capsid polypeptide of both the 35 nm and 30 nm particles.

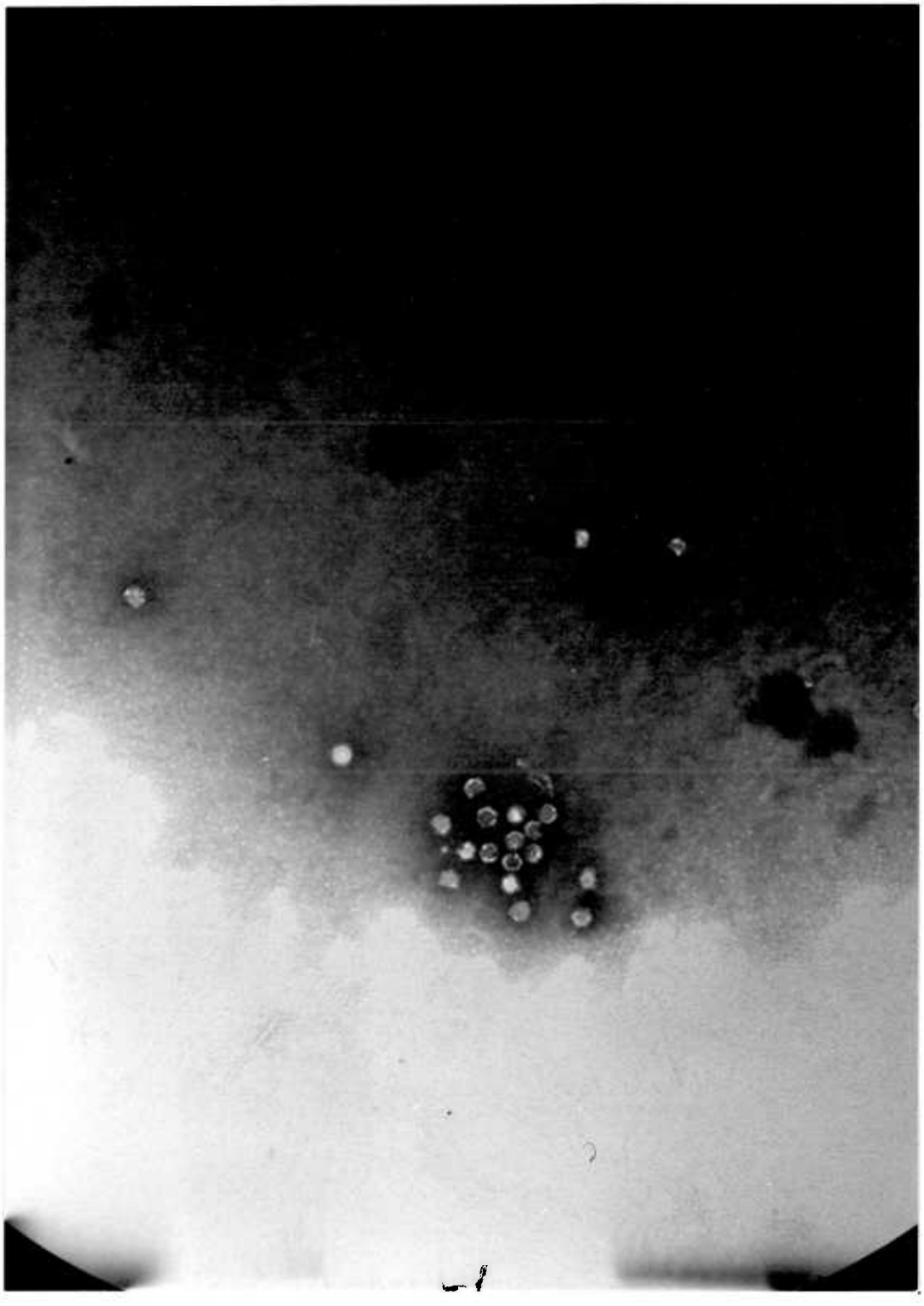


Figure 22 Electron micrograph of Ggt 01-1-4 virus (x 100,000 magnification)

Section 6 - Comparisons between viruses of the complex

(A) Molecular weights of the virus RNA components and capsid polypeptides

Virus capsid polypeptides whose molecular weights differed by 2,000 or less were tested for equivalence of electrophoretic mobility (G.M.7 (c)).

Table 14 Virus polypeptides with mol. wt. difference $\leq$ 2,000 found to have the same electrophoretic mobility

Host isolate	Molecular weights of polypeptides having the same electrophoretic mobility		
3bla	87,000	78,000	54,000
F6	90,000	79,000	55,000*
45/10	72,000		
Aust.T1	74,000		

*Although the smallest polypeptide of the viruses of Ggt F6 was found to have an average molecular weight of 55,000, it did not have the same electrophoretic mobility as glutamate dehydrogenase. The viruses of Ggt 3bla contained a polypeptide of average molecular weight 55,000 which did have the same electrophoretic mobility as the standard.

It was shown that the Ggt 3bla virus polypeptide of molecular weight 127,000 did not have the same electrophoretic mobility as the Ggt F3 virus polypeptide of molecular weight 125,000.

The ds-RNA components of viruses with molecular weights greater than 2.20×10^6 were tested for equivalence of

electrophoretic mobility prior to determination of their molecular weights (G.M.7 (b) and Section 5).

The ds-RNA components of viruses found to be serologically related (Section 7) were also tested for equivalence of electrophoretic mobility.

Table 15 Virus ds-RNA components found to have the same electrophoretic mobility

Host isolate	Molecular weights of ds-RNA components having the same electrophoretic mobility		
45/10	1.64	1.59	1.48
3bla	1.62	1.56	1.46
38-4	1.29	1.21	
019/6	1.28	1.20	
38-4	1.29	1.21	
3bla	1.29	1.20	

(B) Aggregated virus capsid polypeptides

Polyacrylamide gel electrophoresis showed that polypeptide aggregates of high molecular weight occurred in virus preparations from the following isolates: PP2a, Aust. T1, F3, F6 and 3bla. This aggregation prevented the unequivocal assignment of ds-RNA components to specific capsid polypeptides and of sizes to these capsids. The following drastic denaturing conditions were used for sample preparation and during polyacrylamide gel electrophoresis, in order to resolve whether the aggregation was attributable to either covalent or non-covalent linkage of the polypeptides. Virus preparations in

either P, PK, or PNaCl buffer were dissociated by incubation at 37°C for 3 h in a final concentration of 1% (w/v) SDS, 1% (w/v) dithiothreitol, 0.01 M-EDTA and saturation with respect to urea. At the end of the incubation time, dithiothreitol was added to increase the final concentration by 1% (w/v) and the samples were heated at 100° C for three minutes. The polypeptides were then electrophoresed on gels containing 7.92% (w/v) acrylamide + 0.08% (w/v) methylene bis acrylamide and 8 M- urea with a running buffer of 0.1 M-sodium dihydrogen phosphate + 0.01 M-disodium EDTA + 0.1 M-sodium thioglycollate + 0.1% (w/v) SDS, pH 7.2 at 6 ma/tube then stained as in G M.7 (c). Aggregation of the polypeptides was still found, even under these conditions and was therefore almost certainly attributable to covalent linkage.

The covalent aggregation could have been caused by the formation of S linkageswith a quinone nucleus between adjacent capsid subunits similar to those found in a non-infective mutant of cowpea chlorotic mottle virus extracted in the absence of polyphenol oxidase inhibitors (Bancroft et al., 1971). It was therefore decided to prepare virus from isolate PP2a with polyphenol oxidase inhibitors sodium diethyldithiocarbamate and sodium thioglycollate in the extraction buffer. The isolate was grown on basal/corn steep liquor as described in G.M.1 and harvested at the primary stage. Virus was obtained and purified as in G.M.3 but using 0.2% (w/v) sodium diethyldithiocarbamate and 0.2% (w/v) sodium thioglycollate in the extraction buffer, which were the concentrations used by Frick and Lister (1978) in their extraction procedure for Ggt viruses. As a control,

virus was obtained and purified from an equal weight of mycelium using no inhibitors in the extraction buffer. RNA gels (G.M.7 (b)) run of the crude virus prior to purification on sucrose gradients showed that the yield of virus using inhibitors in the extraction buffer was about one fifth of that obtaining using no inhibitors. Polypeptide gels (G.M.7 (c)) run of the velocity sucrose gradient fractions showed that the inhibitors did not prevent aggregation of the virus polypeptides, although the aggregates were lower in molecular weight than those obtained when no inhibitors were used in the extraction buffer. This showed that the aggregation was caused by the action of polyphenol oxidases but that the conditions described by Frick and Lister (1978) for their inhibition were far from optimal. Use of the inhibitors in the extraction buffer on a routine basis for the preparation of the Ggt viruses would therefore have required a five times scale-up of the quantities used. Presumably, the far greater yield obtained when aggregation occurred was due to the stabilisation of the virus to disruption since the non-infective mutant of cowpea chlorotic mottle virus was found to be more pH and salt stable than the wild-type virus (Bancroft et al., 1971).

Section 7 - Serology of the Ggt virus complex

Antisera were raised in mice to purified virus from isolates 38-4, Aust. T1, 019/6, OgA, F3 and 3bla as described in G.M.15. The other virus preparations used in the serological testing were purified from isolates F10, 45/10, F6, 01-1-4 and PP2a. The virus from isolates 38-4, 019/6 and F10 were single viruses, the remaining preparations were mixtures of different viruses (Section 5).

In order to interpret the serological data, it was found necessary to assume that each polypeptide in a mixture of viruses was the capsid polypeptide of a different virus. It was thought reasonable to make this assumption since no minor polypeptide components had been detected in particles of the single viruses. There was least supporting evidence in the case of the Ggt 3bla virus preparation, which was the most complex. Some of the polypeptides present in low concentrations could either have been products of proteolytic degradation or even RNA polymerase molecules.

Standard size polyacrylamide gels were run of the virus mixtures and the polypeptides were visualised with Coomassie Blue G stain (G.M.7 (c)). The concentrations of the different viruses in each mixture were estimated from Gilford densitometer scan traces at 550 nm of the gels (Table 16). For the virus preparations from isolates Aust. T1, F3, F6 and PP2a in which aggregation of the capsid polypeptides had occurred, the assumption was made in the estimation of the virus concentrations that if all the polypeptides had been unaggregated, the relative proportions of the polypeptides would have been the same. It

was also assumed that virus particles which contained aggregated polypeptides would be serologically indistinguishable from those not containing aggregated polypeptides. The justification for this assumption came from the work of Bancroft et al. (1971) who found that the non-infective mutant of cowpea chlorotic mottle virus, which contained aggregated capsid polypeptides, was serologically indistinguishable from the wild-type virus. The viruses used in the serological tests are listed in Table 16.

Table 16 Viruses used in the serological tests

Host isolate	Virus	Molecular weight of capsid polypeptide	E_{260}
38-4		55,000	0.36
Aust. T1	A	73,000	3.00
	B	68,000	0.20
019/6		60,000	0.64
OgA	A	68,000	0.22
	B	55,000	0.24
F3	A	125,000	0.44
	B	55,000	0.12
3b1a	A	127,000	0.02
	B	120,000	0.05
	C	89,000	0.17
	D	83,000	0.18
	E	79,000	0.68
	F	68,000	1.78
	G	66,000	0.50
	H	55,000	0.20
	I	54,000	0.03

Table 16 Viruses used in the serological tests (continued)

Host isolate	Virus	Molecular weight of capsid polypeptide	E_{260}
F10		94,000	0.24
45/10	A	73,000	0.77
	B	68,000	0.22
	C	66,000	0.08
	D	55,000	0.02
	E	51,000	0.03
F6	A	89,000	0.04
	B	86,000	0.08
	C	79,000	0.34
	D	55,000	2.30
01-1-4		55,000	0.58
PP2a	A		0.08
	B		0.08

The gel immunodiffusion tests were performed as described in G.M.16. When tested against their homologous antiserum, the virus from isolates 38-4, Aust. T1, 019/6 and F3 gave one precipitin line, the virus from isolate 0gA gave two precipitin lines and the virus from isolate 3bla gave either two or three precipitin lines (see Figures 23 and 24). The titres of the antisera are given in Table 17. Where a mixed antiserum was raised to a virus preparation containing a mixture of viruses, assignment of the antiserum titre to the single virus was done assuming that immunogenicity was proportional to the concentration of the virus in the mixture injected. The actual titres were double those quoted because of the dilution with

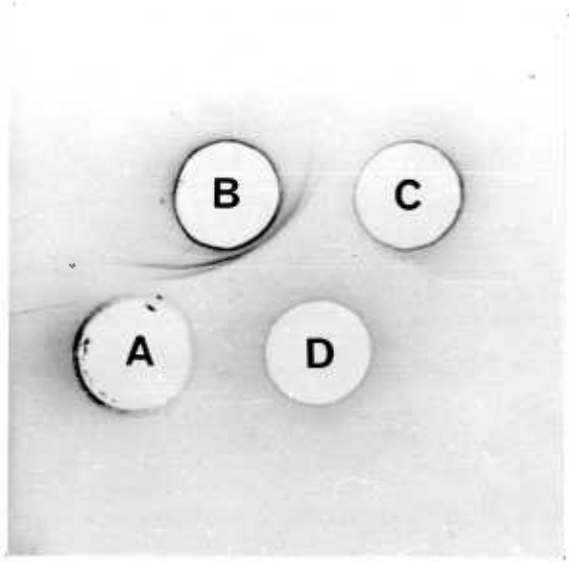


Figure 23 Immunodiffusion test of viruses prepared from Ggt isolates Aust. T1 (well A), 3bla (well B), 45/10 (well C) and Antiserum/Ggt 3bla virus (well D)

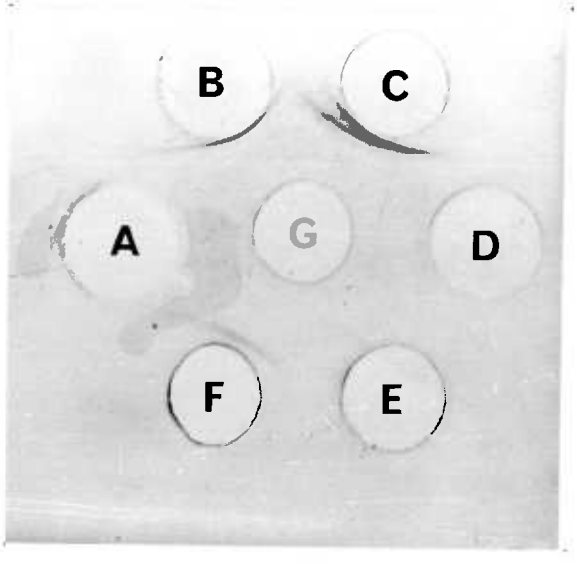


Figure 24 Immunodiffusion test of viruses prepared from Ggt isolates Aust. T1 (well A), 38-4 (well B), 019/6 (well C), OgA (well D), F3 (well E), 3bla (well F) and Antiserum/Ggt 019/6 virus (well G).

glycerol. The loading in each well was 5 µl unless otherwise stated. The virus preparations were used undiluted at the concentrations given in Table 16. None of the antisera reacted with ds-RNA.

Table 17 Antiserum titres

Antiserum	Antigen	Antiserum titre+
As/Ggt 38-4 virus	Ggt 38-4 virus	1/16
As/Ggt Aust.T1 virus A	Ggt Aust.T1 virus A	1/4
As/Ggt 019/6 virus	Ggt 019/6 virus	1/4
As/Ggt OgA virus A*	Ggt OgA virus A	1/4
As/Ggt OgA virus B*	Ggt OgA virus B	1/4
As/Ggt F3 virus A	Ggt F3 virus A	1/32
As/Ggt 3bla virus E	Ggt 3bla virus E	1/4
As/Ggt 3bla virus F	Ggt 3bla virus F	1/128

*15 µl loading of antigen

+See text

Table 18 Virus antigen dilution end-points

Antigen	E_{260}	Virus antigen	E_{260} of virus at virus antigen dilution end-point
Ggt 38-4 virus	0.36	1/4	0.09
Ggt Aust.T1 virus A	3.00	1/64	0.05
Ggt 019/6 virus	0.64	1/4	0.16
Ggt OgA virus B	0.24	1/2	0.12
Ggt F3 virus A	0.44	1/4	0.11
Ggt 3bla virus E	0.68	1/64	0.01
Ggt 3bla virus F	1.78	1/64	0.03
Ggt 3bla virus G	0.50	1/1	0.50

When determining the virus antigen dilution end-points, the loading in each well was 10 µl. The homologous antisera were used undiluted. The values obtained are to be found in Table 18.

Theoretically, it should have been possible to assign precipitin lines to specific viruses in a mixture of viruses directly by the technique of immunoelectrophoresis, thus avoiding the assumption that immunogenicity was proportional to the concentration of the virus in the mixture. However, a number of techniques and different conditions were tried in the attempted electrophoretic separation of the viruses of Ggt 45/10, none of which were successful (Section 1). An attempt to separate the viruses of Ggt 3bla and Ggt OgA using 0.5% (w/v) agarose gels with a running buffer of TAE, pH 8.0 resulted in precipitation of the viruses on the tops of the gels. These results indicated that much more work will have to be devoted towards finding a set of conditions which will effect an electrophoretic separation of a mixture of Ggt viruses.

Each of the Ggt viruses (Table 16) was tested against each of the antisera (Table 17). In all the gel immunodiffusion tests performed (G.M.16), a 10 µl loading per well was used. All antigens and antisera were used undiluted.

The positive serological relationships are listed in Table 19. In all cases, the positive reaction consisted of one precipitin line only.

In the cases where a positive serological relationship was found between an antiserum raised against a single virus and a

mixture of viruses or between a mixed antiserum and either a single virus or a mixture of viruses, the related antibody and antigen could be deduced by consideration of the virus sizes, capsid polypeptide molecular weights and molecular weights of the RNA components encapsidated which were common to both virus preparations. These deductions followed from the observation that serologically related viruses also share many other properties. Such considerations led to the conclusion that the viruses listed in Table 20 belong in two serological groups.

Table 19 Positive serological relationships between Ggt viruses

Antiserum	Antigens
As/Ggt 38-4 virus	Ggt 019/6 virus and Ggt 3bla virus A, B, C, D, E, F, G, H or I
As/Ggt 019/6 virus	Ggt 38-4 virus and Ggt 3bla virus A, B, C, D, E, F, G, H or I
As/Ggt 0gA virus A and B	Ggt 38-4 virus and Ggt 019/6 virus
As/Ggt 3bla viruses A-I (inc.)	Ggt 38-4 virus, Ggt 019/6 virus and Ggt 45/10 virus A, B, C, D or E

It is interesting to note that both Ggt 019/6 virus and Ggt 38-4 virus possess antigenic determinants which are common to Ggt 3bla virus H and Ggt 0gA virus B which themselves were found to have no antigenic determinants in common. Frick and Lister (1978) found a serological relationship between viruses from their isolates No.3 and No.59 but none between these and

Table 20 Ggt viruses belonging to the two serological groups

Serological group	Virus	Particle size (nm)	Molecular weight of capsid polypeptide	Molecular weight of ds-RNA components(s) encapsidated($\times 10^{-6}$)
1	Ggt 3bla virus F	35	68,000	1.63 and 1.58
	Ggt 45/10 virus B	35	68,000	1.48
2	Ggt 019/6 virus	35	60,000	1.29 and 1.21
	Ggt 38-4 virus	35	55,000	1.29, 1.21 and 1.09
	Ggt 3bla virus H	35	55,000	1.29 and 1.21
	Ggt OgA virus B	35	55,000	

virus from their isolate M-10. Viruses from most other isolates from the 1975 collections were variously related to M-10 virus but not to No.3 and No.59 viruses. However, the reactions of viruses from isolates G-1, E-3 and F-2 with the three antisera indicated interrelationships between the viruses of isolates No.3, No.59 and M-10. These results were not due to different reactions of different particle classes in the preparations used. These serological interrelationships are therefore the same as those to be found in serological group 2.

Ggt 3bla virus F and Ggt 45/10 virus B share many properties but encapsidate different ds-RNA components. Ggt 45/10 virus B may be a deletion mutant of Ggt 3bla virus F (see Discussion). Ggt 019/6 virus and Ggt 38-4 virus are identical in all properties tested apart from the molecular weights of their capsid polypeptides. The smaller capsid polypeptide of Ggt 38-4 virus could have been caused by a point mutation to a nonsense codon resulting in premature termination of transcription. It is not a deletion mutant of Ggt 019/6 virus since the RNA genome is the same size.

The viruses listed in Table 21 were serologically different from the viruses to which antisera were raised (Table 17). This deduction followed from the lack of serological reaction despite the fact that the viruses were used in the heterologous tests at concentrations in excess of the virus antigen dilution end-points in homologous tests.

Higher titre antisera may have detected more distant relationships. Relationships may exist among the viruses of

isolates F10, 45/10, F6, 01-1-4 and PP2a but no antisera to these viruses were available.

Table 21 Ggt viruses serologically different from the viruses to which antisera were raised

Virus	Serologically different viruses
Ggt 38-4 virus	Ggt Aust.T1 viruses A and B, Ggt OgA virus A, Ggt F3 viruses A and B, Ggt 3bla viruses C-G (inc.), Ggt F10 virus, Ggt 45/10 viruses A and B, Ggt F6 viruses C and D and Ggt 01-1-4 virus
Ggt Aust.T1 virus A	Ggt 38-4 virus, Ggt Aust.T1 virus B, Ggt 019/6 virus, Ggt OgA viruses A and B, Ggt F3 viruses A and B, Ggt 3bla viruses B-H (inc.), Ggt F10 virus, Ggt 45/10 viruses A-C (inc.), Ggt F6 viruses B-D (inc.), Ggt 01-1-4 virus and Ggt PP2a viruses A and B
Ggt 019/6 virus	Ggt Aust.T1 viruses A and B, Ggt OgA virus A, Ggt F3 virus A, Ggt 3bla viruses C-G (inc.), Ggt F10 virus, Ggt 45/10 viruses A and B, Ggt F6 viruses C and D and Ggt 01-1-4 virus
Ggt OgA virus B	Ggt Aust.T1 viruses A and B, Ggt OgA virus A, Ggt F3 viruses A and B, Ggt 3bla viruses C-H (inc.), Ggt F10 virus, Ggt 45/10 viruses A and B, Ggt F6 viruses C and D and Ggt 01-1-4 virus
Ggt F3 virus A	Ggt 38-4 virus, Ggt Aust.T1 viruses A and B, Ggt 019/6 virus, Ggt OgA viruses A and B, Ggt F3 virus B, Ggt 3bla viruses C-H (inc.) Ggt F10 virus, Ggt 45/10 viruses A and B, Ggt F6 viruses C and D and Ggt 01-1-4 virus
Ggt 3bla virus E	All other viruses in Table 16

Table 21 Ggt viruses serologically different from the viruses to which antisera were raised (continued)

Virus	Serologically different viruses
Ggt 3bla virus F	Ggt 38-4 virus, Ggt Aust.T1 viruses A and B, Ggt 019/6 virus, Ggt OgA viruses A and B, Ggt F3 viruses A and B, Ggt 3bla viruses B-E (inc.) and G-I (inc.), Ggt F10 virus, Ggt 45/10 viruses A, C and E, Ggt F6 viruses A-D (inc.)., Ggt 01-1-4 virus, Ggt PP2a viruses A and B
Ggt 3bla virus G	Ggt Aust.T1 virus A, Ggt 019/6 virus, Ggt 3bla viruses E and F, Ggt 45/10 virus A, Ggt F6 virus D and Ggt 01-1-4 virus

Section 8 - Summary of the biochemical characterisation of Ggt viruses

Full details of the numbers of particles measured and the distribution of particle sizes found in the virus preparations are given in Table 22. The results of the biochemical characterisation of the Ggt viruses are summarised in Figures 25 and 26 and in Table 23. The better characterised viruses are listed in order of decreasing capsid polypeptide molecular weight in Table 23. Where coelectrophoreses of capsid polypeptides and RNA components have been performed and their electrophoretic mobilities found to be identical, the averaged molecular weights are given. Omitted from Table 23 are the less well characterised 35 nm viruses, the 30 nm virus of Ggt 01-1-4 and the 27 nm virus of Ggt 235-1.

Table 22 Number of particles measured and the distribution of particle sizes among Ggt viruses

	Host isolate													
Particle size (nm)	45/10	45/9	235-1	PP2a	019/6	0gA^{+}(I)	0gA^{+}(II)	Aust.T1	38-4	3bla	F3	F6	F10	01-1-4
24			2											
25														
26														
27			35				7		3	2		1		
28			8											
29														
30	1		8						2			1		14
31	1		3			5			3					1
32	3		1			2								
33	7	6	8		5	1			6			1		1
34	7			2	12	4			23	1	1			1
35	252	244		27	182	64	67	195	119	177	64	185		34
36	16				1									
37	23													
38	5										5			
39								2			6			
40	32			1			1	3		20	82	12	19	3
41														
42													1	
Total number of particles measured:	347	250	65	30	200	76	75	200	156	200	158	200	20	54

+ Two separate preparations of virus from the same isolate

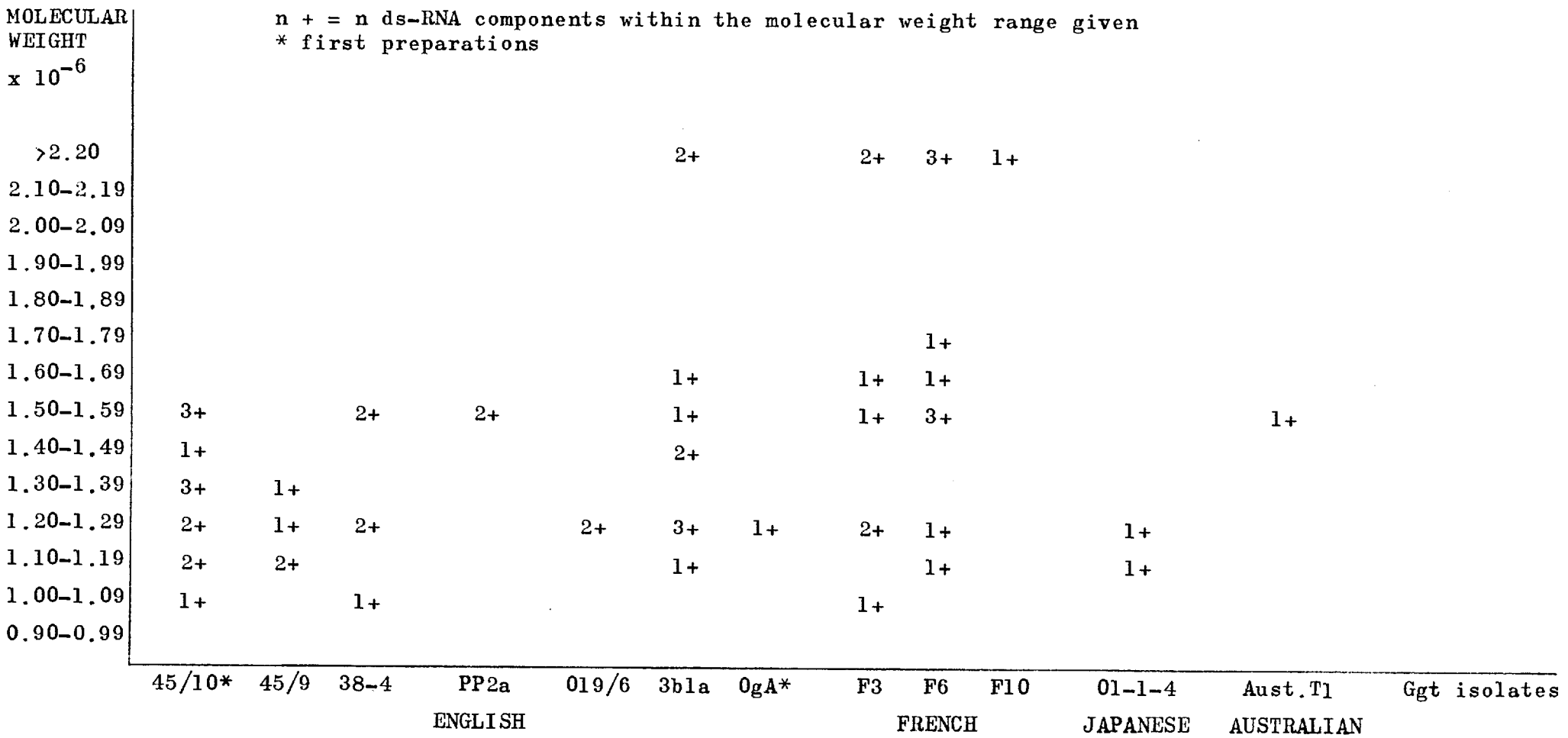


Figure 25 Molecular weights of the ds-RNA components of Ggt viruses

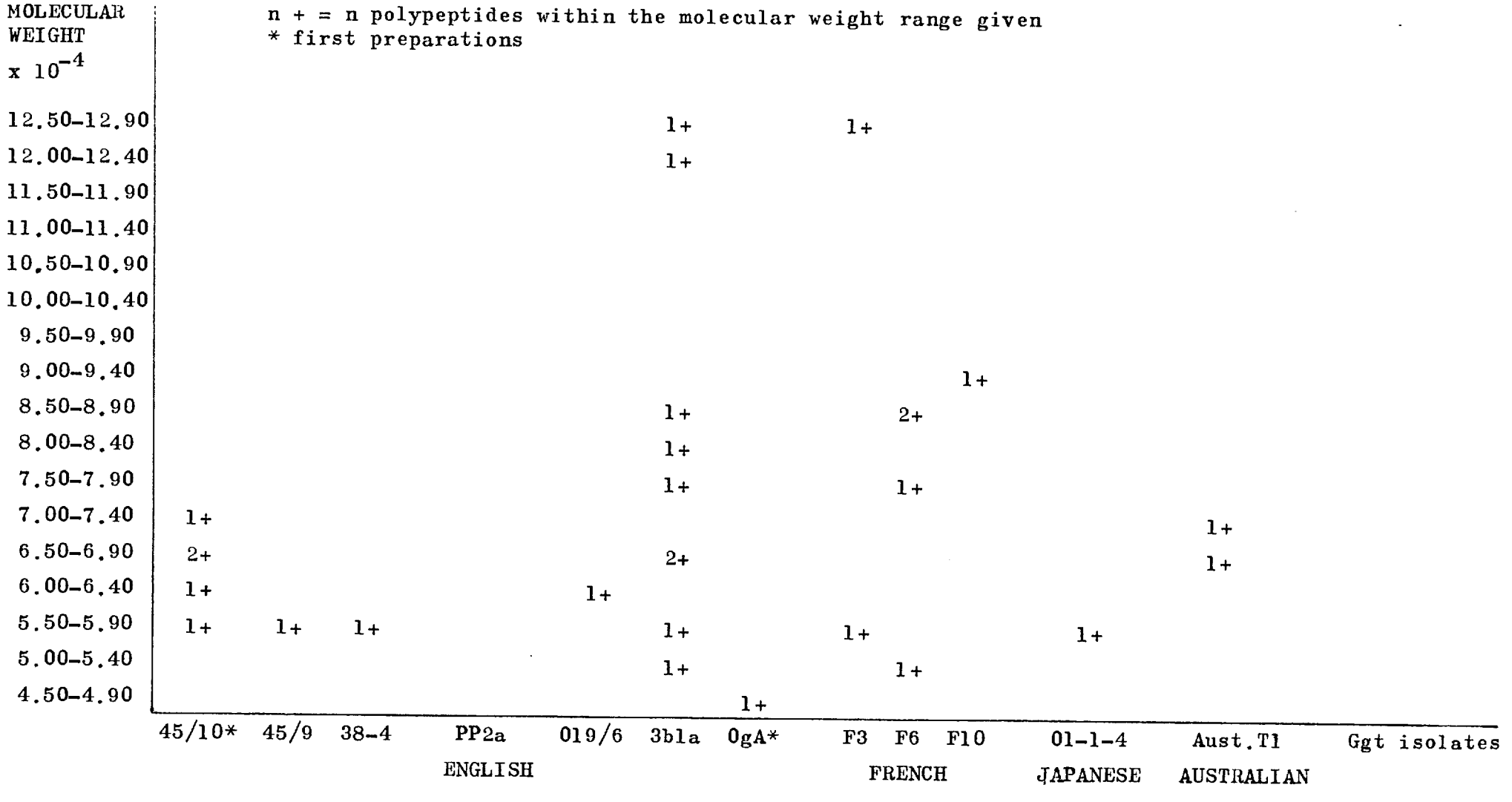


Figure 26 Molecular weights of the polypeptides of Ggt viruses

Table 23 Summary of the biochemical characterisation of distiguishable variants of Ggt viruses

Virus	Particle size (nm)	Molecular weight of capsid polypeptide	Molecular weight of ds-RNA components encapsidated ($\times 10^{-6}$)
Ggt F3 virus A	40	125,000	5.8 to 6 0 and 5.4 to 5.5
Ggt F10 virus	40	94,000	5.4 to 5.8
Ggt 45/10 virus A	35	73,000	1.63
Ggt Aust.T1 virus A	35	73,000	1.54
Ggt 3bla virus F	35	68,000	1.63 and 1.58
Ggt 45/10 virus B	35	68,000	1.48
Ggt 45/10 virus C	35	65,000	1.25
Ggt 019/6 virus	35	60,000	1.29 and 1.21
Ggt 38-4 virus and Ggt 3bla virus H	35	55,000	1.29 and 1.21
Ggt 45/10 virus D and Ggt 45/9 virus	35	55,000	1.33, 1.22, 1.15 and 1.12
Ggt F6 virus D	35	55,000	1.64, 1.59, 1.56, 1.53, 1.27 and 1.18
Ggt OgA virus C	35	48,000	1.22 and 1.17

Section 9 - Incompatibility between isolates

The ds-RNA components of the Ggt 40 nm viruses are probably at least dicistronic and those of the Ggt 35 nm viruses are probably monocistronic (see Discussion). The simplest 40 nm virus is Ggt F10 virus, which has one RNA component and one of the simplest 35 nm viruses is Ggt 019/6 virus, which has two RNA components (Table 23). Consideration of the coding capacity of each genome suggests that each virus possesses only sufficient genetic information to code for virus structural proteins and RNA polymerase molecules. However, similar considerations of the coding capacities of the genomes of the two 35 nm viruses, Ggt 38-4 virus, which has three RNA components and Ggt 45/9 virus, which has four RNA components, suggest that they both contain information other than that required for the structure and replication of the virus. The viruses of isolates 45/10, F3, F6 and possibly 3bla also appear to have an excess of genetic information above that required for virus replication (see Sections1, 2 and 5). It was thought that this "redundant" ds-RNA could code for killer factors as it does in the killer system of *Saccharomyces cerevisiae* (Wickner, 1976) and the killer system of the yeast-like phase of the corn smut pathogen, *Ustilago maydis* (Koltin and Day, 1976). The putative sensitive isolates chosen for the incompatibility testing were ascospore isolate F911-1, which was virus-free and 019/6, which contained virus possessing only sufficient genetic information to code for its own replication.

(A) Incompatibility of F911-1 versus 45/10, 45/9, F3 and F6.

The incompatibility testing was performed as described in G.M.17 with the PDA buffered at pH values 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. The control test was F911-1 paired with itself.

At pH 3.5 isolate F911-1 did not grow and the other isolates showed very little growth. At pH 4.0 colonies of isolates 45/10, 45/9, F3 and F6 showed very slow growth, took at least 21 days at 24°C to reach the F911-1 disc and were little inhibited by F911-1.

At pH 4.5 and 5.0 isolate F911-1 showed negligible growth, even after eleven days incubation at 24°C. Under low power magnification very short young hyphae were seen growing from the periphery of the inoculum disc, indicating that the negligible growth was due to an intolerance of low pH in the medium rather than death or lysis of hyphae. At these pH values, when paired with F911-1, mycelial growth of isolates 45/10, 45/9, F3 and F6 was strongly inhibited when hyphae approached the vicinity of the F911-1 inoculum disc. Hyphae of these inhibited isolates were prevented from growing closer than 3 mm for up to 11 days after their simultaneous inoculation onto agar plates. By 21 days after inoculation, 45/10 and F3 had completely overcome the inhibitory effect of F911-1 at pH 4.5, 45/9 had partially overcome the inhibition while F6 still showed a clear zone of inhibition around F911-1 (Figure 27). At pH 5.0 45/10 had overcome the inhibitory effect of F911-1 while 45/9, F3 and F6 still showed the inhibitory effect.

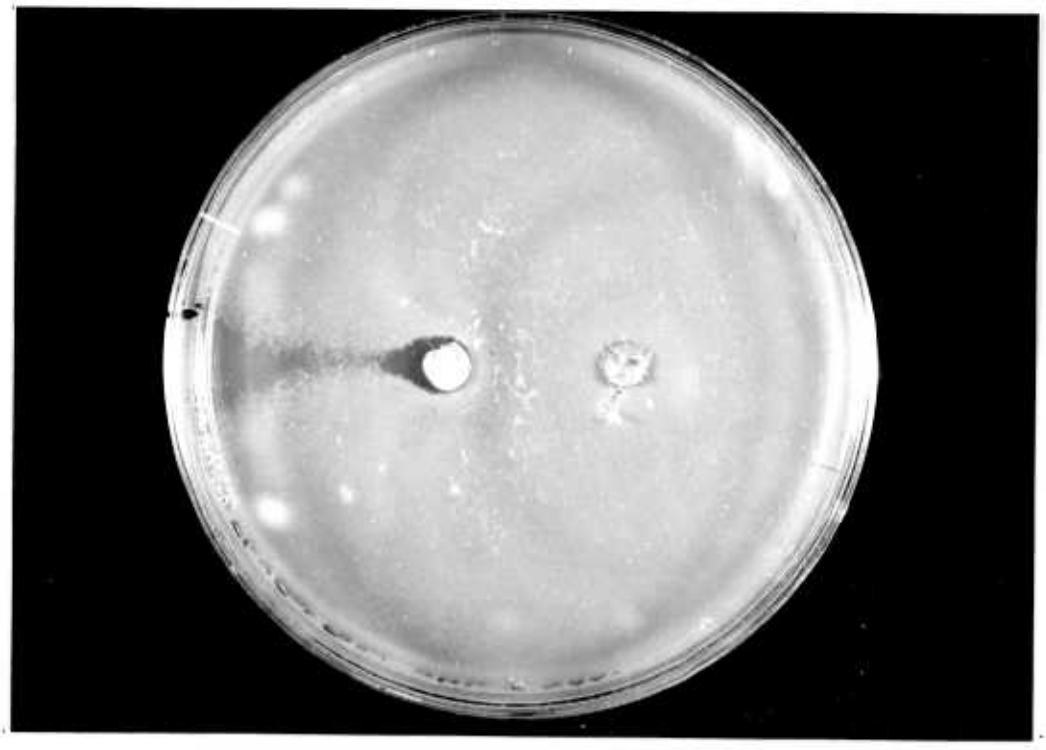


Figure 27 Incompatibility of Ggt F911-1 v Ggt F6 on a PDA plate buffered at pH 4.5

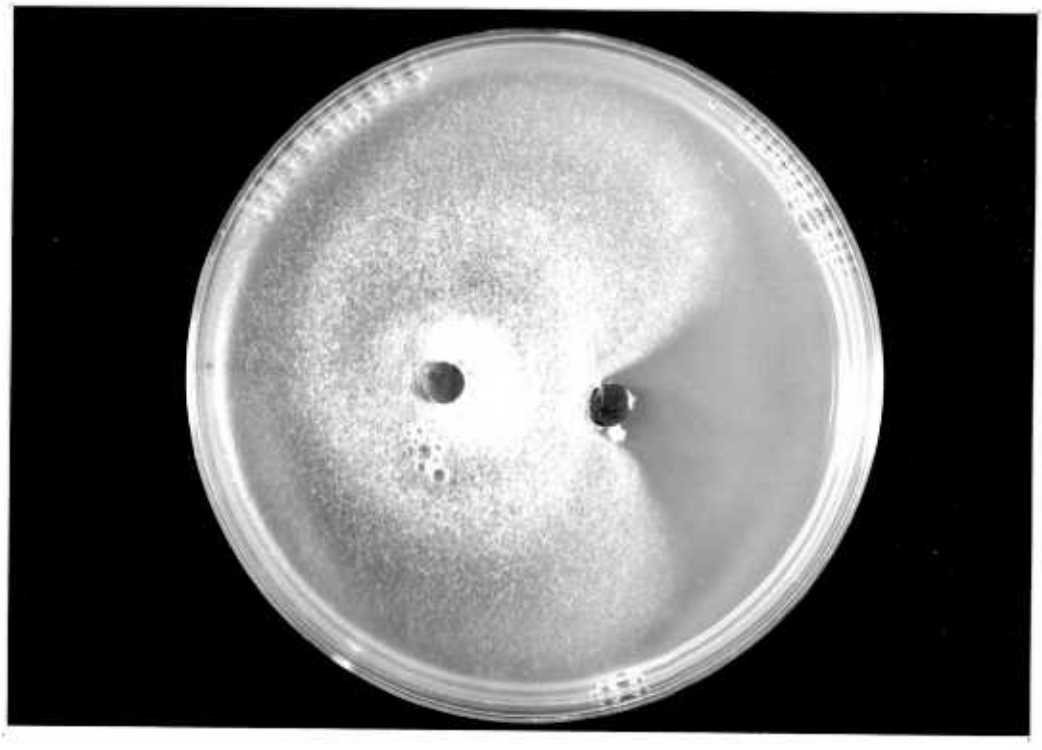


Figure 28 Incompatibility of Ggt 3bla A52 v Ggt 3bla A52 on a PDA plate buffered at pH 5.5

At pH 5.5 to 8.0 inclusive all isolates grew well and six days after inoculation all isolates paired with F911-1 showed free intermingling of hyphae. However, after this stage, two types of response were observed; either intermingled hyphae remained hyaline (F911-1 v 45/9 and F6) or they darkened to form a narrow band along the point of contact between colonies (F911-1 v 45/10 and F3). This latter "barrage" response could have been caused by heterogenic incompatibility (Esser and Blaich, 1973).

(B) Incompatibility of 019/6 versus 45/10, 45/9, F3 and F6.

Incompatibility tests were set up exactly as described above but using 019/6 as the putative sensitive isolate instead of F911-1.

All isolates grew very little at pH 3.5, more growth was made at pH 4.0 in all isolates but interactions within a time interval of twelve days were most conveniently observed at pH 4.5 and above. Twelve days after inoculation the tests showed the following results:

019/6 versus 45/10: Hyphae had intermingled at pH 4.0 to 8.0 inclusive with a dark "barrage" effect between the two isolates at pH 5.0 to 8.0 inclusive.

019/6 versus 45/9: Hyphae had intermingled at pH 4.5 to 8.0 inclusive and appeared compatible at all these pH values.

019/6 versus F3: Hyphae had intermingled at pH 4.5 to 8.0 inclusive with a dark "barrage" effect between the two isolates at pH 6.0 to 8.0 inclusive.

019/6 versus F6: Hyphae had intermingled at pH 4.5 to 8.0 inclusive and appeared to be compatible.

019/6 versus 019/6: Hyphae had intermingled and were compatible at pH 4.5 to 8.0 inclusive.

(C) Incompatibility of a single ascospore culture of 3bla versus 3bla parent culture

In order to determine whether an incompatibility reaction could be attributed to the presence of virus alone and to eliminate the possibility that incompatibility could be caused by host genetic differences, it was decided to test a single ascospore culture of 3bla designated 3bla A52, which was derived from an ascus in which no segregation had occurred and was virus-free (Rawlinson et al., 1973), against 3bla (containing a complex virus system). The incompatibility tests were set up as described above (omitting pH 3.5) with the following pairs of isolates: 3bla A52 versus 3bla, 3bla versus 3bla (control) and 3bla A52 versus 3bla A52 (control). Six days after inoculation, the tests showed the following results:

3bla A52 versus 3bla: At pH 4.0 there was assymetric growth of 3bla away from 3bla A52 which had not grown. At pH 4.5 3bla A52 had grown a little, its hyphae intermingling with no sign of incompatibility with hyphae of 3bla. At all higher pH values, 3bla had grown faster than 3bla A52, the hyphae intermingling with no sign of incompatibility.

3bla versus 3bla (control): At all pH values hyphae had intermingled with no sign of incompatibility with the growth rate increasing with increase of pH value.

3b1a A52 versus 3b1a A52 (control): At pH values 4.0, 4.5 and 5.0, there was very little growth. At pH 5.5 one colony had grown assymetrically away from the other which itself had grown very little (Figure 28). At all higher pH values, hyphae had intermingled compatibly and growth rate increased with increase of pH. Thirteen days after inoculation, at pH 5.5, the colony which had grown still showed inhibition in the immediate vicinity of the colony which had not grown.

DISCUSSION

The Ggt virus complex

This study is one of the first to have screened field isolates of the same fungus of world-wide origin for ds-RNA viruses. A striking observation was that no two isolates contained the same distribution of ds-RNA components and that their molecular weights ranged from 1 x 10^6 to greater than 5 x 10^6. This indicated that, world-wide, Ggt acts either as host to a large number of viruses possessing only sufficient genetic information for their replication or as host to a smaller number of viruses, some of which possess more genetic information than that required for their replication, like the viruses of Ustilago maydis (Koltin and Day, 1976).

This study has found evidence for at least twelve different viruses on the basis of size, capsid polypeptide molecular weights, the molecular weights of the RNA components encapsidated and serological relationships (positive and negative) (Table 23). All these properties were agreed to be of high taxonomic importance by members of the Fungus Virus Sub-committee of the International Committee on the Taxonomy of Viruses (C.J. Rawlinson, personal communication). The distinction between virus strains, "species" and groups can be clarified by studying a range of properties (Gibbs and Harrison, 1976). Table 24 lists characters that differ between virus isolates, which are relevant to mycoviruses. Variant progeny usually differ from the parent in one or another of a restricted range of

properties listed in part C. More rarely they differ in properties listed in part B, but never in those listed in part A except when they are deletion mutants. In the absence of fingerprint analyses of the Ggt capsid polypeptides or ds-RNA components, the possibility that some of the 35 nm viruses are deletion mutants cannot be excluded. Koltin (1977) has recovered mutants with partial genomes for the virus particles of Ustilago maydis following treatment with nitrosoguanidine. However, although some of the mutants lacked certain ds-RNA components, none of their ds-RNA components differed in molecular weight from that of the parent.

Table 24 Characters that differ between virus isolates

A Unrelated viruses usually differ from one another in one or more genetically stable properties such as the:

- Type, strandedness and number of pieces of their nucleic acid genome
- Size, shape, symmetry and gross composition of their particles
- Size and number of polypeptides in their particles
- Hydrodynamic behaviour of their particles
- Ability of their particles to react with one another's antisera

B Viruses in one virus group share most or all properties in A but may:

- Differ in the amino acid composition of their coat proteins so that their particles have different electrophoretic mobilities, and are serologically only distantly related or unrelated

C Virus strains or the variant progeny of one virus usually share the majority of their properties but often:
Differ slightly in the amino acid composition of their coat proteins, and hence differ slightly in the serological specificity, electrophoretic mobility or stability of their particles

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Ramig, Cross and Fields (1977) have found that several RNA and polypeptide species of three mammalian reovirus serotypes have different electrophoretic mobilities from the corresponding RNA or polypeptide species of type 3 Dearing. The term "serotype" ("serological type") was proposed by Kassanis (1961) for viruses that share a few of their antigens in contrast to "strain" for viruses that have most of their antigens in common. The reovirus genome consists of ten functionally as well as structurally discrete segments of ds-RNA, which falls into three size classes. The large size class contains three genome segments (L1 to L3), having molecular weights of approximately 2.3×10^6 to 2.5×10^6. The middle size class has three segments (M1 to M3), with molecular weights of approximately 1.4×10^6 to 1.6×10^6 and the small size class has four segments (S1 to S4), with molecular weights of approximately 0.6×10^6 to 0.9×10^6. Each genome segment is transcribed into single-stranded RNA, which serves as mRNA for the synthesis of the viral polypeptides. At least eleven different virus-specified polypeptides have been detected in infected cells. These polypeptide species fall into three size classes, large (λ) with molecular weights of 143,000 to 160,000, medium (μ)

with molecular weights of 72,000 to 79,000 and small (σ) with molecular weights of 39,000 to 54,500. Ds-RNA components of reovirus type 1 Lang, type 2 Jones, type 3 Abney and type 3 Dearing and polypeptides of reovirus type 1 Lang, type 2 Jones and type 3 Dearing were examined. The difference of the molecular weight of any one ds-RNA or polypeptide component from the average molecular weight for that component varied by up to 6%.

Verly and Cohen (1977) determined the molecular weights of the eleven segments of ds-RNA of rotavirus extracted from wild rotavirus isolates and cell culture-adapted virus. They found that the most important differences in the molecular weight of certain segments was of the order of 6%, the same as that found for reovirus serotypes. Assuming that mycoviruses are comparable with these two other ds-RNA viruses, then a maximum of 6% difference in the molecular weights of the capsid polypeptides and ds-RNA components of mycovirus serotypes might be expected. To take an example: it is possible that the two viruses of Ggt 45/10 with capsid polypeptide molecular weights of 73,000 and 68,000 are serotypes but highly unlikely that the two Ggt 45/10 viruses with capsid polypeptide molecular weights of 73,000 and 55,000 are serotypes. Indeed the large spread of Ggt capsid polypeptide and ds-RNA component molecular weights suggests that it may be necessary to create several virus groups in order to accommodate all the Ggt viruses described here, for example, two groups of 40nm. viruses having capsid polypeptide molecular weights of 94,000 and 125,000 respectively, and two groups of 35nm. viruses, having capsid polypeptide molecular weights of around 55,000 and around 70,000 respectively.

The claim of this study to have found evidence for at least twelve different Ggt viruses is to be compared with that of Frick and Lister (1978), who concluded that Ggt viruses commonly occur in a wide range of serotypes. They examined 22 isolates of Gaeumannomyces graminis, 19 of which were from Indiana wheat, for ds-RNA and virus-like particle content. Histograms of particle diameters of virus from six isolates indicated modes at 35, 39 and 41 to 42 nm. Preliminary analyses of total virus coat protein and nucleic acid for their No. 3 isolate by polyacrylamide gel electrophoresis revealed two major structural proteins with molecular weights close to 70,000 and three major species of ds-RNA. Serological comparisons made using antisera to the viruses in preparations from three selected isolates indicated various serological groupings and degrees of relatedness among the viruses of all the isolates. Their serological work is to be criticised because they did not determine whether the antisera raised were to single viruses or to mixtures of different viruses. No. 3 viruses contained two major structural proteins, gave two precipitin lines with the homologous antiserum and contained three size classes, suggesting that at least two different viruses were present. The workers themselves failed to reach this conclusion since although they suggested the possibility of two distinct particle types within one preparation from some of the gel diffusion test patterns - a suggestion they had already confirmed by electron microscopy anyway - they thought that the band splitting could have been due to either excess of antigen or antibody.

This study has left several capsid polypeptides and RNA components unassigned. Ggt 3bla and Ggt F6 also contain 40 nm viruses whose RNA component molecular weights are different to those of Ggt F3 and Ggt F10. Fingerprinting of the RNA components would probably show that these viruses are different to those already characterised. Similar considerations apply to the 35 nm viruses. The discovery of at least ten different 35 nm viruses illustrates the danger inherent in regarding one parameter as uniquely characteristic of each virus infecting a certain host fungus (Rawlinson et al., 1973).

It is interesting to note that the RNA components with molecular weights greater than 3×10^6 and the largest capsid polypeptides are associated with 40 nm viruses. The characterisation of Ggt F10 virus (Table 23) and a consideration of the coding capacity of its RNA genome indicates that it is least dicistronic, which is probably true of the RNA components of all the 40 nm viruses. On the other hand, similar considerations of the 35 nm viruses indicate that their RNA components are monocistronic. The Ggt 40 nm viruses appear to be similar to the yeast V1 virus which is 39 nm in diameter and contains a ds-RNA component of molecular weight 2.92×10^6, which must be at least dicistronic, since it occurs alone in strains sensitive to killer toxin (Bevan and Mitchell, 1978).

Population shifts in plant pathogens result either from a readjustment of the frequency of the components of an original population, or from the infusion into the latter of a

new character that gives a selective advantage to those strains possessing it (Watson, 1970). In most cases these new characters involve changes in specific genes for virulence, but other features can also be important. Virulence changes are probably arising continually in populations of plant pathogens, but in the field many will not be detected on account of the genotype in which they occur. While it is proposed that a change from avirulence to virulence in a particular host is one involving few genes, it can be assumed from the work on the inheritance of growth rate in other organisms that aggressiveness is under polygenic control. The most dramatic population changes therefore are likely to follow when a variant having a particular gene for virulence arises either sexually or asexually in an aggressive well-adapted genotype. Blanch (1977) investigated the control of pathogenicity in Ggt. Crosses between pathogenically different isolates were made and analysis of the progeny from three successful crosses indicated that pathogenicity is under polygenic control. Variability within field isolates was examined by comparing the pathogenicity of cultures derived from hyphal tips and from older hyphae. Although little variability was found between hyphal tips when a range of isolates were examined, older hyphae were found to be more variable. One of the possible explanations for the observed heterogeneity is that field isolates are simply a mixture of individuals growing in close association, without being heterokaryons. The unreproducible ability of Ggt 45/10 and Ggt 45/9 to produce virus (Sections 1 and 2) and the complete change of the properties of the virus isolated from Ggt OgA

between the first and second preparations (Section 5) would appear to be another characteristic which is subject to variability. Repeated subculturing and growth on synthetic media could cause shifts in the population of individuals within an isolate and hence the variations observed. This heterogeneity could also explain the multiplicity of viruses found in some field isolates. Five differest viruses were found to infect field isolate Ggt 45/10 whereas a maximum of two different viruses has been isolated from single-spore cultures of other virus-infected fungi.

This variation within field isolates demonstrates that any biochemical characterisation of these viruses should use homogeneous genetically defined entities as sourcematerial in the future. This would mean that the virus or viruses isolated from a given source would remain the same and, hopefully, that the virus yield would be more predictable. Microconidial cultures could be used as this source material since, when prepared from cultures of virus-containing isolates, they have been found to contain virus particles morphologically similar to those in the parent mycelium (Rawlinson et al., 1977). The formation of germinable conidia by Ggt, induced by flooding colonies with distilled water, was recently described by Deacon (1976). Ascospore cultures cannot be used, since they are virus-free (Rawlinson et al., 1973). A biological separation of viruses could probably be effected by isolating them from microconidial cultures, since it is probable that they commonly act as host to single viruses, corresponding to the simplest cases to be found in the field isolates, where presumably only one individual is infected with a single virus (Section 5).

Although the complexity of the viruses of Ggt isolates of world-wide origin is daunting, all six viruses in the two groups of serologically related viruses were prepared from English Ggt isolates, of which four came from Rothamsted fields. This indicates that Ggt isolates from a particular locality act as host to a relatively small number of viruses. This conclusion is in agreement with the results of Frick and Lister (1978), who found that viruses prepared from Ggt isolates from the same Indiana wheat field, isolated in the same year, were either serologically related to one or the other of two serologically distinct viruses i.e. different viruses or related to both.

Biological implications of the Ggt virus complex

Since this study has found evidence for at least ten different 35 nm viruses, it is not surprising that Rawlinson et al., (1973) found no correlation between pathogenicity and the presence of 35 nm and 27 nm viruses, which they treated as two different viruses only. It could be argued that there may be a correlation between pathogenicity and specific viruses. However Blanch (1977) concluded that cytoplasmic factors were probably not an important cause of pathogenic variation in Ggt. This possible cause of pathogenic variation was investigated by examining the effects on pathogenicity of storage in culture and repeated inoculation and reisolation from wheat plants; no overall trend in pathogenicity was observed. Further attempts to demonstrate cytoplasmic factors included selecting for high and low pathogenicity within a single-ascospore isolate and comparing mycelial and ascospore

cultures of the same isolates. Lapierre et al. (1970) based their novel claim that a relationship exists between the presence of virus and the reduction of pathogenicity on the discovery of virus in two weakly pathogenic isolates and of its absence in five pathogenic isolates. Their claim therefore had no sound statistical basis, especially in view of the discovery of Rawlinson et al. (1973) of virus in more than half of 145 isolates, differing widely in pathogenicity. It is probable that the association of virus with the two weakly pathogenic isolates was fortuitous; a probability conceded by Lemaire et al. (1975).

Take-all decline has been recently reviewed by Hornby (1978). One of the many possible explanations for the phenomenon is that there is a population change towards avirulent individuals. Given the two observations that Ggt isolates from a particular locality act as host to a relatively small number of viruses and that the virus acts as a marker for Ggt individuals during population changes within field isolates, it could be feasible to use virus markers to monitor the postulated population change towards avirulent individuals. Measurement of the pathogenicity of the marked isolates would then be a convincing demonstration of whether take-all decline can be attributed to a population change towards avirulent individuals and whether there is any correlation between pathogenicity and the presence of specific viruses.

The incompatibility testing failed to demonstrate a killing reaction analogous to those of Saccharomyces cerevisiae

and of Ustilago maydis. Either the isolates containing "redundant" ds-RNA (ds-RNA not required for virus replication) did not produce a killer factor or the putative sensitive isolates used were resistant to the killer factor. Resistance to killer factor could be conferred by nuclear genes as in U. maydis. If the isolates containing "redundant" ds-RNA do not produce killer factor then the question of the role of the ds-RNA remains unanswered. The ds-RNA could be defective deletion mutants of the genome required for virus replication. Fingerprint analysis of all the ds-RNA components associated with the viruses would be the next step in answering the question. Alternatively, any positive killing reaction may have been masked by the differing abilities of the individuals within each field isolate to produce killer factor and to be affected by it. It would be better in the future to use genetically known entities such as microconidial cultures in the incompatibility testing, analogous to the genetically characterised laboratory strains used in the work on S. cerevisiae and U. maydis.

The inhibition at a distance actually observed was produced by single ascospore isolates, not containing viruses but only when these isolates grew very little. This inhibition was found to be non-specific since it affected all the isolates tested and was overcome by some isolates and not limited to as narrow a pH band as that for optimum stability of the yeast killer strain toxins (Rogers and Bevan, 1978). The inocula producing the inhibitory effect were viable, therefore it was unlikely that the inhibition was merely a

trivial effect attributable to autolysis and consequent release of staling factors into the surrounding medium.

A similar kind of inhibition at a distance was observed when field isolates were grown near a Ggt 45/8 disc inoculum on PDA buffered at pH values in the range 3.5 to 4.5 (M.A. Romanos, personal communication). Isolate 45/8 sectored frequently, demonstrating once again the heterogeneity of field isolates; the inhibitory activity was associated with a dark sector growing poorly in low pH media (45/8Q). Subcultures from another sector (45/8P) were consistently non-inhibitory and grew well at low pH.

Isolate 45/8Q inhibited three other isolates tested at pH 4.0 on PDA. The inhibition was overcome after some weeks, when a "barrage" response developed, probably caused by heterogenic incompatibility (Esser and Blaich, 1973).

When 45/8Q was grown on malt extract liquid containing thiamine in shake culture, the mycelium homogenised and the pH adjusted to 4.0, inhibitor was found in the culture filtrate. Although inhibitory activity was dependent on the homogenisation of 45/8Q, no inhibitor was produced when isolate 64/14 was homogenised, demonstrating that the inhibitor was not a normal cell constituent. The inhibitor was inactivated at room temperature, not stabilised by 0.1% gelatin and its activity was not destroyed when up to 1 mg/ml pepsin was incorporated into PDA test plates. It was therefore unlikely to be proteinaceous.

Although 27 nm viruses have been detected in isolate 45/8 by electron microscopy, their ds-RNA, if present, was not

detectable (see Section 3). Clearly this inhibition at a distance is different to the killer activity of strains of S. cerevisiae and U. maydis in that the inhibitor appears not to be proteinaceous and its production is not associated with specific virus ds-RNA components. Such an inhibitory effect has not hitherto been reported for filamentous fungi and is therefore worthy of further study.

The one clear point which emerges from the preceding discussion of the biological implications of the Ggt viruses is the need to develop a reproducible method of infection of fungal cells either by the viruses or their ds-RNA. It is therefore surprising that little published use has been made of the protoplast method described by Lhoas (1971) which allows infection with extracellular virus. In this paper, Lhoas described the preparation of protoplasts from virus-free and morphologically distinct Penicillium stoloniferum strains susceptible to viruses, using the snail gut enzyme "Helicase". After infection of the protoplasts with ds-RNA viruses, the protoplasts were regenerated and virus prepared from subcultures of single spore isolates. The presence of virus in the preparations was proven by polyacrylamide gel electrophoresis and electron microscopy.

Philosophy for future research

The justification for the work presented in this thesis rested in large measure on the need for a thorough biochemical investigation of the viruses in the Ggt system. This was identified as necessary in order that the impasse in research by plant pathologists should be overcome and before any firm

conclusions on the biological involvement of these viruses with their host could be made. The investigation described here was deflected from its original objective by the unforeseen multiplicity of the viruses discovered. Although this change in direction has resulted in a contribution, not only to knowledge of viruses in a plant pathogen but also to an evaluation of criteria that are important in the taxonomy of mycoviruses, it has left untouched the biological questions which were the main stimulus for the project.

To date there is no unequivocal evidence from any source for any biological effect of the Ggt viruses in their host. This is unfortunate since this information is required to validate hypotheses which implicate viruses in both the ecology and biological control of this fungus (see Introduction). The speculation has overtaken the facts and there appears to be a real danger of allowing an interesting but academic approach to the biochemical investigation of Ggt viruses to move too far away from the "raison d'etre" of the project. It becomes imperative now to investigate what, if any, biochemical implications the viruses have for the host. No investigation to date has tackled this problem equipped with all the necessary information for authoritative comment in this field. The whole area of the biochemical implications of Ggt viruses remains essentially unresearched. As already discussed, an answer to the problem could come from the development of a reproducible method of cell-free transmission of mycoviruses, which would allow replacement of the speculation with hard fact. Sir Ernst Chain's philosophy of "biology first, chemistry second" is as pertinent now as it was when the first Ggt virus was discovered eight years ago.

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