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DETECTION AND CHARACTERISATION OF VIRUSES IN CONIDIAL AND ASCOSPORE ISOLATES OF GAEUMANNOMYCES GRAMINIS VAR. TRITICI

by

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ABSTRACT

Methods for producing isogenic strains of the wheat take-all fungus Gaeumannomyces graminis var tritici (Ggt), with and without virus, have been investigated. Such strains are required to evaluate the possible effects of virus infection on any aspect of the biology of Ggt. The following lines of approach were used: (1) Production of genetically homegenous strains from field isolates of Ggt, via single uninucleate germinable conidia, and characterisation of viruses in these conidial isolates. (2) Removal of viruses from the conidial isolates by (a) isolation of hyphal tips and (b) production of ascospores. (3) Development and evaluation of sensitive methods to detect virus particles, double-stranded RNA (dsRNA) or possible DNA proviruses in hyphal tip and ascospore isolates to ensure that such isolates are completely free from viral genetic information.

Ten single conidial isolates were generated from Ggt isolate F6. Three serologically unrelated dsRNA viruses from one of these isolates were isolated and characterised. All the conidial isolates and the parent F6 contained the same three viruses, although two conidial isolates each lacked one of the dsRNA components found in the other nine isolates. The virus and dsRNA content of single conidial isolates derived from Ggt isolates 3b1a and 45/10 were also examined.

Hyphal tip cultures were prepared from single conidial isolates F6 con 8 and single ascospore isolates were produced from single conidial cultures of Ggt isolates 3b1a and 45/10. The following methods were used to detect low levels of virus particles and dsRNA in these isolates: (1) enzyme-linked immunosorbant assay; (2) immune electron microscopy; (3) transfer of dsRNA to nitrocellulose and detection by hybridization with a viral ssRNA probe, produced by in vitro transcription of dsRNA with the virion-associated RNA polymerase. Hyphal tip cultures contained reduced virus levels, but after further subculturing these levels increased. Nine out of eleven ascospore isolates (from 3b1a) contained no detectable virus particles or dsRNA. The remaining two isolates contained only one out of four isolates originally present in the 3b1a conidial isolates and this virus had undergone genomic alterations during ascospore transmission.

Ascospore isolates were also examined for the presence of a proviral form of viruses present in isolate 3b1a. This work entailed characterisation of the genome of Ggt including evaluation of the genome size, repetitive DNA content, and ribosomal RNA gene structure. No proviral form of Ggt viruses was detected in the Ggt genome.

Lastly, with the isogenic strains, with and without virus, generated in this study, the presence of these viruses was shown not to be involved in the production of a diffusable growth inhibitor by Ggt.

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ABBREVIATIONS

The following non-standard abbreviations have been used:

Ggt	*Gaeumannomyces graminis* var *tritici*
ssRNA, dsRNA	single-stranded and double-stranded RNA
ssDNA, dsDNA	single-stranded and double-stranded DNA
ds1, ds2 etc.	dsRNAs 1, 2, etc.
ss1, ss2 etc.	transcript ssRNAs 1, 2, etc.
DMSO	dimethylsulphoxide
EDTA	ethylene diamine tetraacetic acid Na_2 salt
PDA	potato dextrose agar
PPO	2, 5-diphenyloxazole
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid
TEMED	N, N'-tetramethylethylenediamine
tris	tris (hydroxymethyl) aminomethane
R Nase	ribonuclease
D Nase	deoxyribonuclease
Kb	kilobase-pairs for kilobases for single-stranded nucleotides.

INTRODUCTION

Virus particles were first discovered in Gaeumannomyces graminis var tritici (Ggt) by French workers (Lapierre et al, 1970; Lemaire et al, 1970) who associated the occurrence of virus particles with low pathogenicity of Ggt isolates. Further work however (Rawlinson et al, 1973) failed to find any correlation between presence of virus particles and reduced pathogenicity of Ggt. The demonstration of a relationship between the presence of virus in Ggt and pathogenicity of the fungus would most clearly be shown by comparison of the pathogenicity of isolates containing virus with isogenic strains lacking virus. The latter are most conveniently generated as ascospore derived strains generated from homokaryotic virus-containing isolates. Ascospore isolates of Ggt have been reported to be free of virus particles (Rawlinson et al, 1973); however, there is some evidence (Rawlinson & Muthyalu, 1976) that virus may reappear in ascospore isolates in which virus was previously undetectable. This suggests that viral information may be present in a suppressed or eclipsed state in ascospore isolates. The aim of the present study is to resolve this question. Such a study would be an essential prelude to any future work intending to investigate whether there is any correlation between the presence of virus in Ggt and pathogenicity of the fungus.

A review of the biology of Ggt, particularly the evidence of genetic control of pathogenicity is attempted, in order to demonstrate that what is known of the pathogenicity of Ggt does not preclude an involvement of virus in the pathogenic response. A discussion of the properties of Ggt virus and other fungal viruses is presented in order to illustrate various possible models for involvement of virus in the

biology of Ggt. Finally, the possible forms of viral information in ascospores, are outlined, and the methods available for their detection are reviewed.

1.1 Take-all Disease

Take-all (TA) is a root rot disease of cereal crops and is considered to be the second most important disease of wheat causing wheat losses greater than 50% in severe infections. It is, however, being realised that large reductions in grain yield can occur without overt disease symptoms. Stynes and Wallace (1974) reported an average yield loss of 9% in South Australia in 1972 when take-all was not considered a problem. The causal agent of take-all of wheat, Gaeumannomyces graminis var tritici (Ggt), can be isolated from crops showing no obvious disease symptoms; and it seems likely that the pathogen is a common, if not omnipresent inhabitant of graminaceous crops in temperate climates (Walker, 1975).

Little progress has been made in breeding varieties of wheat resistant to take-all. Apparently the host has no resistance genes analogous to the resistance genes of wheat varieties to Puccinia graminis var tritici, the black stem rust fungus of wheat. Chemical control is ineffective against the take-all fungus, and so a method of biological control seems the only alternative. At present, the methods available for controling the disease (bare fallowing, crop rotation with non-susceptible crops) achieve limited success but are often unpractical or uneconomical.

TABLE I.1

Fungi of the Gacumannomyces/Phialophora Complex

Abbreviation	Fungus
GGT	Gacumannomyces graminis (Sacc.) Arx & Olivier var. tritici (Walker) : the wheat take-all fungus.
GGA	Gacumannomyces graminis (Sacc.)Arx & Olivier var. avenae (E.M. Turner) Dennis : the oat take-all fungus.
GGG	Gacumannomyces graminis (Sacc.) Arx & Olivier var. graminis.
PG	Phialophora graminicola (Deacon) Walker = Phialophora radicicola var. graminicola sensu Deacon (PRG, Deacon, 1974). = Phialophora radicicola sensu Scott (Scott, 1970). Distinct from Phialophora radicicola Cain (1952). Probably the imperfect state of Gacumannomyces cylindrosporus (Hornby et al., 1977).
P.sp.(l.h)	Phialophora sp. (lobed hyphopodia) = Phialophora radicicola var. radicicola sensu Deacon (PRR, Deacon, 1974). Distinct from Phialophora radicicola Cain (1952). Probably the imperfect state of GGG.

The biological properties and taxonomy of these fungi are discussed by Walker (1981).

1.1.1 The Pathogen

The ascomycete Gaeumannomyces graminis is the causal organism of take-all disease of cereals, and three varieties are recognised with differing host specificities. Gaeumannomyces graminis var tritici (Ggt) is highly pathogenic on wheat and less pathogenic on rye and barley, while oats are almost immune. Gaeumannomyces graminis var avenae (Gga) is the oat attacking variety and is also virulent on grasses. Gaeumannomyces graminis var graminis (Ggg) is a mild pathogen of rice but usually causes no apparent disease on other cereals or grasses. Ggg has not been recorded from Great Britain. The taxonomy of this group is reviewed by Walker (1981) and is illustrated in Table 1. A related group of fungi is the Phialophora complex, which are predominantly associated with grass leys and pasteurs rather than cereal crops; they do not usually penetrate the stele and so never cause disease of cereals and perhaps only mild disease in grasses. The taxonomy of the group is under review (Walker, 1981).

The symptoms of take-all vary with host plant, soil conditions, seasons and countries, but the major cause of damage is root rot. Wheat plants exhibiting severe take-all are stunted and frequently occur in patches. Infected plants may die before heading or the ears may emerge normally but death may occur at a later stage so that the ears appear bleached ('whiteheads').

During infection mycelium invades the cortex from runner hyphae growing ectotrophically on the root surface, infection pegs penetrate the host epidermal cells and the cortex is invaded. In response to penetration, host cells ensheath invading hyphae in cell wall material impregnated with lignous substances (lignitubers).. However, hyphae may grow through the lignitubers to invade subsequent cell layers and the stele. On reaching the vascular tissue hyphae tend to grow lengthwise within it, blocking the vascular flow and the plant eventually dies. Perithecia are produced on dead tissue and the mycelium reverts to saprophytic growth on plant residues. Reinfection is thought to be primarily from plant residues.

The saprophytic stage of growth of Ggt is poorly understood. The fungus has low saprophytic survival ability and the rapid disappearance of inocula in soils is generally associated with conditions favouring microbial activity (Garrett, 1970). Ggt has not been shown to produce any growth-cessation structures in the soil, although thick walled chlamydospores (Sivasithaparum & Parker, 1976) and sclerotia are produced in culture; their role in nature is not clear. It is not certain that Ggt does have a true saprophytic growth stage; it may be that it survives in dead plant remains with minimal growth.

The infection of wheat by Ggt is thought to be initiated from runner hyphae emerging from dead plant remains. Although Ggt can produce three types of germinable spores: ascorpores, germinable conidia and chlamyclospores, only one of these, ascospores, has been found in nature. However, infection by ascospores has not been

demonstrated, except on sterile soil and their role in nature is unclear. It is possible that they may be able to infect germinating seedlings lying on the soil surface where microbial antagonism is least. Rawlinson et al (1976) indicated that isolates of Ggt from first year cereals in fields that had not carried cereal crops for over 100 years, were virus free. This could be explained if the cereals were infected by (wind borne) virus-free ascospores.

In culture, two types of conidia can be produced, lunate micro-conidia which have never been shown to germinate, and germinable phialidic conidia produced under artificial flooded conditions (Deacon, 1976). The latter have not been reported in nature and Deacon suggests that they may be a vestige of a vascular wilt habit. (Chlamy dospores have been produced under acidic condition in the laboratory (Sivasi-thamparam and Parker, 1976), but again, have not been reported in nature. It seems unlikely that Ggt would retain the fidelity of genetic information necessary to produce three viable types of spores if these had no function, it is therefore likely that their role is not realised at present.

1.1.2 Take-all decline (TAD)

Biological suppression of take-all that occurs in cereal crops has been classified (Walker, 1975) into six types. General suppression is that caused by non-specific antagonism of soil microorganisms which is increased by addition of organic matter and warm soils (greater than 25°C). It is not transferable to non-suppressive soil and is thought to be due to

the development of a wide variety of antagonistic micro-organisms, particularly bacteria, since it is most effective in conditions favouring bacterial growth, and operates in the bulk soil. It is not eliminated by moist heat at 60°C. Five types of specific suppression are listed, but only one of these, the suppression developed within a few seasons in the presence of a severely diseased host, is termed take-all decline (TAD). The essential characteristic of inhibition by specific soils; eliminated by 60°C moist heat, or fumigation, operates in cooler soils (10° - 25°C), its action is in the rhizoplane and rhizosphere, and is fostered by wheat monoculture. The remaining four types of specific disease suppression listed are developed in varying conditions and may overlap with TAD. Perhaps the most interesting of these is antagonism to the parasite developed in the absence of the host but in the presence of a virulent isolate of the pathogen, added to the soil.

The mechanism of specific suppression is unknown but many theories have been proposed (reviewed by Walker, 1975). The evidence suggests the involvement of specific microorganisms, fungi or non-sporing bacteria, perhaps antibiotic or toxin secreting, since water extracts of TAD soils decreased the ectotrophic response and growth of Ggt on roots (Gerlagh, 1968; Pope & Jackson, 1973). Hyperparasitism of Ggt by other organisms such as pseudomonads, actinomycetes and other grain-negative bacteria, has been proposed as a possible mechanism since there is a proliferation of these organisms in TAD soils (Vojinovic, 1972, 1973; Rovira and Wildermuth, 1981), and they have been shown to parasitise Ggt in culture.

Brown, Hornby and Pearson (1793) consider that antagonism alone cannot account for TAD and propose that nutritional changes in the rhizosphere could be involved. Changes in the virulence of the parasite in TAD has been reported by some workers (Shipton, 1973, Cunningham, 1975) but other workers (Asher, 1980) could find no changes. Asher suggests it is possible that changes in virulence might be occurring in TAD but the method of isolation, by surface sterilisin root lesions, could be selecting only those virulent isolates that penetrate into the root cortex. Other methods of isolation such as extensive washing of roots (Asher, 1978) may give a different result.

The involvement of virus particles in TAD was suggested by Lapierre et al (1970). This will be discussed in more detail in Section 1.1.5.

1.1.3 Cross-protection of take-all

Recently, much work has been aimed at achieving protection of wheat against take-all by inoculating with weakly virulent isolates of Ggt and isolates of Ggg and Phialophora spp. Scott (1970) and Ballis (1970) showed that Phialphora graminicola Cain, restricted the colonisation of wheat root by Ggt if the protecting fungus was established on the host prior to its encounting the pathogen and Deacon (1973a, 1973b) has shown the possibility of controlling take-all by encouraging a high population of graminicola in grass leys before cropping with cereals. Wong (1974) has shown that disease

can be reduced by prior colonisation with Ggg and the protection is greater than that achieved with P. graminicola. Hypovirulent isolates of Ggt can be used in glass-house experiments to protect the host from disease caused by virulent isolates (Tivoli et al 1974; Lemaire et al 1976; Asher, 1978); in some cases (Asher, 1978) better protection was achieved than that with Ggg or Phialophora spp. Three possible mechanisms have been proposed for cross-protection of wheat against take-all by weakly virulent fungi. First, direct competition for infection sites may take place during the ectotrophic phase of infection. This would imply that rapid growth on roots would be an important factor in restricting the pathogen. This has been found in some cases using P. graminicola (Deacon, 1974) and Ggg (Wong, 1975) but Asher (1978) found that the degree of protection of wheat by weakly virulent isolates of Ggt was inversely proportional to their growth rate on wheat roots. The pathogenicity of isolates of Ggt was directly proportional to their growth rate on wheat roots. Direct antagonism between fungi due to antibiotic secretion or incompatibility reactions may occur resulting in restricted growth of the pathogen. Such incompatibility reactions have been found between Ggt isolates (Davis, 1925; Chambers and Flentje, 1967; Nilsson, 1969; Rawlinson et al, 1973; Cunningham, 1975) and a diffusible fungal inhibitor produced at low pH by Ggt isolates has been described by Romanos et al, (1980). Asher (1978) found no correlation between incompatibility reactions on agar and suppressive ability of several hypovirulent Ggt isolates; however under the conditions used, the diffusible type of inhibitor described by Romanos et al (1980) may not have been produced. A third mechanism is induction of resistance to the pathogen, in the plant by the protecting fungus. There is good evidence that Phialophora spp, Ggg and hypo-

virulent Ggt which protect wheat against take-all and each grow ectotrophically on wheat roots (relative to the stele for some Ggg and hypovirulent Ggt), elicit considerable ligonfication of surface cells (Speakmgn et al, 1978). A forth possible mechanism would be infection of the virulent isolate with virus by hyphal anastomosis with a virus-containing hypovirulent isolate. Although this was proposed by Lemaire et al (1971) the subsequent failure to correlate virus particles with hypovirulence made this hypothesis unlikely. If, as will be suggested, however, the question of virus involvement in the pathogenicity of Ggt is still open, then this mechanism remains a possibility.

It may be that, like specific suppression of disease, cross-protection is not associated with a single mechanism. It may be that both phenomena are linked: if, as has been suggested, there is a decrease in the virulence of Ggt with continuous cropping, then the resultant hypovirulent isolates may protect the wheat from attack by more aggressive isolates, contributing to TAD.

1.1.4 Genetics of Pathogenicity

The genetic control of pathogenicity in plant pathogenic fungi is complex, and a convenient simplification is to distinguish between 'aggressiveness', defined here as the ability to infect and cause disease to varying disease in the host, such that highly aggressive fungi cause severe disease, and virulence, defined here as the ability

to infect genetically different hosts differently (Finks, 1975; Woods, 1967): such that highly virulent fungi are those with the ability to infect a wide range of host varieties. It should be noted however that the term virulence is used by pathologists in somewhat different ways and in some uses, could be equated with aggressiveness, as defined above. Indeed, in this work the use of the term hypovirulent, to describe isolates causing low levels of disease when the term hypo-aggressive may be more properly used, considering the above definition. This confusion is unavoidable by the use of different definitions by different plant pathologists. Essentially what distinguishes these definitions in practice is the method of measurement of the property. Pathogenicity has been shown for many plant pathogenic fungi to be controlled by major genes in the host and pathogen so that a 'gene for gene' relationship exists, defined by Person and others (1962) as:

> "A gene for gene relationship exists when the presence of a gene in one population is contingent upon the presence of a gene in another population, and where the interaction between the two genes leads to a single phenotypic expression".

This type of interaction is often called 'vertical' and results in one variety of host being resistant to a particular race of the parasite. It has been found for many fungal-pathogen host relationships (Flor, 1971) including some wheat-pathogen relationships. Resistance evenly spread against all races of the pathogen is often termed 'horizontal' resistance and where studied has usually been found to be under polygenic control (Robinson, 1973).

The evidence for G. graminis is that resistance to the pathogen is horizontal. Paddick (1936), using different strains of G. graminis, found that a host highly susceptible to one strain was also highly susceptible to the others. Nilsson (1969) obtained similar results, as did Riley and Macer (1966) with rye. Thus it seems that there are no major and probably no single pathogenicity gene in the pathogen.

Aggressiveness of plant pathogens is less understood at the genetic level. Most fungi show a more or less continuous range of pathogenicity to a single host; this and other evidence suggests that aggressiveness is usually under polygenic control. Blanch (1977) obtained evidence that pathogenicity (aggressiveness) in Ggt was under polygenic control with between 5 and 9 effective factors segregating between sexual crosses.

If the presence of virus can modify some biological properties of Ggt then the modification of these properties should show cytoplasmic (extrachromosomal) inheritence. The operation of extrachromosomal elements has been shown for many fungi in non-mendelian segregation at sexual reproduction when the parental confugate structures have contributed unequal amounts of cytoplasm, but identical amounts of chromosomal material to the cross, often termed matrilinear inheritence. Aspects of the pathogencity of Puccinia graminis tritici show this phenomenon (Newton et al 1930); the 'mi' mutant of Neurospora crassa, behaves similarly; and also 'petite' mutants of yeast (De Dekan, 1966). In many cases the extrachromosomal element is believed to reside in the mitochondrial DNA (petite mutants of yeast 'mi' mutants of N. crassa).

Perithecial frequency in *Aspergillus nidulans* is decreased by successive hyphal tip or conidial transfer; but, if at any stage an ascospore culture is employed, perithecial production is fully restored. This suggests that hyphae that give rise to perithecia are those with the lowest concentration of an extrachrosomal element that inhibits perithecial production; alternatively, there is a reorganisation of such elements in the ascus.

The nature of extrachromosomal elements has been established in a few cases. There is good evidence in yeasts that some extrachromosomal elements are located in mitochondrial DNA (eg 'petite' mutants, Burnet, 1975). Recently defective circular mitochondrial DNA molecules consisting of sets of repeated sequences resulting from amplification of part of the mitochondrial genome have been isolated from *Podospora anserina* and may be associated with senescence in this organism (Jamet-Vierny *et al*, 1980; Stahl *et al*, 1978). Similar DNA molecules have been isolated from 'Ragged' mutants of *Aspergillus armstelodami* (Lazarus *et al*, 1980), and from 'poky' and 'stopper' mutants of *Neuropora crassa* (Mannella *et al*, 1979; Bertrand *et al*, 1980). However, Collins *et al* (1981) isolated plasmid-like DNA molecules from mitochondria of wild-type *Neurospora crassa* that contained no detectable homology with mitochondrial DNA. *Saccharomyces cerevisiae* strains are known to carry a plasmid DNA (2 micron plasmid) but the function of this plasmid is unknown (Guerineau, 1979). Recently, strains of the yeast *Kluyveromyces lactis* were reported to harbour two linear DNA plasmids of molecular weights 8.4 and 5.4 x 10^6 (Gunger Sakagulchi, 1981).

Interestingly, strains possessing both of these plasmids were shown to 'kill' S. cerevisia killer and sensitive strains. Grimaldi et al (1979) reported the isolation and cloning of a plasmid-like DNA molecule from Fusarium oxysporum and claimed its expression in Escherichia coli as a polysaccaride hydrolysing activity which may be involved in the pathogenic process of this fungus. Viruses as extrachromosomal elements represents a special case and will be considered separately.

The evidence for involvement of extrachromosomal elements in the biology of Ggt is slight and equivocal, complicated by the frequent use of genetically undefined field isolates. Many workers have reported variation in pathogenicity (usually a decrease) of isolates of Ggt kept in culture, often associated with a decrease in the frequency of perithecial formation (Blanch et al, 1981a, 1981b, 1977; Chambers, 1970; Skous, 1968; Russel, 1939). Several workers found that pathogenicity could be restored by ascospore isolation or plant innoculation and reisolation. Single-ascospore isolates have been reported to be more pathogenic than the parent (Walker, 1975) and cultures grown from single conidia have been shown to show a wide range of virulence (Deacon et al, 1978). These results could be accounted for by segregation of cytoplasmic genetic elements, however, field isolates were usually used and an alternative explanation would be segregation of nuclei within a heterokaryon.

Blanch et al, (1981a, 1981b, 1977) investigated the possible involvement of extrachromosomal elements in the pathogenicity of Ggt.

No changes in pathogenicity were observed in four isolates of Ggt maintained in culture; however she did observe a loss in frequency of perithecial production on storage of Ggt isolates including an ascospore (homokaryatic)strain; and also noted a linkage between pathogenicity and perithecia production, the highly pathogenic isolates producing the most perithecia.

Repeated inoculation and reisolation on wheat roots produced variable results but no overall trend; this contrasts with the results of other workers (Walker, 1975). A selection experiment was performed in which high and low lines were selected on the basis of pathogenicity (Jinks, 1957). After five cycles of selection no difference was found between high and low lines; however, the use of ascospore and hyphal tip isolates may have excluded or reduced the level of any cytoplasmic factor. No difference was found between mycelial and ascospore isolates of a single strain but again ascospore and hyphal tip isolates were used. Blanch did find a greater variation in pathogenicity of cultures derived from old cells than hyphal tips, which would indicate a higher concentration of cytoplasmic factors in older mycelia than in hyphal tips, but could also be explained by a higher mutation rate or greater frequency of heterokaryosis in older cells. On the basis of her results Blanch concluded that cytoplasmic factors were not involved in the pathogenicity of Ggt.

A phenomenon that shows some similarities to the appearance and behaviour of the 'red' mutant of Aspergillus idulans was discovered

by Romanos et al (1980). An isolate of Ggt designated 45/8, usually showed poor growth and produced a diffusible inhibitor at low pH but occasionally grew well and did not produce inhibitor. This isolate frequently sectored; most sectors showing the same variable growth at low pH, as the parent. However, one sector, 45/8 P was found to grow well consistently and not produce inhibitor at low pH.

In conclusion, there is some evidence that could indicate cytoplasmic factors are involved in the biology of Ggt but the evidence is equivocal and most results could be explained by a heterokaryon condition existing in many field isolates, or by mutation. However, Blanch's observation that an isogenic strain of Ggt lost the ability to produce perithecia in storage could indicate that cytoplasmic factors may influence spore formation in Ggt, as has been shown to occur with Aspergillus nidulars (Jinks, 1958). This may be important in Ggt since there seems to be a correlation between perithecial frequency and pathogenicity. The possible involvement of cytoplasmic factors in the biology of Ggt, and indeed the whole question of the genetics of Ggt, particularly with respect to pathogenicity, is poorly understood at present. Much of the problem derived from the use of genetically undefined field isolates for experiments. This problem could be overcome by the use of strains derived from uninucleate spores (or spores derived from a single nucleus) such as ascospores. However, ascospores contain little cytoplasm and it is possible that cytoplasmic elements may be excluded; also they can take months to produce. Recently Deacon (1976) described the production of germinable conidia from

flooded cultures of Ggt. These were described as predominantly or exclusively uninucleate (Deacon, 1978) and have been shown to be capable of transmitting virus (Rawlinson et al, 1977) and presumably other cytoplasmic factors. The use of these spores should resolve the question of whether the cytoplasm is important in the biology of Ggt. In the present study these spores were generated from virus-containing isolates to produce homokaryotic virus containing isolates.

1.1.5 Virus Particles in Ggt

Virus particles were first detected in two weakly pathogenic isolates of Ggt from wheat crops showing TAD in France (Lapierre et al, 1970: Lemaire et al, 1970). The workers distinguished two types of isolates:

1. Abnormal isolates with virus. These are hypovirulent: come from plants in TAD soils; cause lesions limited to roots: rarely form perithecia; tend to sector; lack diarnal zonation in culture: and are difficult to maintain on agar.

2. Normal isolates without virus. These are strongly pathogenic: produce mature perithecia on plants or agar; do not sector in culture, and form concentric zones of growth in response to light.

Preliminary characterisation of the virus particles showed that they were isometric with a diameter of 29 nm $\pm$ 2 nm, a sedimentation coefficie of 1165, a molecular weight of 5.5×10^6, and were isolated in quantities of 5

5 - 10 /100g dry weight of mycelium (Lapierre et al, 1970: Spire, 1971). The abnormal isolates were considered to have undergone a metabolic change caused by a viral complex (Lemaire and Jouan, 1972) and it was hypothesised that TAD might be caused by the progressive infection of Ggt by the virus. A method was suggested by Lemaire et al (1971) of dual control of take-all involving activation of antagonistic microflora by incorporation of protein rich substrates into soil, and protection of the host plant by introducing hypovirulent virus infected isolates. Significant control of take-all in pots and wheatfield plots was achieved in this way.

Rawlinson and Pearson (1972) confirmed the occurence of virus particles in six out of ten English isolates of Ggt and one isolate of Gga. These particles were larger (35nm) and were not associated with weak pathogenicity or TAD. Further work by Rawlinson et al (1973) revealed virus particles in 114 out of 156 isolates examined but found no correlation of presence of virus, with decreased pathogenicity, growth rate on agar, perithecial formation or morphology. Two sizes of virus particles were found with diameters of 27nm and 35nm and sedimenting at 110s and 148s, respectively. Particles of either size or both, could occur in isolates. No virus particles were found in hyphal-tip cultures or in 56 single ascospore cultures from four isolates containing virus. The proportion of isolates with VLPs of both sizes increased during monoculture.

Two out of eleven isolates from first cereal crops after a 'break' (fallow or non-susceptible crops) contained virus (27nm). After nine years of monoculture all isolates examined contained virus particles (of one or both sizes) but after twelve years the proportion of infected isolates decreased

to 73%. No simple association was found between virus infection and level of take-all disease. Isolates with only one particle size tended to be more pathogenic than those without, and this was especially true of the 27 nm type. Virus could be transferred to "uninfected" isolates by hyphal anastomosis or lost by a combination of hyphal-tip culturing and heat treatment; all tip cultures had similar colonies and growth rates to their parent isolates. These results suggested that the correlation made by French workers between hypovirulence and virus presence was purely fortuitous. This became more likely when Lapierre (1973) revised earlier measurements and reported that the particles found in 1970 were 35 nm: the same size as one of the particles found by Rawlinson, and contained dsRNA (Lapierre et al, 1974) as did VLPs from English isolates (Carpenter and Rawlinson, 1974).

It was later shown that the presence of virus was not an essential property of weakly pathogenic, abnormal isolates capable of protecting wheat plants from attack by highly aggressive isolates (Tivoli et al, 1974): Brun et al, 1976; Lemaire et al, 1975, 1976 and others - see above). This seemed to allow the protection the French workers observed with hypovirulent virus-infected Ggt isolates, without the need for invoking virus involvement. Baker and Cook (1974) dismissed the idea that mycovirus could account for decreased take-all in wheat monoculture soils in Washington because isolates removed from suppressive soils regained their virulence when inoculated into non-suppressive soils. Rawlinson (unpublished) found that isolates taken from a location where cereals had grown continuously for 100 years proved to contain virus and to be pathogenic when tested on wheat grown in sand. Rawlinson (1975) could find no evidence that virus infection

impaired enzyme production (poly galacturonase and cellulase, Holden, 1975) in culture and in a series of experiments began in 1976 (Rawlinson et al, 1977) no significant increase in yield of wheat could be obtained by treating soils and seeds with a perlite inoculum of virus infected, weakly pathogenic isolates of G. graminis and Phialophora spp.

Rawlinson and Muthyalu (1975, 1976) isolated Ggt from French fields growing wheat crops and found many virus-infected (27 and 35 m particles) in Ggt isolates from crops with severe take-all, and discovered a third particle size (40nm) in these isolates. They also found virus in Ggg from Australia and particles of all three sizes (27, 35 and 40 m) in English isolates of Phialophora sp. with lobed hyphopodia. A serological relationship was found to exist between Ggt virus particles from Africa, Australia, Belgium, England, France, Japan and the USA. It therefore seemed that a direc causal relationship between viruses in Ggt and hypovirulence was unlikely. However two new approaches suggested that less apparent relationships might be found.

Frick and Lister (1978) considered that previous failure to demonstrate a consistent association between virus infection and pathogenicity could be due to virus biotype variation. It was demonstrated that VLPs from different isolates from the same field varied in serotype and serologicall unrelated viruses were found. At about the same time Almond et al (1981) found that the number and size of dsRNA and polypeptide components of VLPs from different isolates varied greatly, and no two isolates were found that

contained the same dsRNA components. These results suggested that variations in VLP structure, serotype and possibly biotype might explain the lack of any simple correlations between presence or absence of virus. It is well known in plant and animal virology that different strains and serotypes of viruses can differ enormously in the biological effect that they exert on their hosts.

Clearly a more detailed analysis of Ggt viruses was needed to evaluate any possible role they may have in the control of take-all. An understanding of the implications of the heterogenicity already apparent in Ggt viruses will require consideration of the properties of other mycoviruses.

1.2 Double-stranded RNA Mycoviruses

1.2.1 Discovery and Occurrence

Viruses, or virus-like particles (VLPs) have been found in over 100 species of fungi and Bozarth (1972) estimated that 10-15% of randomly sampled fungal isolates contained VLPs. VLPs able to infect and multiply in a fungus were first detected in the cultivated mushroom Agaricus bisporus and associated with die-back disease (Hollings, 1962). Later, VLPs were associated with the anti-viral interferon-inducing effects of statalon, a cell free extract of a strain of Penicillium stoloniferum and dsRNA was identified as the active agent (Ellis and Kleinschmidt, 1967, Banks, et al, 1968). Soon VLPs of several morphological types were found in many fungal species (reviewed by Buck, 1979; Lemke and Nash, 1974; Hollings, 1978). The most common morphological type found had

small sometric particles containing ds RNA.

The late discovery of VLPs in fungi was probably due to the fact that they are usually latent causing no detectable disease and appear to be transmitted purely by intracellular routes, having no extra-cellular phase. Although fungal isometric VLPs do not satisfy all of Koch's postulates, they may justifiably be termed viruses because (a) these VLPs have the typical structural characteristics of viruses, (b) they appear to contain enough genetic information in the ds RNA to encode all the virus proteins and proof that VLP proteins are encoded by VLP ds RNA has been obtained in a few cases, (c) several VLPs have particle-associated dsRNA-dependant RNA polymerase, (d) extracellula inoculation has been shown in a few cases (Lhoas, 1971, Hollings and Stone, 1971) and they can be transmitted by heterokaryosis (Lemke and Nash, 1974; Koltin et al, 1973). The lack of extracellular infection may be due to properties of fungal hyphae, which have tough cell walls and exist under considerable hydrostatic pressure so that damaged cells, where infection could occur, effectively 'explode' and all adjacent unplugged hyphae are destroyed. An intracellular mode of transmission would be essential for a virus that can not infect extracellularly. In this may lie a clue to why fungal viruses are usually latent: other plant and animal viruses are capable of infecting extracellularly and so death of the host liberates virus particles for further infections. However, any virus liberated from a dead fungal cell would be unable to reinfect and its genetic information would be lost so that a (mutant) virus that did kill its host would be lost from the population. Clearly the survival of a fungal virus depends upon the proliferation of their host and its ability

to disseminate its genetic information by the only available means heterokaryosis. This would entail the virus infecting a minimal damaging effect on the host. It is quite likely that mutant lethal viruses arise regularly and spontaneously in fungal populations, but unless they simultaneously acquire a mechanism of infecting extra cellularly, a very unlikely event, they will be lost from the population. It is also likely that a mutant virus that causes disease would allow its host to be out-competed by healthy fungi. The only situation where this may not occur would be if the diseased fungus is maintained artificially. It is probably for this reason that microvirus diseases (to be discussed) have only been found in commercially grown fungi (mushrooms) or fungi that have been maintained artifically in culture (e.g. Rhyzoctonia solani). This hypothesis is supported by observation that diseased isolates of R. solani do not survive in non-sterile soil for more than one month.

If this reasoning is followed and one assumes, as is likely, that intense competition exists in the natural fungal habitats, then it will be seen that viruses will be maintained in natural populations only if they do not significantly impair the competitive ability of their host. This seems to be borne out by the estimates of virus concentration in fungal cells. In the case of Penicillium stoloniferum which produces high virus yields, virus accounts for only 0.5% of fungus dry weight. This is unlikely to be a significant load on the fungus. Alternatively or additionally, the virus could confer some competitive advantage to the host, as may account for yeast viruses and Ustilago maydis, to be discussed later. A different situation may be found in some plant

pathogenic fungi where the parasitism of the pathogen is so damaging it virtually eliminates its host from a population. If this occurs, then a virally modified change in the pathogen towards low aggressiveness might allow the host to re-establish itself as a habitat for the pathogen. However, unless this new population is isolated, the newly emerged host is likely to be attacked by remaining aggressive isolates in the population. It is possible that an equilibrium could be achieved in which virus could modify the aggressiveness of the pathogen to a level which allows both host and pathogen to be proliferate. This could be relevant to virus infection in Endothia parasitica the causal agent of chestnut blight and may be relevent to Ggt

Fungal viruses appear to be diverse in their structural and physio-chemical properties, and it is likely that several virus families will be needed to accomodate them. Most contain dsRNA. The other families of dsRNA viruses are the Reoviridae (including reoviruses, cytoplasmic polyhedrosis viruses, dsRNA plant and insect viruses and rotaviruses), the cystoviridae (with $\phi 6$ as the only known member) and the recently discovered group of animal viruses with bisegmented dsRNA genomes (Pobos et al, 1978). Fungal viruses have been reviewed extensively since 1969, most recently by Buck (1980) and Hollings (1978).

1.2.2 Physical Structure and Genome Organisation

The isometric dsRNA microviruses do not form a homogenous group and only limited information is available on some viruses. Virus capsids

are usually constructed from single major polypeptide species with molecular weights ranging from 25,000 to 130,000, have a particle size of 25-50nm and either 60 or 120 polypeptide copies per virion. Small amounts of minor polypeptides have been detected: these may be RNA polymerase molecules or proteolitic cleavage products of the major polypeptide (or the RNA polymerase) or some other specific protein of unknown function. RNA polymerase molecules have been tentatively identified in two cases Penicillium stoloniferum virus and P. stoloniferum virus F(Buck and Kempson-Jones, 1974).

The number of dsRNA components varies from 1 to 8 with sizes varying from 0.27 to 6.3 x 10^6 daltons. In the viruses with more than one dsRNA component, each dsRNA is usually encapsulated separately. Buck (1980) has made a preliminary classification of dsRNA mycoviruses into those with individual genomes and those with divided genomes (Tables 2 and 3).

It is postulated that, in order to replicate in a fungal cell a dsRNA virus genome must encode a capsid polypeptide, and since as far as is known fungi do not possess a dsRNA dependent RNA polymerase, the virus must encode such an enzyme. It has been demonstrated the genomes Saccharomyces cerevisiae virus (Hooper et al, 1977) Penicillium chrysogenum virus and P. stoloniferum virus S (Buck, 1979, et al), encode their own capsid Polypeptide. Virion associated RNA polymerase activity has been demonstrated for viruses from S. cerevisiae, Aspergillus foetidus, P. stoloniferum, P. cyareofulreum and Allonyus arbuscula (reviewed by Buck, 1979) however in no case has formal proof been obtained that the Polymerases are virus coded,

Table I.2 Physical properties of isometric dsRNA mycoviruses with undivided genomes

Virus	Particle diameter (nm)	Major capsid polypeptide ($mol.wt.X10^{-3}$)	Virion dsRNA ($mol.wt.X10^{-6}$)	Calculated coding capacity of dsRNA ($mol.wt.X10^{-3}$)
Helminthosporium maydis virus	48	121	6.3	350
Gaeumannomyces graminis virus F10 A	40	94	6	330
Saccharomyces cerevisiae L virus	40	88	3.4	189
Ustilago maydis virus	41	75	4.1	229
Mycogne perniciosa virus	42	69	4.3	239
Helminthosporium victoriae virus (190S particles)	35-40	88(+83?)	3.0[a]	-

Table from review by Buck (1980).
[a]Almost certainly an underestimate since value obtained by linear extrapolation from reovirus dsRNA standards.

Table I.3 Physical properties of isometric dsRNA mycoviruses with divided genomes

Virus	Particle diameter (nm)	Virion polypeptides (mol.wt.X10^{-3})	No. of each polypeptide per virion	Virion dsRNAs (mol.wt.X10^{-6})	Calculated coding capacity of each dsRNA (mol.wt.X10^{-3})
Penicillium chrysogenum virus	35-40	130	60	2.2 2.1 2.0	123 117 111
Aspergillus foetidus virus	35-40	125 100 87	1 1 120	2.7 1.87 1.70 1.44 1.24	150 104 94 80 69
Mushroom virus 4	35	64	-	1.5 1.4	83 78
Penicillium stoloniferum virus F	30	59 47	1 120	0.99 0.89 0.46	55 49 27
Penicillium stoloniferum virus S	30	56 42	1 120	1.10 0.94	61 52

From Buck (1980).

The polypeptide coding capacity for individual dsRNA components has been calculated for several mycoviruses assuming that (a) only one strand codes (b) three base pairs encode each amino acid (average molecular weights 310 and 110 respectively), and (c) the whole length of one strand codes and that non coding regions are negligible (Buck, 1980). The calculation makes the assumption that there were no overlapping genes and that there is no splicing of pre-mRNA, that translation initiates and terminates at one site and that there is no post-translational modifications. On the basis it can be deduced that mycovirus dsRNA components are monocistronic in some virus and at least diastronic in others. In the virus with undivided genomes (Table 3) it can be seen that the major capsid polypeptide accounts for only one third to one half of the total dsRNA coding capacity implying that it is at least dicistronic. In the multi-component viruses (Table 2) the calculated coding capacity often corresponds quite closely to polypeptide molecular weights, so these dsRNAs are probably monocistronic: however in some cases the coding capacity exceeds the amount calculated to code for the known polypeptides. Aspergillus foetidus virus has three dsRNA components, a major and minor polypeptide, the largest dsRNA has sufficient coding capacity to code both polypeptides. Mushroom virus (Barton and Hollings, 1979) has a major polypeptide of molecular weight 24,500 and 2 dsRNA components, one each with a coding capacity of 78 daltons.

In some cases more than one capsid polypeptide has been reported (Helsinthosporium victoria virus Sanderlin and Ghabrial, 1978). However it is not clear whether one polypeptide results from protolytic cleavage

of the capsid polypeptide, such as occurs with Aspergillus foetidus virus S. (Buck and Ratti, 1975).

The isometric dsRNA mycoviruses differ from dsRNA viruses from other host taxa. Members of other groups have divided genomes. Human type 3 reoviruses have a complex double-shelled capsid composed of ten polypeptides and contains ten dsRNA components. Phage 6 has a nucleo capsid composed of ten polypeptides and contains three polycistronic dsRNA components. Drosophila x virus, and other similar viruses have biseg mented genomes; virions are composed of capsids in three size classes. In all these cases virus particles contain one copy of each dsRNA component. Isometric dsRNA mycoviruses are generally smaller have simple capsids composed of a single major polypeptide, total genome size of 3-9 Kb, and in the case of those viruses with divided genomes, each dsRNA component, with rare exceptions is contained in a separate virion.

There is no known cellular enzyme which will transcribe dsRNA efficiently, so dsRNA viruses must carry their own dsRNA-dependent RNA polymerase. All dsRNA viruses that have been examined have been found to carry virion-associated RNA polymerases. Genetic evidence indicates that the polymerases of reovirus (Cross and Fields, 1977) and phage 6 (Pimon and Haselkorn, 1978) are virus-coded. In reovirus and ϕ6 dsRNA is never released from virus particles during the entire replicative cycle. It is possible that dsRNA is never released from isometric dsRNA mycoviruses either, particularly as it is known that dsRNA inhibits the initiation of protein synthesis in some systems.

1.2.3 Transmission

The transmission of fungal viruses appears to be only by intracellular routes, there is no evidence for the release of virus particles followed by infection. Fungal cells cannot usually be infected with cell-free virus; although some success has been achieved using fungal protoplasts (Loas, 1971; Pallet, 1976). This contrasts with the viruses of bacteria higher plants and animals. Some plant viruses are sufficiently contagious to infect by foliage contact or during cultural operations: others are released into the soil from healthy roots and can infect adjacent roots of healthy plants. Other plant and animal viruses are transmitted by invertebrate vectors. There is no evidence of any fungal virus being transmitted by a vector. DsRNA viruses of plants can be transmitted by invertebrate vectors (clover wound tumour, maize vough dwarf and others) and capable of replicating in their vectors; so it does not seem that the possession of a dsRNA genome is an obstacle to this type of transmission.

Transmission of fungal viruses from one host to another is termed horizontal transmission and occurs by hyphal anastomosis during heterokaryosis Transmission during heterokaryosis has been demonstrated for Aspergillus nigers, Penicillium stoloniferum (Lhoas, 1970), Ustilage maydis (Wood and Bozarth, 1973), Schizophyllum commune (Koltin et al, 1973), and others. In fungi, heterokaryosis between strains is greatly restricted by vegatative incompatibility: two strains may not anastomose (fusion incompatibility), anastomosed cells may die (post-fusion death) or strains may be inhibited from making contact. However, hyphal anastomosis without heterokaryosis between incompatible strains of Schizophyllum commune was sufficient, for virus transmission to occur (Koltin et al, 1973). Similarly, vegetative

incompatibility did not prevent transmission of dsRNA and hypovirulance between some strains of Endothia parasitica. (Anagnostakis and Day, 1979).

Transmission of virus from parent to progeny is termed vertical transmission. Transmission during vegatative growth probably occurs with translocation of protoplasm between unplugged hyphal compartments in the peripheral growth zone. Transmission of virus to spores was first shown for mushroom viruses 4 where virus particles could be seen in thin sections of mushroom spores (Dielemon van Zaayen, 1972, 1975). Since then viruses have been shown to be transmitted in conidia of Penicillum species (Banks et al, 1968, 1969b· Lemke et al, 1973), Aspergillus species (Wood et al, 1974) and others· in zoospores of Allomyces arbuscula (Roos et al, 1976): aleurospores of Mycogone pernicioso (Albany and Lapierre, 1972): basidiospores of Lentinus edodles (Ushiyama and Nakai, 1975); teliospores of Ustilago maydis (Day, 1981) and ascospores of the yeast Saccaromyces cerevisiae, (Mitchell et al, 1976). However, virus or dsRNA has not been found in ascospore strains of Helminthosporium maydis (Bozarth, 1977), derived from virus infected strains. Similarly, as will be discussed further, ascospore strains of Ggt appeared to be free from detectable dsRNA or virus particles (Pawlinson, et al, 1973) although Lapierre (1973) stated that loss of virus by sexual reproduction did not always occur in Ggt. In cases where spore transmission has been established, it does not always seem to occur, invariably. In an investigation of transmission of virus S and F during conidiogeneses of Penicillium stoloniferum (De Marini, et al, 1977) it was found that where 43 random single conidial isolates derived from the same strain were examined for virus particles, most cultures contained virus levels similar

to that of the parent strain, a small number contained higher or lower levels; three isolates contained no detectable virus F and a further two isolates contained no detectable virus particles.

1.2.4 Host Range

Since dsRNA mycoviruses are transmitted by only inter-cellular routes and horizontal transmissions by heterokaryosis, and since vegetative incompatibility greatly restricts transmission of cytoplasmic elements even within the same species; then it would be expected that mycovirus host range would be very limited. Serologically related viruses have been found in Penicillium brevicompactum, P. cyanofulvum and P. chrysogenum (Wood and Bozrath, 1972; Buck and Girvan, 1977) in Aspergillus foetidus and A. niger (Buck, et al, 1973) and in numbers of the Gaeumannomyces, Phialophora complex (Rawlinson and Muthyalu, 1975, 1976).

1.2.5 Replication

Replication of dsRNA microviruses has been reviewed in detail by Buck (1979b). Viruses appear to replicate in the cytoplasm in parallel wit the growth of their host, but may continue to replicate when host growth ceases, Barber et al, (1972) examined thin sections of fungal hyphal for virus. Viral particles were found to be absent from the apex to 0.1nm behind: virus particles could be seen 0.1 to 0.2mm behind the hyphal tip in aggregates of up to several hundred in the cytoplasm. In the

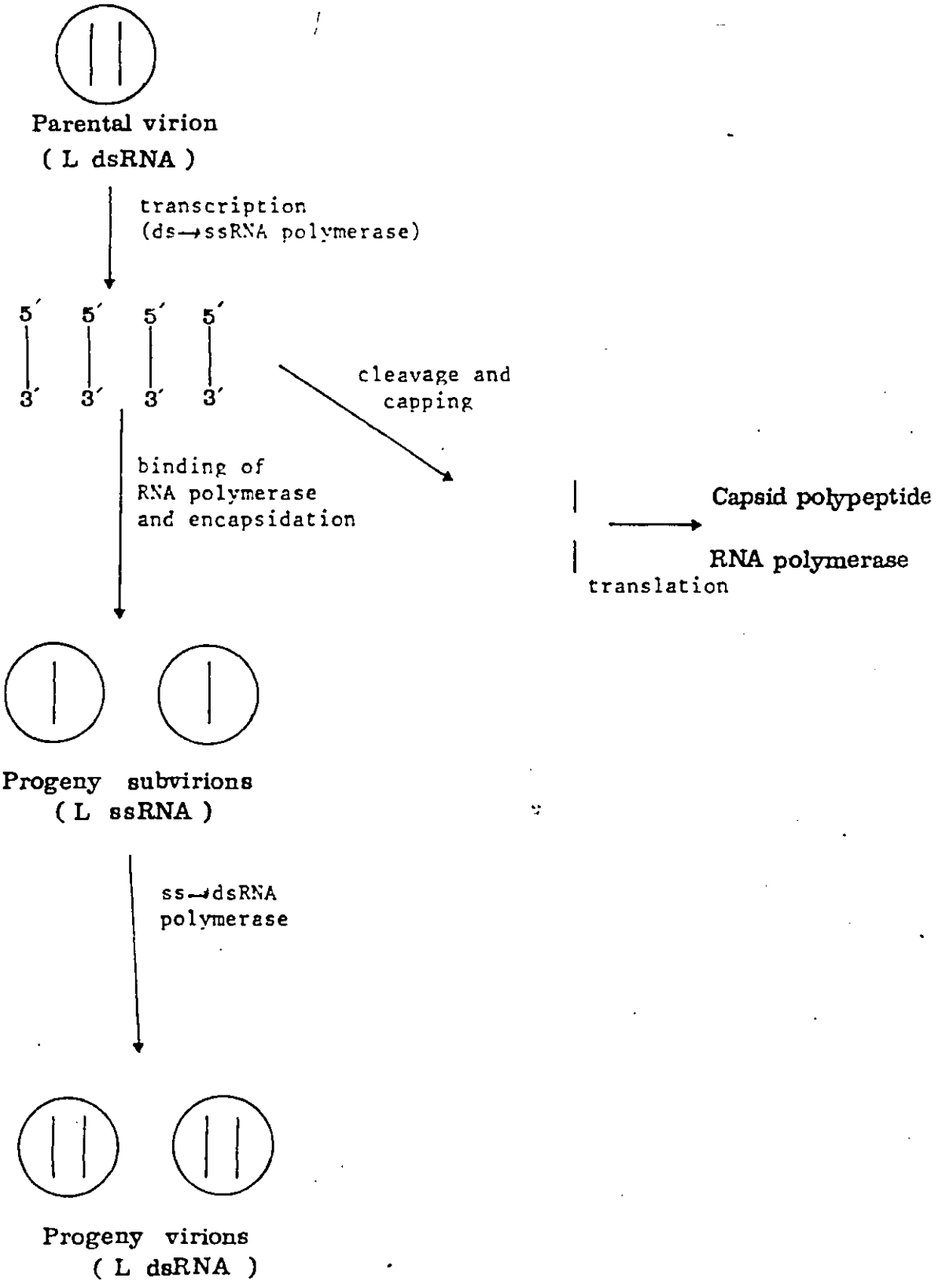


Fig.I A model for the replication of Saccharomyces cerevisiae L virions (from Buck, 1980).

region of 2 to 3 mm from the tip, aggregates of viral particles were found bounded by a single membrane, as well as free in the cytoplasm, further back, virus particles in paracrystalline arrays were seen in large vacuoles. Similar results have been obtained with P. chrysogenum and P. funiculorum.

The replication of mycoviruses in vitro has been studied for Saccharomyces cerevisiae virus (ScV), Penicillium stoloniferum virus S. (PsV-S) and Aspergillus foetidus virus S. (AfV-S), which have an undivided genome, a divided genome, and a genome of unknown structure respectively.

Two types of RNA polymerase activity are associated with ScV virions: (a) ss → dsRNA polymerase activity associated with particles isolated from log phase cells (Bevan and Herring, 1976); (b) ds → ss RNA polymerase (transcriptase) activity associated with virions containing dsRNA isolated from stationary phase cultures (Herring and Bevan, 1977): the product of the reaction was a full length of one of the strands of dsRNA. On the basis of these two types of activity, Herring and Bevan (1977) postulated that ScV dsRNA duplicate asynchronously like reovirus dsRNA (Silverstein et al, 1976) i.e. transcription and replication occur at separate stages, however Buck (1980) pointed out several differences with the reovirus system and suggested an amended model shown in Fig.1 in which full length transcripts of dsRNA are produced and there may be either cleaved and capped to act as message or packaged in progeny virions to act as transcrip for ss → dsRNA polymerase activity.

AfV-s virions from stationary phase cultures possess transcriptase activity producing full length ssRNA copies of all three dsRNAs, if the virions are treated with proteinase K. Without proteinase K treatment transcripts of mainly dsRNA 2 are produced. Transcription takes place by a semi-conservative displacement mechanism (Ratti and Buck, 1978).

PsV-S virions from stationary phase cultures have dsRNA replicase activity in vitro (Buck, 1975). Progeny dsRNA is not released giving rise to diploid particles. This may be an artifact of the in vitro reaction. Replication is semi-conservative and probably consists of synthesis of daughter (+) strand with displacement of parental (+) strand followed by synthesis of daughter (-) strand on the displaced parental template. Dipolid virions have not been isolated from cultures although particles containing one molecule of dsRNA and one molecule of ssRNA have been isolated as well as particles containing only ssRNA. Since PsV-S dsRNAs are probably monocistronic, cleavage of primary transcrips is probably unnecessary.

It is possible that multi-component RNA viruses arose from those with undivided genomes, as a convenient method of production of monocistronic mRNA. Separate encapsidation of individual segments of dsRNA would not be an impediment to transmission if transmission is intracellular and may be an advantage allowing intrastrand recombination in mixed infections. A similar situation may exist for influenza virus which has a segmental genome consisting of 8 RNA segments: recombinants have been obtained from mixed infections in mice of two strains, one adapted

to grow in mice but non-neurovirulent and the second a human strain highly neurovirulent in humans but non-neurovirulent in mice. Recombinants were obtained that were highly neurovirulent in mice and consisted of segments from both parents (Scholtissek et al, 1979). This recombination by RNA segments reassortment has been reported for single stranded RNA viruses replicating by use of an RNA dependent RNA polymerase, in plants and vertebrates (Simpson and Hirst, 1968; Webster et al, 1973).

In summary, it appears that many dsRNA mycoviruses replicate asynchronously by a semi-conservative mechanism. Virions isolated from different stages of fungal growth show different RNA polymerase activity: and in vivo the relative rates of transcription and replication may be controlled by host cell factors and be dependent on the physiological stage of the host.

1.2.6 Biological Effects

1.2.6.1 Virus diseases of fungi

The dsRNA micoviruses are usually latent causing no overt effects on their host. However, virus diseases have been described in at least four species: Penicillium chrysogenum, Agaricus bisporus, Helminthosporium victorae and Rhizortonia solani.

Die-back disease of Agaricus bisporus is caused by virus infection and several different viruses may be involved. Infection of healthy mushrooms by cell-free virus has been achieved (Hollings, 1962: Dieleman -van Zaayen and Temink, 1968). Two particle sizes (25mm and 35mm)

of the five known to occur in mushrooms have been detected in infections though the dsRNA components associated with them vary. Disease may depend on host factors since latent virus infections accompanied by very low concentrations of virus particles are quite common in cultivated mushrooms and can often be experimentally induced by, for instance, heat treatment (Last et al, 1974).

The involvement of host factors is shown in Penicillium chrysogenum. Certain mutant strains produce lytic plagues when grown on surface culture. Lemke (1975) has shown that plague formation depends on (a) the presence of dsRNA virus, (b) host mutation (wild type strains of nuclear gene for resistance to lysis dominant in diploids), and (c) the use of high lactose medium.

A disease of Helminthosporium victoriae, the causal agent of Victoria blight of oats transmissable by heterokaryoses is correlated with the presence of 145S virus (Ghabrial, et al, 1979) which may be a satellite to a serologically unrelated 190S virus since the 145S virus never appears on its own. Recently the disease has been transmitted, using cell free 145 plus 190 virus, during fusion of virus free protoplasts of H. victoriae (Gabrial, 1980). About 1.5-2.5% of resulting colonies from regenerated protoplasts were diseased and contained virus.

Rhizoctonia decline is a degenerative disease of R. solani a plant pathogenic fungus, cytoplasmically transmitted and associated with dsRNA. Diseased isolates are hypovirulent on hosts. In diseased

isolates three dsRNA components were present and these were usually absent from hyphal tip cultures which were healthy. However, some dsRNA preparations of hyphal tip cultures showed trace amounts of the three dsRNA segments and basidiospore culture of one of these gave all healthy colonies but after successive transfer, some colonies became diseased, indicating that some of the healthy hyphal tip cultures may contain the disease agent (Lastenko and Butler, 1978). Only diseased isolates of 12 isolates examined contained dsRNA but in each case the three dsRNA components were of different electrophoretic mobility. Virus particles have not been detected in diseased isolates: this could be because virus particles are unstable or difficult to detect or naked dsRNA may be present in R. solani. Castenko and Butler (1979b) have used diseased isolates of R. solani to protect plants from attack from healthy virulent hyphal tip cultures derived from the diseased isolate. Total biological control of the disease was achieved in pot tests and since protection was shown to depend on both isolates actively growing, it was thought to result in the conversion of the healthy isolate to the disease type by anastomosis with the diseased isolate. However, the diseased isolate failed to survive in sterile soil for one month. Also, attempts to transmit the disease to four isolates failed (possibly due to vegetative incompatibility) and so the use of R. solani for biological control of disease is still problematic.

1.2.6.2 Double-stranded RNA and hypovirulence

The fungus Endothia parasitica is the causative agent of chestnut blight and is responsible for the decimation of the American chestnut

(Castanea dentate) in the United States. The disease was first reported in 1906 and in 40 years every major stand of American chestnut in the Eastern United States was reduced to stumps. In 1938 the disease spread to Europe and a similar epidemic ensued. However, in 1951 Antonio Biraglii, an Italian plant pathologist found trees that seemed to be recovering from the disease (Biraghi, 1951). Grente (1965) isolated from bark of these trees forms of the blight fungus that had reduced virulence; these he called hypovirulent. Grente showed that these hypovirulent (H) strains cured existing blight when they were inoculated into cankers infected with the virulent (V) strains. Van Alfen et al, (1975) showed that hypovirulence is cytoplasmically transmitted and virulent strains can be converted to hypovirulent character by hyphal anastomosis. Since their discovery H strains have gradually spread in Italy where chestnut blight has lost its epidemic character, and a host - pathogen balance appears to have been established (Mittempergher, 1979). Hypovirulent strains have since been isolated in France (Grente, 1965) and in the USA (Anagnostakis, 1978).

All H isolates of Endothia parasitica so far examined have been found to contain dsRNA (Day et al, 1975), whereas most, but not all, V strains lack dsRNA. On examination by polyacrylamide gel electrophoresis several patterns of dsRNA components have been observed, but all H strains appear to contain at least one component in the mol. wt. range 5-7 x 10^6, which is very high for a multi component mycovirus. In at least one strain the dsRNA is associated with club shaped virus like particles (Dodds, 1979); particles of similar morphology have been reported to

be associated with a severe disease of Agariccus bisporus (Lesemann and Koenig, 1977). H strains do not have reduced vigour as saprophytes: however, they have never been found to produce ascospores and in axenic culture will frequently revert to the V phenotype presumably due to the loss of the cytoplasmic H determinant. Hypo-virulent strains are usually white,but these strains even if derived from H strains are usually red-orange. Single conidial isolates derived from H strains do not necessarily give rise to the clonal type: some progeny are more pathogenic than the parent but still hypovirulent and have a reduction in the quantity and number of dsRNA components: some are indistinguishable from normal V strains and lacking dsRNA, some are identical to the parent in hypovirulence and dsRNA content. The hypovirulent character seems to be sensitive to uv radiation. When conidia are irradiated with uv to kill 90 - 99% of conidia, virulent strains (pigmented) gave rise to very few white avirulent colonies; a pattern typical of genetic mutations. However, if hypovirulent (white) conidia are similarly irradiated then the number of pigmented colonies produced, increases from 10% (without irradiation) to 70% (with irradiation), (Van Alfen, et al, 1979). H. strains have been shown to be less sensitive to killing by uv irradiation than these strains so it seems that the high hypovirulent determinant is sensitive to uv. One Italian virulent strain EP.49 contains dsRNA and is virulent. This strain which is pigmented yields only pigmented colonies when conidia are plated. However, if conidia are irradiated with UV light from 1-20% of the surviving colonies were white. The white colonies contained dsRNA and were typically hypovirulent. This type of behaviour is similar to that of some virulent strains derived from H strains. EP-11 was derived by heterokaryosis between a French H strain and an American strain and

is hypovirulent. Some conidia derived from this are V type pigmented, however on subsequent replated transfer 78% of resultant colonies reverted to white hypovirulent type, the remaining pigmented V colonies remained stable on repeated transfer, and colonial colonies were all pigmented. However, after uv irradiation of conidia 1-10% of surviving colonies were white and hypovirulent. This is much higher than what would be expected from genetic mutation and Van Alfen et al. (1979) suggests that in these Ep-1' derived virulent strains and in Ep-49 hypovirulence is in a suppressed state and can be induced by UV irradiation.

Although the evidence for dsRNA being responsible for hypovirulence in Endothia parasitica is persuasive it is possible that other cytoplasmic factors are involved. Proof of the role of dsRNA would require extracellular infection of a virulent isolate with dsRNA or virus and its conversion to hypovirulent character.

1.2.6.3 Killer factors of yeasts

Killer strains of Saccharomyces cerevisiae secrete a toxin or killer factor (Woods and Bevan 1968) to which they themselves are insensitive but which is lethal to certain other strains of the same or related species designated sensitives. Toxin has been purified from 1 strain K 12/1 and shown to be a protein of molecular weight 11500 (Palfrey and Burrey, 1979). Genetic studies have shown that killer and immunity character are cytoplasmically inherited (Bevan and Somers, 1969) and several lines of evidence (reviewed by Wickner, 1979a:

Bevan and Mitchell, 1979; Vodkin, 1979) have indicated that the killer determinent is a dsRNA plasmid. Most sensitive strains are infected with virus particles which contain a single dsRNA molecule (L dsRNA molecular weight 3.4×10^6) and which are able to replicate autonomously. L killer strains are also infected with L virions but also contain a dsRNA component, designated M dsRNA of molecular weight 1.4×10^6. M dsRNA is found only in strains also infected with L dsRNA and virus, suggesting it is unable to replicate autonomously: it is not a cleavage product of L dsRNA since no sequence homology can be detected by finger printing or hybridization studies (Bruenn and Kane, 1978). M dsRNA is enclosed in virus particles of the same size as L virions and is a capsid constructed from a polypeptide with the same molecular weight as, and serologically related to, the capsid polypeptide of L virions (Herring and Bevan, 1975). It is therefore likely that the L dsRNA encodes the polypeptide for its own encapsidation and that of M dsRNA; it may encode an RNA polymerase for the replication of both RNAs. M dsRNA is therefore probably a satellite dsRNA, which replicates only in the presence of helper yeast L virus.

Under a variety of conditions where sensitive clones have been derived from killer parents, it was found that loss of both function and immunity was accompanied with loss of M dsRNA. Recently, unequivocal proof that M dsRNA encodes killer toxin has been obtained by *in vitro* translation of denatured M dsRNA (Bostian, *et al*, 1979).

No yeast mutants are known which are unable to support L dsRNA replication. However, the killer phenotype and maintenance of M dsRNA, though independent of mitochondrial DNA and several yeast plasmids, is dependent on at least 29 nuclear genes (mak 1-27, pet 18, and spe 2), the so called maintenance genes (Bevan et al, 1973: Wickner, 1978; Cohn, et al, 1978). Recessive mutations of any of these (none of which are required for L dsRNA replication) results in loss of M dsRNA and the killer phenotype. Two chromosomal genes (kex 1 and 2) are required for secretion of toxin, another (rex 1) is required for expression of resistance to toxin (Wickner, 1974). The biochemical function of most of these genes is unknown but some may have an indirect effect on the killer character.

Several killer mutants have altered M dsRNA. Some have lost ability to produce killer but are still immune; immunity minus mutants are also known, suggesting that killer and immunity character are determined by separate genes on M dsRNA. Suppressive non-killer mutants suppress killer phenotype on mating with wild type killers. These mutants contain small (s) dsRNAs, of mol. wt. less than 1.0×10^6, lack M dsRNA, but contain L dsRNA. In crosses with wild type killers, the resulting diploids are frequently non killers and all four meiotic products are sensitive, lack dsRNA but contain L and S dsRNAs. Examination of these S dsRNAs by heteroduplex mapping and RNA finger printing showed that they were derived from M dsRNA: one of these S 1 was shown to be derived from internal deletion of M (Fried and Fink, 1978: Bruenn and Kane, 1978); another S 3 arose by duplication

of S 1; yet another by internal deletion of S 3. Kane et al (1979) examined several of these defective interfering dsRNAs and found them all to be internal deletions of M dsRNA with tandem duplications in some cases. The S dsRNAs diverged in sequence from the parental M dsRNA as do defective interfering RNAs of animal viruses in persistent infections (Holland et al, 1979). Denatured S dsRNAs give rise to polypeptides but do not suppress in vitro translation of M or L dsRNA so suppression probably occurs at the level of RNA replication, possibly by competition for RNA polymerase or other replication factors. S dsRNA have 3-terminal sequences in common with each other, and M dsRNA, probably involved in RNA polymerase recognition (Bruenn and Brennan, 1980)

Killer strains in yeast of several genera (Phillis Kirk and Young, 1975) and killer yeasts have been classified in four groups (Rogers and Bevan, 1979). Tox 1-4 or ten groups (Young and Yagui, 1978, K 1-10, K 1, K 2, and K 3 corresponding to tox 1, tox 2 and tox 4 respectively) the K 1, K 2 and K 3 killers contain L and M dsRNA (different molecular weight in each group) and could be cured of killer phenotype by treatment with cycloheximide. The K 4 to K 10 toxins appear to be proteins but none of these killers contain dsRNA or could be cured by treatment with cycloheximide. Woods, Ross and Hendry (1974) obtained a killer strain of Saccharomyces cerevisiae which arose as a spontaneous mutation from a K 1 sensitive strain and which contained only L dsRNA. K 1 killers were able to kill 'Woods' killers and vice versa. Crosses of K 1 sensitive and 'Woods' killers suggested that in the latter strains killer toxin may be encoded by a nuclear gene, raising the question of a possible dsRNA 'provirus'.

1.2.6.4 Killer factors of Ustilago Maydis

Killer strains of Ustilago maydis (Puhalla, 1968) the corn smut pathogen, whilst in the yeast-like phase, secrete toxins which kill or inhibit the replication of strains from the same or related species (Koltin and Day, 1975). Ability to produce toxin is cytoplasmically inherited and occurs only in strains carrying dsRNA virus particles (Wood and Bogarth, 1973; Koltin and Day, 1978a). Immunity to killer is also cytoplasmically transmitted on dsRNA determinants but can also be determined by nuclear genes. Nuclear resistance genes are recessive to their sensitive alleles (Koltin and Day, 1976a); they may be analogous to the killer resistant mutants of Saccharomyces cerevisiae, lacking cell wall toxin binding sites (Al-Aidroos and Bussy, 1978). Three killer specificities (P1, P4 and P6) are known in U. maydis: each killer is immune to its own killer substance but sensitive to the other two. All three types of toxin have been purified and are proteins of about 10000 daltons, but differ in their electrophoretic mobility at pH8.3, in their thermolability, pH dependence and biological activity.

Each killer strain contains between five and seven dsRNA components. Two dsRNA components (4.1 and 3.1 x 10^6 daltons) are common to all three killer types and additional dsRNA components are present (Koltin and Day, 1978). In crosses between killer and sensitive strains, most progeny are killers but a low frequency of progeny are non-killers but killer sensitive. The killer progeny gave dsRNA patterns identical to the killer parent; the non-killer immune progeny all carry dsRNA and share

components with the killer parent. Immunity was shown to be cytoplasmically inherited (Koltin and Day, 1975a). The non-killer immune progeny had dsRNA patterns which contained some bands of identical mobility to the killer parent but some bands were lost, and in some cases, new dsRNA bands appeared. One of these progeny lost three dsRNA components (retaining two), another lost two components and gained a dsRNA component of molecular weight of about the sum of the two components that were lost: another lost three dsRNA components and gained two new components. In general, loss of killing and retention of immunity was associated with a change in dsRNA pattern but no specific functions could be assigned to any dsRNA component to account for all the results.

In another study, Koltin and Day, (1976b) crossed nineteen non-killer sensitive cell lines of U. maydis with three killer strains. Crosses involving eleven of these lines gave results typical of cytoplasmic inheritance (71-100% progeny killers and 4.0 segregation in tedrads). However segregation in some crosses involving each of the remaining eight lines gave killer frequencies from 0% - 58% and mixed tedrads contained both non-killer and killer meiotic products. Many of these killers were unstable on further culture. This result, together with the results of backcrossing experiments, could most simply be explained (at least for one of the suppressor strains) if the strains which give low killer frequencies in crosses contain a dominant cytoplasmic suppressor. The suppressor action operated on immunity function as well, despite the fact that the information for killer and immunity function may reside on different dsRNA components (Koltin and Day, 1976a). Two suppressor strains contained dsRNA, two others contained no detectable dsRNA. Kolton and Day suggested that in those dsRNA-containing

suppressors, a restriction mechanism may prevent the occurrence of more than one killer specificity in a cell.

The functions of individual dsRNA molecules have been examined by using mutants with partial genomes. The dsRNAs are of three size classes: H (1.8 - 4.1 x 10^6 daltons) and M (0.6 - 1.0 x 10^6 daltons) and L (0.3 x 10^6 daltons) and genetic studies have indicated that H components are independently viable and may act as helpers for the replication of M and L components, which are associated with the killer expression (Koltin, 1977: Kiltin and Kendel, 1978). One mutant killer had only one H, one M and one L dsRNA component.

No nuclear genes have been found which are required for killer expression or RNA replication, in contrast to the yeast killer system. Killer strains of different specificities may also be suppressive to each other, for example, a P4 x P6 cross yielded only P4 progeny among the killers. This exclusion, phenomenon is abolished from P4 strains by deletion of the specific dsRNA determinants for killer expression: it may be that P4 toxin somehow restricts P6 toxin determinants, possibly by nuclease action (Koltin et al, 1978). Nuclease activity has been found in purified toxin protein (Levine et al, 1979).

Koltin and Day (1975) suggested that U. maydis killer may be used in controlling cereal smuts, possibly by introducing the genetic determinants for toxin production into the host. However, the significance of U. maydis killer in nature is unclear since killer strains do not adversely

effect sensitive strains in the vegetative mycelial phase in the host plant. Killer strains of U. maydis are quite rate, though dsRNA is common and toxin resistant strains are abundant. Some strains may have partial viral genomes, and Day and Dodds (1979) considered that killer strains could arise de novo by mating or heterokaryosis between strains carrying complementary dsRNA genomes.

Day (1981) investigated the contribution of cytoplasm containing different dsRNA components on pathogenicity of Ustilago maydis. Day transferred dsRNA to strains lacking dsRNA, by heterokaryon transfer and measured the effect on the pathogenicity of the isolate. It was found that some cytoplasm (with dsRNA components) have a small (not significant) effect on the pathogencity, appearing to promote pathogenicity and Day concluded that there was no obvious role for dsRNA in the biological control of smut, as there is in Endothia parasitica.

1.2.7 Physicochemical Properties of Ggt Viruses

Attempts to correlate the presence of any of the three sizes of virus particles found in Ggt (27, 35 and 40nm) have failed (reviewed by Rawlinson and Buck, 1981). Frick and Lister (1978) suggested that this might be due to virus biotype variation. They examined 22 isolates of G. Graminis by testing isolated nucleic acid preparations with antisera to synthetic double-stranded polynucleotides and examined for the presence of virus by electron microscopy. Twenty isolates were shown to contain

dsRNA and nineteen of these contained virus particles. Virus preparations from three of these (No. 3, No. 59 and M - 10) were purified by caesium chloride density gradient centrifugation and virus banded in the region 1.33 - 1.39 g/ml. Electron microscopy of virus preparations indicated three modal particle diameters: 35, 39 and 41-42 nm. Antisera raised to purified virus preparation of these three isolates were titrated against both homologous and heterologous virus preparations. Antiserum titres for the heterologous reactions ranged from zero to the same as the homologous titres, indicating the existance of unrelated serological groups and serological variations within a group (confirmed by the formation of a spur in gel immunodiffusion plates). Virus particles from an isolate of Cga (No. 59) obtained from oats, were found to exhibit various degrees of relationship with virus preparations may have contained more than one type of virus (virus from isolate No. 3 contained two major capsid polypeptides and gave two precipitin lines in immunodiffusion tests) and so the serological data should be interpreted with care.

Almond (1979) purified virus from 12 isolates of Cgt by sucrose density gradient centrifugation and examined the viral dsRNAs and polypeptides by polyacrylamide gel electrophoresis. The number of dsRNA components from a single isolate varied from one to 14 and, although some components from different isolates had molecular weights in common, no two isolates contained the same distribution of dsRNA molecules. Numbers of capsid polypeptides from the same isolate varied from one to nine and similarly no two isolates contained the same polypeptide components.

Table 4. Physicochemical properties of the GGT viruses

irus	Group[a]	Particle dia.(nm)	S_{20}[b]	Virion ds RNA components (mol.wt. x 10^{-6})[c]	Capsid polypeptides (mol.wt. x 10^{-3})[c]
9/6-A	I	35	126	1.27,1.19	60
-4-A	I	35	115	1.27,1.19,1.09	55
-1-4A	I	35	109	1.22,1.14	55
/9A	I	35	117	1.30,1.22,1.14,1.11	55
A-B	I	35	125	1.30,1.22	55
1a-C	I	35	115	1.27,1.19,1.11	55
-C	I	35	128	1.27,1.19	54
/10-D	I	35	-	1.14,1.05	55
-A	II	35	133	1.49,1.47	73
-B	II	35	133	1.60,1.56,1.45	73
A-A	II	35	135	1.49,1.39	68
1a-B	II	35	140	1.60,1.54,1.45,1.43	68
/10-A	II	35	-	1.60[d]	73
/10-B	II	35	-	1.43[d]	68
/10-C	II	35	-	1.25[d]	65
1a-A	III	40	163	4.1,3.5	87,83,78
-A	III	40	159	4.3,3.2	87,83,78

om Buck et al., 1981.

Grouping by physicochemical properties corresponds to grouping by serology.

Determined in PBK buffer at very low concentration (A_{260} = 0.5 to 0.7); values are a mean of 3 determinations.

All dsRNAs and polypeptides tested for identity or non-identity of mol. wt. by co-electrophoresis. dsRNAs differing in mol. wt. by 0.02 x 10^6 and polypeptides differing by 1 x 10^3 are distinguishable.

45/10 contains other viral dsRNAs not yet assigned to each virus. Each virus probably contains two dsRNAs.

sRNAs of Group I and II viruses are probably monocistronic, and of oup III viruses oligocistronic. Calculated polypeptide coding pacities are: Group I 55 to 65,000; Group II 70 to 80,000; and oup III 160 to 220,000 Daltons, approx.).

Some of the polypeptides may however have been proteolytic products, aggregates or contaminating host proteins. Partial fractionation of the virus preparations by sucrose density gradient centrifugation and fractional precipitation methods has enabled allocation of polypeptides and dsRNA components to single viruses in some cases. A summary of the properties of individual viruses, some of which have been only partly characterised at the present time, is shown in Table 4. The viruses are grouped according to the molecular weight of the major polypeptide: other properties such as dsRNA molecular weights, particle sizes, and sedimentation coefficients, fit in with this grouping. Using mouse antisera to Ggt viruses T1-A 0196-A, 38-4-A, and OgA-A + OgA-B, related to each other and less closely related to OgA-A or OgA-B virus, no other relationships were detected. However, mouse antiserum gave low titres with homologous antisera, so more distant relationships were probably undetected.

A comparison of the isolates shows that some viruses from different isolates contain dsRNA components of common molecular weight, for example, viruses 38-4-A and 0196-A, which are serologically related, have capsid polypeptides of similar molecular weights and appear to have two dsRNA components in common. The extra component of 38-4-A may therefore be unnecessary for replication of the virus, and may be a deletion product of one of the two dsRNAs, or a satellite dsRNA. This possibility was investigated further by Romanos (1981).

Comparison of the coding capacities of the dsRNA components with the capsid polypeptide molecular weights of those viruses having a particle diameter of 35nm and those of 40 nm diameter reveals a fundamental difference between their dsRNAs: the former and probably monocistronic whereas the latter are probably at least dicistronic.

Romanos (1981) compared the genomes of four related Cgt viruses from group 1 (Table 4): 45/9-A, 38-4-A, 019/5A and 01-1-4-A by RNA fingerprinting and by hybridization analysis with tritiated transcripts, synthesised in vitro as probes. The results taken together show that 38-4 dsRNA components are unrelated to each other but the higher molecular weight components of 38-4-A, ds1 and ds2 (1.27 and 1.19 x 10^6 respectively) were closely related to ds1 and ds2 of 019/6-A virus (96% and 54% homology respectively). This suggested that ds3 of 38-4-A virus (1.09 x 10^6 daltons) is a satellite dsRNA.

Evidence to support this was obtained by in vitro translation of RNA transcripts (Romanos, 1981). Each transcript produced one major polypeptide, designated P1, P2 and P3 of molecular weights 63,000, 55,000 and 57,000 respectively. P2 co-migrated with 38-4-A capsid polypeptides in gels and the two were shown to be virtually identical by tryptic peptide analysis and partial proteolysis peptide mapping. P1, P2 and P3 appeared to be unrelated. Since genome components ds1 and ds2 of 38-4-A and 019/6-A are related and ds2 encodes the major polypeptide, ds1 probably encodes RNA polymerase, and ds3 (not present in 019/6) is probably a satellite RNA, but its function is unknown.

Analysis by RNA fingerprinting 45/9-A virus genome, which has four dsRNA components suggested that ds3 and ds4 were derived from ds1 by deletion and subsequent mutation.

1.3 Background to the Present Work

The ultimate goal towards which the present work was directed was to discover whether the presence of virus in Ggt has any effect on the biology, particularly the pathogenicity of the fungus. Previous attempts to correlate presence of virus particles with a biological effect (virulence) have failed (Rawlinson et al, 1973). However, a consideration of the cases where virus has been implicated in a biological phenomenon leads one to conclude that such an approach is over simplistic. Viruses are commonly found in healthy isolates of Penicillium chrysogenum (Lemke et al, 1973), Helminthosporium victorial (Ghabrial, 1979) and Agaricus bisporus (Last et al, 1974; Pussmore and Frost, 1974) such that any attempt to correlate presence of virus, in randomly selected isolates, with a biological property would probably lead one to conclude that virus is always latent. However virus diseases, as outlined above, have been found in each of these fungi. Similarly,killer, neutral, and sensitive strains of yeasts all contain dsRNA virus particles but differ in their dsRNA components; dsRNA is extremely common in U. maydis whereas killer strains are quite rare, and there is no simple association of dsRNA pattern with killing ability (Day, 1981). DsRNA-containing strains of Endothia parasitica have a wide range of virulence from hypovirulent to virulent: hypovirulence can not be simply correlated with dsRNA pattern or quantity of dsRNA (Van Alfen et al, 1979). In each of these fungi, the type of study carried out on Ggt may have concluded that presence of virus has no effect, particularly if, as in yeast (and possibly U. maydis) virus in some cases confer an advantage on the host. The

wide variation in serotype, dsRNA and polypeptide components of Ggt discovered by Frick and Lister and Almond clearly indicates a complex situation where it would probably be naieve to expect a simple relationship between the presence of virus particles and some property such as virulence of the fungus.

Classically, infection of an organism by isolated virus is the usual criterion for establishing the effect of virus on its host. As discussed earlier infection of fungal hyphal with extracellular virus has not been report presumably because the fungal cell prevents virus uptake. This problem was overcome with some fungi by infecting isolated protoplasts in vitro (Lhoas, 1971; Pallet, 1976) but other workers have generally found this technique unsuccessful (Bozarth, 1975). Rawlinson and Muthyalu (1974) found no difficulty in generating protoplasts from Ggt with isolates containing virus by removing cell wall with 5% 'helicase' snail gut enzyme. However virus-free ascospore cultures produced few protoplasts and none were able to regenerate mycelium on a variety of media.

Another approach to this problem is to compare cultures which have been freed of virus with those that are still infected. Growing the fungus at, or near the thermal death point reduces the virus titre (Dieeman-van Zaayen, 1972) and if this is combined with isolation of hyphal tip then virus free cultures can sometimes be obtained. This method has been used for Penicillium chrysogenum bispores (Lemke, et al, 1973; Banks et al, 1978; Hollings, 1972) and shown that virus freed cultures have increased growth rate and stability. However, mycelial cultures of apparently healthy virus free mushroom can also show increased growth rate and vigour after heat treatment (Rasmussen et al, 1972). High temperature itself can be mutagenic or can act as a selective agent for spontaneous mutations (see Burnett, 1975, p. 35); and so differences observed after heat treatment

may be due to mutation rather than absence of virus: it is even conceivable that mutations may mark a difference in biology, after freedom from virus. It is possible that repeated hyphal tipping without heat treatment may be sufficient to completely free the mycelia of virus and this method is attempted in the present study.

A related method would be to free the fungus of virus using natural mechanisms, such as the production and isolation of ascospores. In the case of virus-infected strains of Cgt (Rawlinson et al, 1973) and Helminthosporium maydis (Bozrath, 1977), all ascospore progeny appeared to be free from detectable dsRNA or virus particles. However, one intriguing possibility is that dsRNA mycoviruses could be transmitted as DNA proviruses, so that absence of dsRNA and virus particles from ascospore cultures need not necessarily mean that these cultures do not contain any viral information. This possibility is discussed by Buck, (1980).

There are several observations for which the presence of provirus and its induction would offer an attractive explanation. Adler (1979) detected no dsRNA or virus particles in several isolates of Histoplasma capsulatum grown in the mycelial phase; however, after conversion the yeast phase, dsRNA and virus particles were readily detected. In one case, dsRNA and virus particles were maintained after conversion back to the mycelial phase, but with another no dsRNA could be detected after such reconversion. Provirus induction offers an explanation for this phenomenon, but gross alterations in virus or dsRNA levels as a result of cellular differentation are also possible.

The occurrence of empty isometric capsids (27nm diameter) constructed of a single polypeptide of mol. wt. 18300 in a strain of Aspergillus flavus, in which no dsRNA or other 'viral' nuclei acid could be detected (Wood, et al, 1974; Bozarth, 1979) could also be explained by a DNA provirus if the genetic information for capsid production could reside in endogenous provirus DNA. The production of empty capsids in this fungus is a stable characteristic, transmissible through conidia.

The production of killer toxin by yeast strains lacking dsRNA (K4 to K10 strains: Young and Yalgiu, 1978) raises the possibility of a proviral form of M dsRNA. As mentioned earlier a spontaneous mutant from a K1-sensitive strain of Saccharomyces cerevisiae contained only L dsRNA and evidence from crosses indicated that the toxin may be encoded by a nuclear gene (Woods et al, 1974). These results could be explained by a proviral form of M dsRNA residing in the K4 to K10 strains and the 'Woods' strain; however it is also possible that the K4 to K10 strains contain an unrelated nuclear gene coding for a killer toxin: and that the K1 sensitive strain that the 'Woods' killer was derived from contained a similar gene in a suppressed (or even mutated) state.

A related possibility is that M dsRNA was derived from both strands of a DNA gene encoding toxin. A similar situation appears to exist for the transforming genes of the RNA tumour viruses, and will be discussed later. Vodkin (1977) reported sequence homology between

Saccharomyces cerevisiae virus L dsRNA, derived from several yeast strains and S. cerevisiae DNA. This work will be discussed in detail after a review of the properties, and methods of detection of proviral forms of RNA viruses.

The possibility that hypovirulence exists in a 'suppressed' state in virulent isolates of Endothia parasitica, that have been derived from single conidia of hypovirulent strains; and that this hypovirulence can be induced by uv irradiation (unpublished results of Van Alpen, Janes and Bowman, referred to by Day and Dodds, 1979), could be explained by a proviral hypothesis; however no information on dsRNA content of these strains is available and gross alterations in levels of dsRNA would seem a more likely explanation.

Buck (1980) suggested that there are several observations for which a proviral form of Ggt virus, would offer an attractive explanation. Rawlinson et al, (1973) found that isolates of G. graminis from first year cereals are generally free from detectable virus particles. This is consistent with the hypothesis that initial colonisation of fields previously free from G. graminis could be by virus-free ascospores (Brooks, 1965). In a study of the natural introduction of G. graminis and its viruses into cereal crops grown in a field which had carried no cereals for over 100 years previously, Rawlinson and Muthyalu (1976) found as expected, no virus particles in the first G. graminis isolates from spring crops grown in alternate years of a period of three years. However, within a further period of three months, virus-like particles, 27nm in diameter and apparently mainly empty as judged by electron microscopy, were found in 17 out of 38 isolates taken from widely separated plants

in the same field now growing a consecutive crop of winter wheat. After a further peiod of nine months, larger (35nm) particles, later shown to contain dsRNA, appeared in many isolates, many of these also appeared to be empty capsids. A possible explanation is that G. graminis carries endogenous DNA provirus which is transmitted to ascospores when dsRNA and virus is excluded, is initially not transcribed but is then induced first to produce m RNA for capsid protein, resulting in appearnce of capsids which could be empty or contain just one segment of the m RNA for capsid production. At a later stage, m RNA for viral RNA polymerase could be transcribed, possibly from a gene residing in a different portion of the host genome than the gene coding for the capsid RNA segment; the RNA polymerase would be incorporated into capsids that could now contain the positive strand of both dsRNA segments of a bisegmented dsRNA virus and a viral dsRNA could be produced resulting in independently viable virus.

In another experiment comparing the survival of virus-free and virus-infected isolates on colonised wheat straws buried in the soil, all isolates survived better in soil carrying a first year wheat crop than in soil after fourteen wheat crops, presumably because of the build up of anatagonistic microflora. However, when a fast growing highly pathogenic isolate, initially virus-free, was re-isolated from both soils three months from burial both cultures contained 27nm virus particles. Another field isolate which contained only a small number of empty capsids of the 35nm and 27nm types prior to exposure to soil, was found to contain numerous complete particles of both types, upon re-isolation from soils

(Rawlinson and Muthyalu, 1975 and unpublished data). These observations could be explained by the occurrence of a DNA proviral form of Ggt virus that is replicated independently of virus, is transmitted with the host genome to ascospores and can either be in a latent state, be induced to produce only capsid protein or be induced to produce complete virus. Alternative explanations would be the occurrence of low undetected amounts of virus in ascospore isolates that can be stimulated to produce normal amounts of virus. Such stimulations have been reported for certain insect (Smith, 1967) and plant (Smith, 1977) viruses and fungal viruses. Latent virus infections accompanied by very low concentrations of virus particles, are quite common in cultivated mushrooms (Last et al, 1967; 1974) and can often be experimentally induced by heat treatment or growth on different media (Last et al, 1974; Hollings, 1972). Barton (1978) reported that repeated sub-culture on synthetic media of virus-infected mycelium of Mycogone perniciosa led to a marked reduction of virus concentration. The concentration could be restored by growing the fungus on healthy mushrooms or on mushroom extract media. This possibility of ascospore isolates containing low undetected levels of virus is tested in the present study. The explanation does not account for the appearance of only apparently empty particles.

Another possibility is that ascospore isolates contain low levels of viral dsRNA, but no virus particles. Relevant to this may be the observation that strains of Ustilago maydis contain dsRNA but no detectable virus particles (Koltin and Day, 1976); this may however be due to instabilit

of the virus during purification. Since fungi do not (as far as is known) contain a dsRNA-dependent RNA polymerase then the viral activity must also be present, presumably as a dsRNA-polymerase complex capable of replicating dsRNA. Since no dsRNA-dependent RNA polymerase protein has been isolated from viruses it is likely that the activity depends on intact virus particles. Also, free dsRNA may be inhibitory to host translation. For these reasons free replicating dsRNA in fungi seems unlikel however the possibility of low levels of dsRNA in ascospore isolates is tested in the present study.

The presence of a DNA provirus offers the most attractive explanation to these observations and the testing of this hypothesis was the principal aim of this study. The question of whether, so-called virus-free isolates are completely free of viral information, is crucial to any study of the effect of virus on the biology of the organism.

Most structural genes in cells are present at only single-copy level (which actually means between one and several copies per cell) and can of course exert a considerable effect on the organism: similarly single or near single-copy proviral genes can produce profound effects on the host. The presence of viral dsRNA in cells at levels of 1 to 3 molecules per cell could produce similarly dramatic biological effects as the presence of the stringently controlled bacterial plasmids that determine multiple drug resistance (Falkow, 1975). Similarly low levels of virus may exert a considerable effect on their host. The principal aim of the study is to confirm the absence or otherwise of virus or viral information in ascospore isolates of Ggt. Three possibilities are investigated:

firstly that viral information is present in DNA of the host, that there is a proviral form of the virus, second that ascospore isolates contain low, previously undetected, levels of viral dsRNA, and third that ascospore isolates contain low, previously undetected, levels of virus particles.

The answers to these questions are crucial to any further study that intended to determine whether the presence of virus has any effect on the biology of Ggt. The intention of this work was therefore to produce homokaryotic strains of Ggt containing virus, to then produce ascospore progeny from these and confirm or otherwise the absence of virus. If the absence of virus was confirmed then these (isogenic) strains could be used in subsequent studies to investigate the involvement of virus in the pathogenicity of the fungus. A method of producing homokaryotic virus containing strains of Ggt became available when Deacon (1977) described a method of producing germinable, uninucleate, virus containing conidia, and the first stage of this work involved production of these spores.

1.3.1 Occurrence and Detection of Proviral Genomes

The occurrence of a prophage form of temperate DNA phages such as phage is well established. Phage λ does not form plagues on lysogenic strains of Escherichia coli but in such a strain, DNA is integrated into the bacterial chromosome and is inherited as prophage to all daughter bacteria, none of the viral genes needed for productive

infection is transcribed so that no virus particles are produced and very little viral m RNA is detectable. Most of the m RNA that is formed codes for a repressor protein which prevents transcription of the rest of the viral genome. Inactivation of repressor induces phage production and plague formation. This can be achieved by heat treatment, uv radiation or with various chemical agents such as 5 iododeoxyuridine, or by mating with an irradiated F^+ bacteria. Because repressor is present, lysogenic bacteria are specifically immune to superinfection by phage of the carried type; when repressor is inactivated, this immunity is lifted. Lysogeny is ver common in bacteria but because of its latent nature went undetected in even the intensely studied K12 strain of Escherichia coli until 1951. Some isolate of almost all bacterial species have proven to be lysogenic for one or more phages. Many other phage types establish stable lysogenic complexes by insertion of prophage into bacterial DNA. Some of these can alter the biological properties of their host, such as the antigenic properties, when present as prophage. The ability of Corynebacterium diphtheriae to produce the diphtheria toxin has been shown to be coded by the phage genome (Murphy et al, 1974).

Animal cells transformed by certain DNA tumour viruses, such as SV 40 or polyoma virus, carry a provirus comprising a complete viral genome. Like bacteriophages there is no evidence that integration of the provirus plays an obligate role in the life cycle although integrated DNA has been found in cells productively infected by DNA viruses. Cells that are transformed by DNA virus produce either low amounts of virus or no detectable virus but produce virus-specific RNA and viral antigens can be detected.

Usually virus will transform cells in which virus reproduction is not possible, these are termed nonpermissive cells. Permissive cells in which virus reproduction is possible are usually killed. An exception to this is Epstein-Barr virus which establishes an apparently productive infection whilst simultaneously transforming the cells.

The most persuasive evidence that viral DNA is linearly integrated into cellular DNA was first obtained by Bothan et al, (1976) who isolated DNA from SV40 transformed cells, cleaved it with restriction endonucleases, separated the fragments on agarose gels, transferred directly to nitrocellulose filters and hybridised with SV40 DNA (Southern' blotting described in detail below). By this method Bothan was able to prove the presence of integrated SV40 DNA and show that in different cell lines the DNA was integrated at a different site in the genome.

Retroviruses are viruses which contain RNA but their intracellular state is an integrated DNA, which then behaves as a cellular gene that can be passed from parent to progeny cells. By using the nondestructive budding process common to many RNA viruses, retroviruses can maintain a positive infection without killing cells. The genome of retroviruses consists of two identical molecules of 35s RNA linked noncovalently to produce a 70s molecule. The RNA codes for between five and eight virion polypeptides and a DNA polymerase, termed reverse transcriptase, which synthesises a DNA strand on an RNA template. The RNA can also code for a transforming protein (src in Rous sarcoma virus).

Infection consists of synthesis of a linear DNA molecule on a template of 70s RNA; this is a precursor to a circular DNA which can be isolated separately and is infectious. This circular DNA seems to be the precursor to the integrated complete viral DNA. Integration of viral DNA is essential to the life cycle of retroviruses, there is no evidence of translation of viral RNA prior to integration. Indeed the original evidence for a DNA intermediate in the life cycle was the sensitivity of retroviruses to inhibitors of DNA-dependent reactions, such as actinomycin D. Direct evidence for the existence of a provirus came with the isolation from infected cells of high molecular weight DNA, that can transmit the infection to other cells (Hill et al, 1974). Unintegrated infectious proviral DNA can also be isolated early in infection (Smotkin et al, 1975).

Indirect evidence for provirus came from nucleic acid hybridization studies (to be described in more detail later) where labelled virion RNA was used to detect complementary DNA. Also, using the in vitro reverse transcriptase reaction to label a single-stranded DNA complementary to 70s RNA, the positive proviral DNA strand was detected in host high molecular weight DNA.

The integrated viral DNA occurs at a level of one to five copies per cell. Once integrated the viral DNA is transcribed and message is translated to produce viral proteins. Newly synthesised proteins along with 35s RNA, molecules organise themselves at the surface of the cell, and bud from the plasma membrane to form the infectious virions.

One of the most surprising aspects of retrovirus biology is the demonstration that in many species of animals, proviral DNAs are inherited, presumably reflecting a germ line infection of an ancestral animal (Todavo et al, 1976; Baltimore, 1974; 1976). Nucleic acid hybridization studies have shown that in most species of animals, copies of retrovirus genomes are inherited by every animal of the species. It is likely that many of the inherited viral genomes are defective and cannot code for the synthesis of a complete virus particle, since mutations could accumulate that would inactivate their ability to produce infectious particles. In most cases inherited viral genomes have been shown to be transcriptionally silent. However, treatment of AKR mouse embryo cells with bromodeoxy uridine can induce the synthesis of virus from an inherited provirus.

The transforming properties of Rous sarcoma virus has been shown to be due to the presence of a protein which is the product of the src gene. Deletion of the src gene does not alter the ability of the virus to integrate and replicate, indeed some strains of Rous sarcoma virus lack the src gene but can replicate efficiently. By isolating a portion of the src gene and using this to probe cellular DNA it was shown that src-related DNA sequences occur in the DNA of most vertebrate species even though the src sequences are restricted to the avian sarcoma viruses (Stehelin et al, 1976). It thus seems that the src sequence may be derived from a cellular sequence, perhaps by inexact excision of the provirus.

For detection of proviral DNA of RNA viruses, the most widely used method has been the technique described by Gillespie and Spiegelman (1965). This involved binding denatured host DNA to nitrocellulose filters, annealing with a radiolabelled RNA probe, removing unhybridized probe and determining the amount of hybridized probe by scintillation counting. Detection of very low levels of hybridized radiolabelled probe can be achieved by autoradiography (capable of detecting less than 50 disintegrations per minute if the RNA is labelled with (^{32}p). The technique has been used to detect host DNA complementary to viral RNA for many retroviruses including avian mycloblastosis virus (Baluda Nayak, 1970) avian sarcoma Virus (Stehelir et al, 1976) and others. The sensitivity of technique depends on the specific activity of the probe and the size of the host genome, but can be used to detect less than one viral equivalent per mammaliar cell.

RNA-DNA hybridization can be performed in liquid without immobilisation of DNA on a solid support. The technique, introduced by Nygaard and Hale (1974) depends on removal of unhybridized strands by nuclease treatment or use of columns. The technique suffers from self-annealing of the DNA but this can be minimised by use of high formamide concentrations which favour the formation of RNA-DNA hybrids (Barke and Sharp, 1977). The sensitivity of the technique is again dependent on the specific activity of the probe but can be used to detect below one viral copy per cell. The technique is more amenable to quantitisation than the use of bound DNA and has been widely used to determine values such as the percentage of DNA represented in stable RNA

or transcribed into message (see Kennel, 1971). The method is however tedious and requires careful interpretation.

DNA-DNA hybridization in solution is a much used technique that has been widely used to analyse proviral DNAs of DNA viruses. The most sensitive variation of the technique is to measure the rate of reassociation of viral DNA in the presence of a large excess of an unrelated DNA, and then with a similar excess of the host DNA under question. If the host DNA contains viral sequences then the total concentration of viral sequences will be increased, and therefore the rate of reassociation will increase. If the viral DNA is radiolabelled then the rate of reassociation can be measured by binding of either double or single-stranded DNA to columns or filters, or more commonly by digestion of unhybridized single-stranded DNA by S1-nuclease (Britten & Kohne, 1971; Ando, 1966). The technique is capable of very high sensitivity.

Since the presence of proviral forms of DNA tumour viruses and retroviruses was proven, another technique has become available which is capable of high sensitivity, is simply performed, and can give valuable information on the structure of integrated provirus. The technique described by Southern (1974) involves digestion of DNA with a restriction endonuclease that produces a discrete (though for large genomes - very high) number of specific DNA fragments. The fragments can be separated by electrophoresis on agarose gels and transferred directly to a nitro-

cellulose filter strip after denaturation of the DNA. The filter strip is then probed with radiolabelled DNA or RNA and unhybridized probe removed by washing. If the probe is labelled with (^{32}P) or (^{125}I) then the filter strip can be autoradiographed to reveal discrete bands of DNA complementary to the probe. By the use of different restriction enzymes information on the size and distribution of the complementary DNA can be obtained. The technique gives a clear visual result and possible sources of error implicit in other techniques can be easily elimited. Contamination of host DNA with cellular RNA will not give bands since RNA will not bind to filters under these conditions. The possibility of the viral probe being contaminated with cellular ribosomal RNA can be eliminated by using a control of cellular ribosomal RNA so that the ribosomal DNA bands can be located. Also, whether the DNA copy is integrated into cellular DNA or present as unintegrated DNA can be determined since in the former case hybridizing bands should, with some restriction digests, be of higher molecular weight than the viral DNA alone. The technique was used successfully to detect integrated SV40 DNA (Botcham, 1976) and show the DNA to be integrated at a different position in the host genome in different cell lines. The sensitivity of the technique was determined and discovered to be capable of detecting 10^{-7} μg of SV40 DNA, using probe labelled to specific activity of $5 \times 10^7 - 1 \times 10^8$ cpm per

Although the technique does not lend itself to quantisation as does solution hybridisation, it is possible to estimate values, such as amount of homologous DNA by applying known quantities of homologus DNA

to the gel and comparing the intensity of hybridizing bands. Although a DNA copy of Ggt viral DNA was not available a related technique, whereby RNA (and DNA), including dsRNA, can be bound to nitrocellulose and is available for hybridization, is described in the present study, and is used to determine the sensitivity of the filter hybridization technique.

The determination of the sensitivity of the hybridization technique, to detect proviral DNA, in terms of number of copies of viral genomes detectable per host cell genome, depends on knowledge of the size of the host cell genome. Although estimates of DNA content per cell can be made calormetrically (Bainbridge, 1971) these values are very approximate; a more accurate and informative method of determining this value is by DNA: DNA reassociation kinetics in solution as described by Britten et al, (1968, 1970). The method has been used to determine the genome size (or more correctly sequence complexity) and repetition frequency and pattern for organisms including fungi such as Aspergillus nidulans (Timberlake, 1978); Neurospora crassa (Dutta, 1973); Saccharomyces cerevisiae (Hereford & Rosbash, 1977) and Dictoyostelium discoidium (Firtel et al, 1975).

In the present study, the method of assay chosen is determination of labelled double stranded DNA remaining by digestion with the single-strand specific nuclease S1 and precipating the nuclease-resistant duplexes with acid (Goldberg et al, 1978).

1.3.2 The Detection of Homology between dsRNA and Nuclear DNA of Yeast

The report by Vodkin (1976) of homology between dsRNA and nuclear DNA of yeast was an inspiration to the aims and objectives of the present study. However, shortly after the inception of this work Hastie et al, (1978) reported their failure to detect homology between dsRNA and nuclear DNA from yeast. Both these studies illustrate many of the principles and pitfalls of nucleic acid hybridization techniques and will be discussed more fully.

Vodkin (1976) prepared DNA from yeast cells lacking mitochondrial DNA (petite, rho mutants), by the basic protocol of Marmur (1961). The DNA was further purified by digestion with ribonuclease 111 (dsRNA specific) as well as pancreatic ribonuclease and was banded in caesium chloride gradients; some preparations were additionally purified on CF11 cellulose columns (Robertson et al, 1968). Total RNA was in vivo labelled by growing cells in the presence of (^{32}p) inorganic phosphate. RNA was extracted; ssRNA was precipitated with lithium chloride; and the dsRNA was further purified by chromatography on CF11 cellulose.

The purity of DNA and RNA prepared is crucial to these experiments, particularly important is the demonstration that the DNA and dsRNA are not cross contaminated. Vodkin confirmed the purity of DNA by adding to the DNA preparation, amounts of (^{32}p) labelled dsRNA and ensuring it was removed by the last stages in the purification. In addition, DNA was purified from a strain lacking dsRNA. Purity of the dsRNA was checked by nuclease tests and by gel electrophoresis, in particular, it was shown that the amount of radioactivity in the bands corresponding to the

dsRNA components accounts for between 95 and 99% of the total radioactivity in the sample. The residual activity barely entered the gel and may have been due to aggregation of dsRNA.

The evidence for homology between dsRNA and DNA came from the results of experiments in which it was shown that the rate of conversion of denatured (^{32}p) labelled dsRNA, to ribonuclease resistant form (double-stranded) was increased by addition of denatured yeast DNA, including DNA from strains lacking dsRNA. This implied that the dsRNA was forming DNA-RNA hybrids, that is, that the DNA contains sequences homologous to dsRNA. Evidence for the existence of DNA-RNA hybrids in the hybridization mix was obtained from the appearance of a band, containing radioactivity, lower than that of dsRNA, on applying the hybridization mix to isopycmic caesium sulphate gradients. The results implied several copies of dsRNA per yeast genome.

However, Hastie et al (1978) have been unable to duplicate these experiments and found no effect of denatured yeast DNA (prepared from one of the strains used by Vodkin) on the Kinetics of reassociation of yeast dsRNA. They used (^{32}p) labelled dsRNA purified from agarose gels and checked the purity of the dsRNA. The amount of RNA contamination was estimated from the amounts of 2-O- methyl-dinucleoside diphosphates present in the total RNase T2 digest, and was found to be less than 0.1%; and by showing the T1 fingerprint of L revealed the

presence of no major contaminating species. By measuring the rate of renaturation of single-copy yeast DNA, and finding the reannealing of denatured dsRNA and with a large excess of denatured yeast DNA proceeded at a rate six times slower than that of yeast single-copy DNA, it was concluded that there is, at most, one copy of the L sequences per six yeast genomes. In addition, these investigations found that the rate of hybridization of a ssRNA copy of yeast L dsRNA (prepared by in vitro transcription with the virion-associated RNA polymerase) to denatured L dsRNA was unaffected by addition of excess denatured yeast DNA. On the basis of the results obtained it was concluded that there is at most one copy of L dsRNA per 13 yeast genome equivalents. Using similar methods, Wickner and Leibowitz (1977) and Hooper (unpublished results referred to by Hastie et al, 1978) have also failed to detect sequence homology between yeast DNA and L dsRNA.

These results from independent laboratories are difficult to reconcile. The same strain was used for DNA purification and both workers claim to have minimised the amount of mitochondrial DNA present so the differences could not be due to isolation of different DNA fractions. Vodkin used dsRNA purified from both non-killer and killer strains, the latter presumably containing M dsRNA in addition to L dsRNA and obtained similar results using both dsRNAs. Hastie et al, used purified L dsRNA for their experiments. As, in Vodkin's hands L plus M dsRNA, produced identical results to the use of L dsRNA alone, the answer ca not lie in differences here.

The possibility of contamination of dsRNA by DNA and visa versa, in the Vodkin experiments was ruled out by nuclease tests and by monitoring the fate of labelled dsRNA added to the DNA preparation. Buck (1980) suggests that Vodkin's results may be due to steric hindrance of the ribonuclease by networks formed by the addition of host DNA. This effect would also be apparent in the controls where calf thymus DNA was added, unless the yeast DNA contained a very much higher proportion of long DNA molecules than the control DNA. Vodkin gives no information on the size distribution of any of the DNAs, or whether they were sheared before use. If the DNA, were not sheared then since commercially prepared calf thymus DNA is usually of very high molecular weight this effect would be unlikely. In any case, the size and distribution of nucleic acid molecules is crucial in solution hybridization experiments and any experiment in which these parameters are not controlled is suspect. Another possibility is that Vodkin's yeast DNA preparations were contaminated with polysaccharide. Vodkin gives no information on the melting properties of his yeast DNA, particularly the hyperchromicity; if the yeast DNA were contaminated with polysacccharide, it would be detectable in the melting behaviour of the DNA. Contamination of DNA preparations by polysaccharide is common, particularly DNA preparations from fungi and it is quite possible that polysaccharide in yeast DNA would form networks on cooling that would sterically hinder ribonuclease action, producing the observed increase in ribonuclease resistance on addition of DNA. Alternatively, the yeast DNA could have been contaminated with metal ions which are known to be capable of producing marked effects on hybridization rates.

In the Hastie et al (1978) experiments, the size of yeast DNA is controlled. Apart from this, the only major difference between their work and Vodkin's experiments is the use of dsRNA purified from agarose gels by Hastie et al, and the confirmation that the dsRNA was a single species. It is possible that Vodkin's dsRNA preparations were contaminated with ribosomal RNA and that what Vodkin was measuring was the formation of hybrids between r RNA and DNA coding for ribosomal RNA. It is known that preparations of dsRNA can contain r RNA that behaves as double-stranded and elutes as double-stranded RNA from cellulose CF11 columns. However, Vodkin claimed all the radioactivity of his dsRNA preparation migrated as dsRNA in polyacrylamide gels, apart from a small fraction that did not enter the gel.

Previous to Vodkin's work, Shalitin and Fischer (1975) claimed to have obtained evidence of homology between yeast nuclear DNA and viral dsRNA by isolating poly-A containing m RNA from yeast cells and demonstrating homology between this and nuclear DNA, after showing that viral dsRNA contains poly-A sequences. These conclusions must be considered with extreme sceptism since the workers nowhere show that yeast cellular RNA does not contain poly-A sequences. These criticisms are summarised by Bevan and Mitchell (1979).

The failings and ambiguities of some of the above experiments would have been avoided if similar experiments to those intended in the present work, had been performed. If viral probe is contaminated with ribosomal RNA sequences, then the DNA bands that hybridize will be those that code for r RNA. These bands can be located by use of

a r RNA probe. If any unspecific effect was causing adherance of the probe to host DNA then no bands would be seen. If homology existed between 'control' regions of viral RNA and host DNA (such as sites responsible for binding of message to ribosomes) then hybridization would probably not occur with any specific DNA bands. It was for these reasons and the ability to extract more information from 'Southern blots' than solution experiments, that the 'Southern blot' procedure was chosen to detect provirus in the present study.

1.4 Summary of the Aims of the Present Work

In order to study the effect of virus infection on any aspect of the biology of Ggt, e.g. inhibitor production, the ability to cross-protect; hypovirulence, it is necessary to produce isogenic strains with and without virus. Direct infection of a virus-free strain by cell-free virus would be the best way of achieving this; however many workers have found this an intractable problem with fungi. Therefore experiments to free Ggt isolates from virus were investigated. Field isolates of Ggt may be mixtures or may be heterokaryotic, therefore germinable conidia were prepared from an isolate of Ggt and were shown to be uninucleate. Using isolates derived from single conidia two methods of freeing the mycelium from virus were attempted, (i) hyphal tipping, and (ii) deriving ascospore cultures. Since Ggt is a homothallic ascomycete (although preferentially out-breeding), this is possible with homokaryotic isolates.

Previous methods used for detecting virus in Ggt were not sensitive enough to be certain that isolates were completely free from virus; therefore more sensitive methods of detecting virus and viral dsRNA, e.g. ELISA, immune electron microscopy, dsRNA hybridization blotting, were investigated. However, in cultures shown to be free of dsRNA and virus particles it was necessary to also establish the absence of a proviral form of the virus.

Finally, using isogenic strains of Ggt with and without virus, the effect of virus infection on inhibition production was tested.

1.4.1 Choice of Isolates

Isolates selected for study were (a) F6, a hypovirulent isolate capable of protecting wheat from infection with a virulent isolate. (Rawlinson & Buck 1981). It was known from previous work (Buck, et al 1981) that this isolate contained virus particles and dsRNA, but individual viruses had not been separated or characterised. It was not known whether the isolate was capable of producing conidia or ascospores. (b) 3b1a, an isolate of low virulence known to produce conidia and ascospores, and containing virus particles and dsRNA (Buck et al, 1981). The viruses in 3b1a, (A, B and C) were separated and characterised from this isolate by K.W. Buck (Buck et al, 1981) using methods established for F6 viruses in this thesis. (c) 45/10, an isolate of medium virulence known to produce perithecia and conidia, containing virus particles and dsRNA. Single conidial isolates derived from 3b1a and 45/10 by Deacon were available for this work.

These isolates of low to medium virulence were chosen since if by analogy with Endothia parasitica, virus infection can cause a a reduction in virulence of Ggt then virus-containing isolates of low virulence would be good candidates for a further study on the effect of virus on the virulence of the fungus. The isolates had 7 to 10 dsRNA components and it was felt that such isolates would be more likely to show a phenotype caused by dsRNA, than those with fewer components.

2. METHODS

2.1 Reagents

All reagents used, unless otherwise stated were analytical grade. Phenol, for use in phenol extractions was redistilled and stored at -20°C. It was saturated with TNE before use. All water used was glass distilled.

2.2 Buffers commonly used

MOPS	0.02M-sodium morphopholimopropane sulphonate (Sigma), 5mM-Na-acetate, 1mM-EDTA pH 7.4.
PB	0.03M - NaH_2PO_4, pH 7.6
PBK-1	0.03M - NaH_2PO_4, 0.15M-KCl, pH 7.6
PBK-2	0.03M - NaH_2PO_4, 0.3M-KCl, pH 7.6
PBS	0.03M - NaH_2PO_4, 0.15M-NaCl, pH 7.6
PIPES	0.01M - piperazine - NN bis (2-ethane suphonic acid), pH 7.7
SSC	0.015M - citric acid, 0.15M-NaCL, Ph 7.0
SET	0.05M-tris-HCl, 0.1M-NaCl, 1mM-EDTA, pH 6.9
STE	0.005M-tris-HCl, 0.1M-NaCl, 1mM-EDTA, pH 6.85
TAE	0.04M-tris-acetate, 0.02M-sodium acetate, 1mM-EDTA, pH 8.0
TBE-1	0.09M-tris base, 0.09M-boric acid, 2.5mM-EDTA, Ph 8.2
TBE-2	0.03M-tris base, 0.02M-boric acid, 0.1mM-EDTA, pH 8.3
TNE	0.05M-tris-HCl, 0.015M-NaCl, 1mM-EDTA, pH 7.9
TSE	0.1M-tris-HCl, 0.1M-NaCl, 10mM-EDTA, pH 7.0

Others are listed as they arise. The concentration of these buffers is indicated in the following way: 0.1 x TNE buffer is TNE diluted ten-fold in water; 10 x TNE is ten times the concentration of TNE.

2.3 Miscellaneous

(1) Nuclease precautions

To minimise nuclease contamination, all glassware was oven-baked overnight at 180°C, before use. Plastic containers, such as eppendorf reaction tubes, and plastic pipette tips, were autoclaved for 20 minutes at 121°C, 15 psi. Containers, or apparatus that could not be autoclaved, were soaked overnight in 1% SDS, then rinsed thoroughly in water. Water and all stable buffer solutions, for use in RNA or DNA work, were autoclaved (20 minutes at 121°C, 15 psi) and stored at 4°C.

Disposable vinyl gloves were used for work with RNA or DNA.

(11) Dialysis tubing

Visking dialysis tubing was treated before use, by boiling for 1 hour in 10m M-EDTA, pH T.4, then again in fresh EDTA, rinsing several times in water, and finally, boiling in water. The tubing was stored at 4°C.

(111) Bentonite

Bentonite was used as a ribonuclease inhibitor, particularly where long incubations were necessary, as in virion polymerase assays. (Dunn-Mitchbour, 1965).

Crude bentonite powder (technical grade, BDH) was treated in the following way: Bentonite (2g) was suspended by stirring for several hours in 40 ml of water to allow full hydration. The suspension was centrifuged at 2500 rev/min for 15 minutes and the pellet was discarded. The supernatant was centrifuged at 8500 rev/min for 20 minutes and the sediment was resuspended in 40 ml of 0.1 M-EDTA, pH 7.0. The suspension was centrifuged twice, as before, and the sediment was taken up in the TNE buffer to a concentration of 1.5 to 6% W/V. The bentonite suspension was autoclaved and stored at $4^{o}C$.

(iv) Scintillation counting

The cocktail used for all scintillation counting contained 30g, 2- (4 -t Butylphenyl) -5- (4"-biphenyl) - 1,3,4-oxadiazole (Butyl PBD, scintillation grade), 250g naphthalene (scintillation grade) in 5 litres toluene (analar grade). Samples were counted under 5ml scintillation vials. All results are expressed as counts per minute (it/min) after deduction of background.

(v) Spectrophotometry

Spectrophotometric measurements were made on a Carey Model 19 spectrophotometer, except when polyacrylamide gels were scanned, when a Gilford 240 spectrophotometer was used. The absorbances of nucleic acid and virus preparations were measured at 260nm in silica cells of 1 cm path length. In buffers near pH 7.0, it was assumed that a 1 mg ml solution of double-stranded RNA or DNA had an A of 20; and a 1mg/ml solution of ssRNA had an A of 2.5. These are approximations, the true

value of the extinction coefficient depending on the pH and ionic strength of the medium.

Assuming that all the A_{260} of a virus solution is due to dsRNA and that the mass of a Ggt virion is about five times that of its dsRNA, then an A_{260} of .1.0 corresponds to a virus concentration of 250 g/ml (Almond, 1979). This value was used as a guide.

For more exacting work, the concentration (moles per litre) was calculated from the Lambert-Beer law: $A = \varepsilon cl$, where A is the absorbance, ε is the extinction coefficient, c is the concentration in moles per litre, and l is the path length in centimetres.

(vi) Proteinase K preparation

Stock proteinase k solution was prepared by dissolving proteinase K to 10mg/ml in water. Contaminating nuclease activity was inactivated by incubating the enzyme solution at 37°C for 30 minutes prior to use.

Vii) Deoxyribonuclease preparation

Stock deoxyribonuclease type I (Sigma) solution was prepared by dissolving deoxyribonuclease type I to 1mg/ml in 0.1M-NaCl and stored in 50 µl aliquots at -20°C. Stored in this way it was stable for several months.

(viii) Ribonuclease preparation

Stock pancreatic ribonuclease A (Sigma) solution was prepared by dissolving pancreatic ribonuclease A in 0.15M-NaCl, pH 5.0 to 1mg/ml. In order to eliminate any contaminating deoxyribonuclease activity the preparation was heated to 80°C for 10 minutes and stored in 50 µl aliquots at -20°C.

(ix) Handling of high molecular weight DNA

In order to minimise mechanical shearing of high molecular weight DNA, DNA preparations were never drawn through fine pipettes or syringe needles. Vortex mixers were never used and DNA solutions and precipitates were subjected to a minimum of mechanical agitation.

2.4 Names and Addresses of Firms

Chemicals and equipment were supplied from the following firms:

(a) BDH Chemicals Ltd., Poole, Dorset, BH12 4NN, UK

(b) Bochringer Corp. London, Bell Lane, Lewes, E. Sussex, TN7 2LG, UK

(c) Difco Labs., P.O. Box 14B, Central Ave. W. Mosely, Surrey, UK

(d) Gilford Instruments, 46 Church Road, Teddington, Middlesex, UK

(e) Miles Laboratories Ltd. (UK), P.O. Box 37, Stoke Poges, Slough, SL2 4LY

(f) Millipore (UK) Ltd., Abbey Road, London, NW10 75P, UK

(g) M.S.E. Ltd. Manor Royal, Crawley, Sussex

(h) Oxoid Ltd., Wade Road, Basingstoke, Hants. RG24 0PW

(i) P-L Biochemicals (Windsor Laboratories), Hanover Way, Vale Road Windsor, Berks. SL4 4NJ, UK

(j) Polaron Equipment Ltd., 60 Greenhill Crescent, Holywel Estate, Watford, Herts. UK

(k) The Radiochemical Centre (RCC), Amersham, Bucks, HP7 GLL, UK

(l) Serva: distributed by Uniscience Ltd., 8 Jesus Lane, Cambridge

(m) Sigma (London) Chemical Co. Ltd., Fancy Road, Poole, Dorset, BH17 7NH

(n) Schielman & Schul distributed by Anderman Co. Ltd., Central Av. East Mosely, Surrey

(o) Sterelin Ltd., Teddington, Middlesex, TW11 8QZ

(p) Sorval: Du Pont UK Ltd., Wedgewood Way, Stevenage, Herts.

(q) Whatman Lab. Sales Ltd., Springfield Mill, Maidstone, Kent, ME14 2LE

(r) Carl Zeiss (Oberkochen) Ltd., P.O. Box 4YZ, 31 Foley Street, LONDON.

2.5 Fungal Isolate Source and Code Numbers

(i) Ggt Isolates

M. Chu Chou, 1970, Little Knott, Rothamsted, England: 1-9 (first year wheat), 3b1-a (3rd year wheat), 5-9, 5b3 (5th year wheat), 1061 (10th year wheat).

J.M. Lemaire, 1969, Rennes, France: F911-1 (Single ascospore isolate from 12th year wheat).

C.J. Rawlinson, 1972, Highfield, Rothamsted: 019/6 (barley after wheat).

C.J. Rawlinson, 1972, Little Knott, Rothamsted: 64/4, 64/14 (third year wheat), 45/8, 45/9, 45/10, 45/13 (11th year wheat).

C.J. Rawlinson, 1974, Lerheu, nr. Rennes, France: F3, F6 (2nd year wheat).

D.B. Slope, 1973, Hoosefield, Rothamsted: 14-4, 38-4, 40-4, 49-1 (continuous barley)

S. Yamashita, 1950, Gunma, Japan: 01-1-4 (wheat)

Single conidial isolates from 3b1-a: 3b1a con C, con D and con E and 45/10 con B were supplied by C.J. Rawlinson, prepared by the method of Deacon (1976).

Ascospore isolates from 3b1a: A49, A51, A52, A56 and A59 were supplied by Hornby (Rothamsted Experimental Station).

(ii) Other Isolates

D.B. Slope, Rothamsted, England, Phialophora sp. with lobed hyphopodia (isolate 2-2) and Phialophora graminicola (isolate 1348-2)

P.T.W. Wong, Rydalmere, Australia: Gaeumannomyces graminis var graminis isolate G1.

2.6 Culture of Isolates

Stock cultures were maintained in loosely capped McCartney bottles on potato destrose agar (PDA, Oxoid) stored at 4°C.

Cultures on PDA Petri dishes were used as sources of inoculum for tests on solid media. Ggt isolates were grown in darkness at 24°C.

2.7 Buffered Growth Media

Citrate-phosphate buffer (Gomori, 1955) was used to buffer solid growth media from pH 3.0 to 8.0. The buffer was prepared by titration of 0.04M-disodium hydrogen phosphate with 0.1M-citric acid to the required pH. Buffer and media were made up at 2 x concentration, autoclaved separately and mixed.

2.8 Media Commonly Used

Lilley and Barnet: D. glucose 20g, L asparagine 2g, KH_2PO_4 1g, $MgSO_4$, $7H_2O$ 0.5g, $FeCl_3$, $6H_2O$ 1 mg, $ZnSO_4$, $7H_2O$ 0.9mg, $MnSO_4$, $4H_2O$ 0.4mg, thiamine hydrochloride 100µg, biotin 10µg, agar 15g, water to 1 litre.

Haskins MB-50: sucrose 50g, urea 0.6g, yeast extract (Oxoid) 0.6g, KH_2PO_4 1 g, $MgSO_4$, $7H_2O$ 0.4g, $FeSO_4$ o.03g, agar (Difco) 20g, water to 1 litre.

Malt extract (ME): malt extract (Oxoid) 20g in 1 litre of water.

Malt extract broth (MEB): malt extract 17g, yeast extract 1.5g, bacteriological peptone (Oxoid) 1.5g in 1 litre of water.

Media was sterilised by autoclaving (15 psi 20 min).

2.9 Restriction Endonuclease Digestions

Purified DNA was digested by the following restriction endonucleases:

(i) Eco R1 (from Escherichia coli RY13) 100 units/μl (Worthington)

(ii) Hind III (from Haemophilus influenzae) 10 units/μl (Worthington)

(iii) Bam HI (from Bacillus amylotiguefaciens) 6 units/μl (Worthington)

Digestions were carried out in the following buffers:

(i) EcoR1 buffer, 100 mM-tris-HCl, 50mM-NaCl, 5mM-$MgCl_2$, 100 g/ml bovine serum albumium.
The enzyme was diluted from stock solution in .EcoR1 dilution buffer: 10mM-K H_2PO_4, 7mM-mercaptoethanol, 1mM-EDTA, 0.2m-NaCl, 0.1% Nopidet P-40, 0.1% gelatin (autoclaved), pH 7.0.

(ii) Hind III buffer, 6mM-tris-HCl, 50mM-NaCl, 6mM-$MgCl_2$, 100 g/ml bovine serum albumi n, pH 7.4.

(iii) Bam I buffer, 0.1M-tpis- HCl, 0.1M-$MgCl_2$, pH7.5

Digestions were carried out in sterile buffer at 37^{o}C for usually 1 hour. Reaction was terminated by addition of 0.1 volume of 10%-SDS and the mixture heated to 65^{o}C for 10 minutes.

2.10 Inhibition Testing of Ggt Isolates

(i) Standard test

Interactions between pairs of isolates of Ggt or Phialophora isolates on solid media were examined by placing 6mm diameter discs of each isolate 2 to 2.4cm apart in the centre of a Petri dish. The plates were incubated at 24°C in darkness and examined regularly.

(ii) Mycelial lawns

Mycelial lawns were used for inhibition tests. The isolate to be used was grown in liquid culture, the medium was drained and the mycelium was rinsed with sterile water. The fungal mass was homoganised in 5 x its weight of sterile water for 3 minutes in a sterile Sorval Omnimixer, and the homogenate was passed through one sheet of muslin cloth to remove large mycelial fragments. The filtrate was then applied to Petri dishes containing solid medium spreading 0.5- 1ml evenly across each plate and allowing the plate to dry.

2.11 Growth Measurements

The linear growth rate of isolates on PDA was measured in Petri dishes containing exactly 25 ml of agar medium. The diameter of the colony was measured across two radii at right angles to each other. The periphery of the colony was taken as where individual hyphal strands were no longer visible to the eye. Plates were kept at 20°C in darkness and measurements were taken, using the same radii, daily.

2.12 Production of Germinable Conidia and Isolation of Single Conidia

The medium used contained, 10µg thiamine, 100µg biotin, 0.4mg $MnSO_4$, $4H_2O$, 0.9mg-$2nSO_4$, $8H_2O$, 1.0mg$FeCl_3$, $6H_2O$, 0.5g $MgSO_4$, $7H_2O$, 1g KH_2PO_4, 2g-l-asparagine, 20g-glucose and 15g-agar (Difco) in 1 litre. After disolving by boiling, 3 ml aliquots were poured into 250ml conical flasks. The flasks were then autoclaved and the agar allowed to set. In later experiments the pH of the medium was adjusted (to pH6.0, 6.5 or 7.0) using 1M-NaOH, prior to autoclaving.

Flasks were inoculated centrally with a 6mm diameter plug of mycelium taken from a colony growing on PDA, and then placed beneath a sunlit window and the fungus was allowed to grow for 7 to 14 days, or until the mycelium had grown across two thirds of the agar surface. The mycelium was then flooded by addition to the flask of 70ml of sterile water. The flasks were left as before but were examined daily for the presence of spores (this was performed by shaking the flask to dislodge spores and extracting a sample of liquid). All the subsequent manipulations were performed in a sterile cabinet with sterile equipment.

When the mycelium was producing abundant spores the flask was shaken vigorously and the spores harvested by filtering the contents through four layers of muslin gauze This retained most mycelial fragments (but allowed conidia to pass through giving a suspension of

conidia plus a small amount of mycelium. Single conidia were isolated by streaking the suspension onto the surface of 1% PDA in a Petri dish, and allowing the suspension to dry on the agar plate in sterile air. The agar surface was then examined using the X40 objective of a microscope fitted with a Meopta Monosporic Isolator. (type no. 5960, obtained from D.R. Grey Ltd. Norwich, UK). This instrument consisted of a X10 objective to the end of which was attached a steel cylinder tapering to a fine tube of internal diameter 1mm. When a single conidium was located under X40 objective, the monosporic isolator was rotated to beneath the microscope and the spore again located with the X10 objective focussed through the cylinder. The dish was adjusted to place the spore centrally beneath the cylinder and the cylinder carefully lowered to cut a 1mm cylinder into the agar around the spore. The isolator was withdrawn and the X40 objective placed into position. The 1mm plug was examined to ensure it contained only one conidium and no mycelial fragments. The agar plug was then carefully removed, using a fine needle onto the surface of 1% PDA in a Petri dish and incubated at 24^{o} until germinated. Colonies were usually seen after 3-5 days.

Later in this work it was discovered that germinable conidia could be produced more easily and in much greater abundance, simply by growing mycelium (from fragmented mycelium inoculum prepared by scraping the surface of a slope culture under water) in 500 ml flasks containing 100 ml of MEB, shaken for 5 days. The MEB was then decanted off and the mycelium flooded with 200 ml of sterile water. Abundant germinable conidia were produced in abundance after 3 - 6 days using isolates F6, 3b1a, 45/10 and F911-1. A later refinement of

the technique (with at least one isolate, F911-1) simplified this process without loss of conidia; after shaking for five days, the cultures were simply allowed to stand for a further 5 - 7 days in MEB.

2.13 Production of Perithecia and Isolation of Single Ascospores

Attempts were made to induce various isolates to produce perithecia by the following methods, suggested by M. Holden (Rothamstead Experimental Station). The methods fall into three categories: (a) involving growth of the fungus in Petri dishes containing various solid media- 1% PDA plus 0.2% yeast extract; 1.5% PDA plus 1% yeast extract; 1% agar plus 0.4% malt-extract broth (Oxoid); 1% agar plus 4% malt-extract broth, 1% agar plus 4% 'Cracklin Bran' (Kellogs - an oat based cereal); 2% agar plus 1.5g ground fresh wheat roots, 1% agar plus 1.5g of ground seedling wheat leaves; Weste and Thrower (1063) medium. The cultures were grown in the dark for two days and then placed beneath a window. (b) methods in which the fungus was grown in liquid culture in 50ml conical flasks containing 10ml of 2% malt extract broth. The flasks were inoculated with a plug of the isolate and shaken at 20°C for 7 days; then each of four treatments was used on individual flasks: (i) the liquid was drained and the mycelial mass was left in the flask; (ii) the liquid was drained and the mycelial mass was flooded with 40ml of sterile distilled water; (iii) the mycelial mass was transferred to a plate containing

1% agar; (iv) the mycelial mass was placed on a single sheet of filter paper in a Petri dish. In all cases the flask or dish was kept at room temperature beneath a sunlit window. (c) Growth of the fungus on young, growing wheat seedlings roots. This involved germination of sterile wheat seeds, growth of the seedlings in sterile tubes, in oculating with the fungal isolate and allowing the fungus to infect for about three months. 300g of expanded aluminium silicate ('Perlite' grade EUP 6X, Johors-Manville (GB) Ltd. Richmond, Surrey) were seived through a 3mm sieve, collecting the larger particles that fail to pass through. 50ml glass tubes were half filled with these particles and water was added so that a small amount of free water was present. The tubes were bunged with cottonwool and autoclaved. Wheat seeds (cultivar Cappelle Desprez) were washed with distilled water and then soaked in 10% sodium hypochlorite for 10 minutes. The seeds were then copiously washed with sterile distilled water and placed on moist filter paper in a sterile Petri dish and allowed to germinate. When the seeds had germinated (1-3 days) a few were transferred onto dishes of 1% agar + 0.5% malt extract broth and these were incubated at 37°C and 24°C to check for any contamination. The rest were placed one in each tube of perlite. A plug of the fungal isolate was inoculated with the seedling and the tubes supported at an angle of 30° to the horizontal and incubated in a 19°C growth room with alternating light and darkness for a period of one to three months, and periodically examined for perithecia.

If perithecia were produced and single ascospore cultures were required then the perithecia were allowed to mature such that the perithecia were seen to have a pink tip of almost mature asci at the neck of each perithecium. A perithecium was then removed, placed on a microscope slide and any mycelium scraped away. The perithecium was then transferred to 0.4ml of sterile water until the asci were extruded through the ascocarp pore and left for 15 minutes so that asci dehisced releasing single ascospores (Fig. 32) The perithecium was removed and the liquid was streaked onto the agar surface of a dish of 1% agar. The Petri dish was then examined under a microscope, fitted with a Meopta Monosporic Isolater and 1mm plugs were cut around single ascospores. The plugs were examined under the X40 objective to check that only one ascospore and no mycelium was present. (The former was usually, and the latter invariably the case). The plug was then transferred onto a dish of 1% PDA and allowed to germinate.

2.14 Isolation of Hyphal Tips

All attempts to induce Ggt isolate F6 to produce perithecia were unsuccessful so attempts were made to obtain a virus free culture by isolation of successive growing hyphal tips. These have been shown in other fungi to contain lower amounts of virus, as observed by direct election microscopy and the method combined with heat shock treatment has been used by Rawlinson et al, (1973) to obtain virus free cultures. Since heat can act as a mutagen or as a selective agent for mutations (Burnet, 1975) it was thought unwise to use this treatment because any observed change in virulence could be ascribed to induce mutagenesis. It was therefore decided to isolate successive hyphal tips with the intention of reducing and eventually eliminating virus. Although the hyphal tip cell would be expected to contain the lowest amount of virus it is impossible to isolate this as the leading hyphal cells do not have plugged septa; the terminal two or three cells were therefore isolated.

Petri dishes containing 10ml PDA were inoculated centrally with a plug of the isolate and the fungus was allowed to grow over one third of the agar surface. The dishes were placed beneath a microscope fitted with a Meopta Monosporic Isolater, as described above, locating a hyphal tip such that the cutting tube imprinted a circle that isolated a length of the hyphal tip of about one third of the diameter of the tube, that is about 0.3mm. The Isolater was withdrawn, the dish was removed and, using a sharp sterile scalpel, an incision was made at a tangent to the inner edge of the circular impression made by the Isolater. The Isolater was

again placed above the same hyphal tip and brought down so that it cut an agar plug within the impressed circle. The isolated plug was then examined under an 40x objective to ensure the tip was still intact and the plug contained no other hyphae. If this was the case the plug was carefully removed and transferred onto fresh PDA. The procedure was repeated to isolate several hyphal tips from the edge of the mycelium. These were then incubated at 24°C and if the excision of hyphal tips yielded successful single colonies after a few days (a 30% success rate was common) further hyphal tips were excised from these colonies. This procedure was repeated ten times and hyphal tip cultures 9 and 10 were retained for further work.

2.15 Small-Scale Virus Extraction from Ggt

Virus was extracted from fungus grown in 500 ml conical flasks, containing 100 ml MEB medium, on a rotary shaker (200 rev/min. 4.5 cm throw) at 24°C in darkness for 10 to 14 days. The flasks were inoculated with 6mm discs of mycelium taken from cultures on PDA.

The fungus was harvested by filtration, washed with water, then PBK buffer and homogenised by passing twice through a Pascall roll-mill, and then blending (MSE Ato-mix) with cold PBK buffer. 50 to 100g of fungus (wet weight) was used in most preparations. The homogenate was suspended in cold buffer (up to 10 ml/g wet weight of fungus) by stirring for four hours at 4°C, then cleaned by low speed centrifugation at 23000g for 45 minutes.

The supernatant was centrifuged at 65,000g (27000 rev/min in a Beckman type 30 rotor) for 16 hours. The resulting pellet was resuspended in 80 to 100 ml of PBK buffer by stirring for 4 hours at 4°C, cleaned by centrifugation at 3000 g for 45 minutes, and pelleted again at 65000g for 16 hours. The virus pellet was then resuspended in 1 ml PBK buffer and cleaned by low speed centrifugation to give a crude virus preparation.

Further purification, if required, was by rate-zonal centrifugation in a 30 ml linear secrose density gradient (10% to 40% w/w in PBK). Crude virus (0.2 to 1.0 ml) was loaded onto each gradient and centrifuged at 170,000g (43000 rev/min on a Beckman SW 50.1 rotor) at 4°C for 3 hours. The gradient was then dripped out in 0.2 ml fractions into Eppendore tubes. Virus particles containing nucleic acid could be detected by spotting 5 l aliquots onto ethidium bromide plates (0.5. agarose, 2 g/ml ethidium bromide added just before the agarose gelled), after drying on irradiation with 254 nm ultraviolet light, virus fractions containing dsRNA fluoresced. These fractions were dialysed against PBK and stored at 4°C.

2.16 Double-Stranded RNA Extraction from Ggt

Fungus was grown and harvested as already described, and homogenised in chilled TSE buffer (5 ml/g wet weight). The homogenate was made up to 1% SDS and heated at 60°C for 20 min, then extracted at room temperature with 100 ml of phenol solution (90g phenol/10 ml TSE/0.1g 8-hydroxyquinoline). After breaking the emulsion by spinning at 12000 g

for 45 minutes, the upper aqueous layer was removed and re-extracted with phenol. Solid sodium acetate was added to a final concentration of 0.2M followed by 2.0 vol. of ethanol cooled to -20°C. Nucleic acid was precipitated overnight and collected by centrifugation at 16000g for 30 minutes. The precipitate was taken up in 1 ml TNE buffer and cleaned by centrifugation at 16000g for 30 minutes. The nucleic acid was either reprecipitated with ethanol, dried in vacuo and dissolved in 0.2 to 1 ml of TNE buffer or the dsRNA separated from DNA and ssRNA by passing through a CF11 column as described below. The nucleic acid preparation was dialysed against several changes of TNE buffer and stored in small aliquots, at -20°C.

2.17 Purification of dsRNA on CF11

dsRNA could be separated from ssRNA and DNA using a column of Whatman CF11 cellulose (Franklin, 1966).

20g of Whatman CF11 cellulose was suspended in 200 ml of SET buffer and allowed to equilibrate overnight at 4°C. The powder was resuspended in the buffer and about 10 ml of the suspension poured into a 20 ml column fitted with a glass fibre bung. The cellulose was allowed to settle and washed with SET buffer. Immediately before use the column was washed with five bed volumes of SET buffer, 1% mercaptoethanol, 0.01M EDTA: then washed with 20 bed volumes of 65% SET, 35% ethanol v/v.

The sample was dialysed against SET overnight and the A_{260} measured. The capacity of CF11 is 40 A_{260} units per ml of column but was usually used at one tenth of this. The sample was made 35% ethanol v/v and applied to the column. The column was washed first with 65% SET, 35% ethanol v/v to wash through DNA which does not bind under these conditions; then with 85% SET, 15% ethanol to wash through ssRNA; then with 100% SET and fractions collected. All fractions containing nucleic acid, as detectable by fluorescence of a 1 µl drop on an ethidium bromide plate under a uv lamp, were ethanol precipated by using 5 µg of yeast total RNA source as carrier and taken up in a small volume of 0.1 x ssc.

2.18 Extraction of Nucleic Acid from Virus

Double-stranded RNA was extracted from virus preparations by phenol extraction of the SDS - disrupted virions. Virus solutions were made up to 1% -SDS and heated at 60°C for 20 minutes, then phenol extracted twice and the aqueous phase removed and extracted with ether five times. 0.1 vol. 2M-sodium acetate, pH 5.5 and 2.5 vol. of cold (-20°C) ethanol was then added. The dsRNA precipitate was collected by centrifugation, dried, disolved in 0.1 x TNE and stored at -20°C.

2.19 Large-Scale Virus Extraction from Ggt

Large quantities of virus were prepared from pilot plant scale fermentations of Ggt.

Six 500 ml shake flasks each containing 100 ml of MEB were inoculated with 1 ml aliquots of fragmented mycelium suspended in sterile water (prepared by scratching the surface of slope cultures under water). After 7 to 10 days growth these were used as inoculum for secondary cultures (1 litre medium in 3 litre conical flasks) which were grown for 3 to 5 days at 24°C. These were then used to inoculate one or two 60 fermenters (Banks et al, 1971). The medium for the 1 litre and 60 litre cultures was basal/corn steep liquor (1% glucose monohydrate, 0.1% KH_2PO_4, 0.03% $Mg\ 5O_4$, $7H_2O$, 0.001% $FeSO_4$, $7H_2O$, 3% corn steep liquor, pH adjusted to 6.0 with NaOH, then autoclaved.

Fermentations were carried out in jacketed stainless steel, spanger-aerated fermenters of total capacity 100 litres and working capacity 60 litres. The agitator shaft speed (183 to 367 rev/min and air flow rate (30 to 60l/min) facilitated heat transfer and culture aeration. Fermenters were maintained at an air pressure of 15 lb/in^2 and fermentations were terminated after 3 or 5 days by cooling to 4°C and filtration through a filter press.

A typical wet weight of mycelium after filtration was 5 kg. This was suspended in 2- litres of cold PBK buffer and passed through a Manton-Gaulin homogeniser (The APV Co. Crawley, Sussex, England) at 7500 lb/in^2. The homogenate was mixed with a further 30 litres of cold PBK. The degree of hyphal breakage was checked microscopically and, if sufficient, the insoluble debris was spun off on a Sharples Super centrifuge (Sharples Centrifuges Ltd. Camberley, Surrey, England) at 13200g av. and a flow rate

of 4 l/min. The supernatant was made up to 10 litres with cold buffer and 5.8 kg of sodium chloride was added. 30l of 30% w/v aqueous PEG 5000 was then added dropwise with stirring over 2 to 3 hours and the solution was left overnight. The PEG precipate was collected by passage through a Sharples Super centrifuge and resuspended overnight in gradually increasing volumes of PBK buffer, up to 2.5 litres at 4°C. The suspension was cleaned, by centrifugation at 23,000g for 1 hour and the supernatant was centrifuged at 17,000 rev/min. for 16 hours in a Beckman Type 19 rotor. Virus pellets were taken up to 100 ml of PBK and this solution was cleaned by centrifugation for 1 hour at 1800 g, to give a crude viral preparation.

Virus was purified in volumes of up to 60 ml by rate-zonal sucrose density gradient centrifugation 600 ml 20 to 50% w/w sucrose gradients in PBK in an MSE BX1V zonal rotor. The gradient was formed in a rotor spinning at 3000 rev/min the virus sample was then loaded and flushed through with 20 ml of buffer. After centrifugation at 40000 rev/min. for 3.5 hours, the rotor was slowed to 3000 rev/min. and the gradient was pumped out with 5.5% w/w sucrose solution containing bromophenol blue as market dye, through an ISCO ultraviolet optical unit (model UA) to record the A_{254} trace. The gradient was collected in 10 to 20 ml fractions, and kept on ice. Fractions adsorbing at 254 nm were analysed by (a) spotting on ethidium bromide plates, (b) polyacryamide gel electrophoresis of dsRNA (c) SDS-polyacryamide gel electrophoresus of protein, (d) agarose gel electrophonesis of intact virus and (e) electron microscopy. Sucrose was removed by exhaustive dialysis of the virus fractions against PBS buffer. Virus was stored for several months either at 4°C with 0.01% sodium azide, or was dislysed against PB5-50% glycerol and stored at -20°C.

2.20 Purification of Virus by DEAE - Cellulose Chromotography

Virus was purified on DEAE - cellulose, essentially as described by Buck and Kempson-Jones (1973). A number of buffer systems were compared in order to determine which system gave the maximum separation of viruses.

Whatman DE-52 cellulose was suspended in 20 volumes of water. The acidic component of the buffer to be used was added dropwise until the pH of the suspension reached pH 2.0. When TBE buffer was used, this was not possible since boric acid is a very weak acid, and in this case, hydrochloric acid was added (after maximum reduction in pH using boric acid) until the pH reached pH 2.0. The suspension was degassed by evacuating in a Buchner flask with stirring. The pH was then adjusted by addition of the basic component of the buffer until it had reached the required pH of the buffer. The suspension was then washed twice with 10 x buffer concentration, twice with 5 x buffer concentration and twice with buffer. The pH of the washes was checked and washing was continued until the pH of the washes was exactly the pH of the buffer.

Virus was usually purified using about 50 ml of slurry in a wide column of about twice the packed volume. For test assays however, small columns were poured (2 ml slurry). If gradient was to be used then virus was applied to the column in column buffer and washed through with the same buffer. The gradient was then applied, in a volume of about 20 x the packed column volume. Fractions were

collected and samples spotted onto ethidium bromide plates for detection of virus.

DEAE - cellulose columns, for virus purification, were always run at 5°C in order to avoid virus disruption. One gram of dry DE-52 powder was used per four A_{260} units of virus.

2.21 Electron Microscopy of Viruses

(a) Standard Sprayed Grids

Virus preparations to be used for electron microscopy were dialysed against low concentration buffer (15mM - sodium phosphate, pH 7.0). A few drops of virus solution were mixed with an equal volume of negative stain (2% potassium phosphotungstate pH 7.0) and one drop of albumin solution (10 mg/ml) was added to help spread the sample. The mixture was poured into a high velocity spray-gun (Aerograph Super 63) and droplets sprayed from a distance of about 30 cm, onto 200 mesh copper grids coated with collodion and stabilised with carbon. The grids were examined in a Siemens Elmiskop 1A electron microscope, or a Hitachi H600 electron microscope, and photographs were taken on Ilford EM plates, or in the case of the Hitachi microscope, on Kodak technical pan 2415 film.

(b) Immune electron microscopy

Immune electron microscopy (IEM) involving the use of serum activated grids (SAGs), was used to detect low levels of virus in crude virus preparations. Grids were coated with antisera and then

incubated on a drop of virus preparation such that virus particles specifically bind to homologous antisera. Contaminating material was then washed off the grids, and for greater ease of detection of virus particles a further incubation allowed the particles to be visibly decorated with antibody molecules. The method used was that of Derrick (1977). The procedure consists of, (i) grids (200 mesh copper grids coates with collodion and stabilised with carbon) were incubated for 5 minutes over a drop of antiserum, diluted 1:10 or 1:100 in PBK, at room temperature in a humidity chamber, (ii) grids were washed with 20 drops of 0.1M - sodium phosphate, pH 7.0, (iii) grids were incubated over a drop of virus preparation for 15 minutes as above, (iv) grids were washed as before, (v) grids were incubated over a drop of antisera, diluted 1:100, for 15 minutes as above, (vi) grids were washed with twenty drops of 0.1M - sodium phosphate, pH 7.0, then 30 drops of 0.003M - sodium phosphate. The grids were then stained with 5 drops of 2% potassium phosphotungstate, pH7.0 and dried. In some cases 1% bovine serum albumin was added to the negative stain in order to improve spreading of the stain.

2.22 Determination of Sedimentation Coefficient by Boundary Sedimentation

Sedimentation coefficients were determined for viruses by boundary sedimentation in sector-shaped centrifuge cells, in a Beckman model E analytical ultracentrifuge equiped with a uv optical system, using a titanium AN-H rotor, as described by Buck and Kempson-Jones (1973), or in a MSE Centriscan using a 6 hole analytical rotor. The method of centrifugation is essentially the same for both instruments.

Virus sample (0.4 ml, A_{260} 0.5-0.8) was placed in one sector of a double-sector centerpiece and buffer was placed in the other sector. The centerpiece was placed in one of the cell holes of the rotor and a counterbalance placed in the opposite hole. The rotor was positioned in the centrifuge chamber, the chamber evacuated, the uv lamp and optics switched on and the rotor temperature read. The rotor speed was set at 14000 rev/min: when this speed was reached the rotor temperature was again noted. The cell was then scanned at 256 nm every four minutes and the scan recorded on chart paper. When the MSE Centriscan was used each 4 minutes scan was recorded on the same sheet of paper (Fig .12) when the Beckman centrifuge was used, each scan was recorded separately on a continuous sheet of chart paper.

Determination of sedimentation coefficient from the trace of the MSE analytical ultracentrifuge was performed by Markham's (1960) graphical method. When the Beckman centrifuge was used, measurements were made of the distance of the axis of rotation from the mid point of the boundary trace. The natural logarithm of these values was then plotted on a graph against time. Since the sedimentation coefficient, s is defined as the velocity per unit field, that is:

$$s = \frac{v}{w^2 r} = \frac{1}{w^2 r} \cdot \frac{dr}{dt}$$

where v ($=\frac{dr}{dt}$) the velocity of the molecule (cm/sec) w is the angular velocity (radient GEC) r is the distance from the centre of rotation (cm).

If the boundary is at r_o at time t_0 and r_1 at some time t_1 then:

$$\int_{t_o}^{t_1} s\,dt = 1/w^2 \int_{r_o}^{r_1} dr/dt$$

or, $$s(t_1 - t_o) = 1/w^2 \left[\ln(r_1) - \ln(r_o)\right]$$

therefore, $s = 1/w^2$ [slope of a plot of $\ln(r)$ vs time]

Therefore, if the slope of the above graph is measured the angular velocity can be calculated from the rotor speed, and the sedimentation coefficient s (sec) can therefore be determined. This can be corrected for temperature and viscosity of solvent so that a standardised value of 20°C (in water) is calculated. This can then be converted to Svedberg units by dividing the value by one Svedberg unit (10^{-13} sec).

Three determinations were performed for each virus and a mean value obtained.

2.23 Preparation of Antisera and Gel Immunodiffusion Tests

Antisera to viruses were raised in rabbits using the following injection schedule: 1 ml of virus in PBS buffer (A_{260} 1 to 2) was injected intravenously: 1 ml of virus emulsified with 1 ml Freund's complete adjuvant was injected intramuscularly 1 week and 3 weeks after

the first injection. A week after the last injection, test bleeds were made and the antibody titre was found. If the antibody titre was high enough the rabbits were bled by cardiac puncture. The whole blood was incubated at 37°C for 1 hour, then at 4°C overnight, and the clots were removed by centrifuging twice at low speed. The supernatant was mixed with an equal volume of glycerol, as a bacteriostat and stored at 4°C or in small aliquots at -20°C.

Serological relationships between viruses were examined by gel immunodiffusion on microscope slides. The gels were prepared by boiling solutions of 1% purified agar (Ionagar, Oxoid) in PBS buffer, filtering through Whatman filter paper, cooling to 60°C and adding sodium azide to 0.01% final concentration. The molten agar was pipetted in 3 ml aliquots onto clean 3" x 1" microscope slides on a flat surface, and allowed to set for 15 minutes. The slides were placed in plastic Petri dishes.

To find the antibody titre against a virus antigen, a central trough 2 mm wide was cut in the gel, with circular wells (7 mm dia.) placed on either side, 3 mm from the trough. The trough was filled with 200µl of virus (A_{260} 1 to 2) and the wells with serial two-fold dilutions of antiserum. The slides were allowed to develop at room temperature in a humid atmosphere for 1 to 2 days, then washed for 24 hours in several changes of PBS. Precipitin bands, faintly visible before staining, were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue R, 10% 5-sulphosalicylic acid, 10% TCA) and destained in 5% acetic acid. The antiserum titre was the highest dilution factor of antiserum where a precipitin line was observed.

To examine the type of serological relationship between viruses, Ouchterlony plates were used (Crowle, 1961). A pattern of wells (4mm diameter) was cut in gels using a template: a central well was cut surrounded symetrically by six wells (5mm edge to edge). The central well was filled with 25 µl of antiserum and the surround wells with virus. The concentrations of antiserum and antigen were varied to give the best results, i.e. a precipitin line roughly equidistant from the antiserum and antigen wells. The slides were developed, washed and stained as before. If the dsRNA content of the bands was to be examined then the slides were not stained and the bands excised. The excised bands were homogenised using a fine scalpel, mixed with SDS containing buffer and applied directly to a dsRNA gel slot.

Antisera prepared in mice against 3b1-a virus, by described methods (Buck et al, 1981) was obtained from K.W. Buck and used in some experiments.

2.24 Preparation of Serum Gamma Globulin

The gamma globulin fraction was purified from antiserum preparations for use in Enzyme-linked Immunosorbart Assay by the method of Stanworth (1960).

One ml of antiserum was taken from aliquots stored at -20°C and diluted to 10 ml with water. 10 ml of saturated ammonium sulphate

was added and the precipitate formed was collected by centrifugation and washed with 5 ml of 18% (w/v) ammonium sulphate. The precipitate was again collected and dissolved in 2 ml of 0.5 x PBS and the resulting solution dialysed overnight against 4l of 0.5 x PBS, then against two changes of 0.01M-sodium phosphate pH 7.5 for 24 hours to give a crude gamma globulin preparation which was further purified by the removal of contaminating proteins by their absorbtion on DE-52 cellulose at pH 7.5.

50g of Whatman DE-52 were equilibrated with 0.01M - sodium phosphate as described for column chromotography (p. 115) 2 ml of the crude gamma globulin was mixed with 14g (wet weight) of the DE-52 slurry and left for 3 hours at 5°C with occasional stirring. A further 2 ml of buffer was mixed with the DE-52 and the suspension was filtered through a sinterred glass funnel. The filtrate was collected and mixed with a further 7g of the DE-52. The suspension was then stirred, left at 5°C for 20 minutes and filtered similarly and the filtrate was again collected. The absorbance at 278nm was measured and the purified gamma globulin was stored at -20°C in small aliquots.

2.25 Preparation of Enzyme-Linked Gamma Globulin

Gamma globulin was covalently linked with the enzyme alkaline phosphatase using gluteraldehyde as coupling agent by the method of Avramees (1969) for use in enzyme-linked immunosorbant assays.

2 ml of purified gamma globulin (A_{278} = 1.4) was mixed with 0.2 ml of alkaline phosphatase solution (Sigma, type VII, 1075 units per mg of protein, 5 mg per ml in 3.2M-$(NH_4)_2SO_4$, pH 7.0). The solution was dialysed overnight at 5°C against two changes of 4l of PBS. 13 µl of an 8% solution of gluteraldehyde was added (to make 0.05% gluteraldehyde) and the solution was incubated at 24°C for 4 hours then dialysed extensively against PBS in order to remove gluteraldehyde and stored at 5°C.

2.26 Enzyme-Linked Immunosorbant Assay (ELTSA)

The ELISA technique was used to detect virus present at low concentrations. The technique (Clark and Adams, 1976; Voller et al, 1976) consists of coating microtitre plate wells with purified gamma globulin prepared from antiserum directed against the virus to be detected; applying the test solution to the wells to allow the plate-bound antibody molecules to 'fix' the virus (antigen) to the wells; and then applying enzyme-linked gamma globulin to the wells such that only virus specific enzyme-linked antibody binds, via the fixed virus, to the wells, allowing unreacting antibody to be washed off. The amount of virus bound enzyme-linked antibody can be determined by incubation of the wells with enzyme substrate and determing the amount of product formed (photometrically). If the substrate concentration is kept constant then the rate of product formation is directly proportional to the amount of enzyme bound to the wells. This in turn will be

proportional to the amount of virus bound to the wells. If the incubation time is kept constant then the amount of product formed, measured photonetrically, can be directly correlated with the amount of virus, and if known concentrations of virus are used a standard curve can be produced with which to determine the concentration of virus present in unknown samples. The enzyme products used was p-nitrophenol and inorganic phosphate. The concentration of p-nitrophenol can be determined by its absorbance at 405nm.

The outer wells were not used as it has been reported that anomalous results can occur in these. Gamma globulin was diluted in PBS to between 1-100 µg per ml, assuming an absorbance of 1.4 at 278 nm is equivalent to a protein concentration of 1 mg per ml and applied in 200 µl aliquots to the microtitre plate wells. The plates were allowed to stand at room temperature for 4 hours at 37°C. The solution was removed and the plates were washed once with PBS then twice with PBS-Tween, each for 15 minutes. It was found that coating the plates with 3% BSA at this stage, in order to 'fill' any remaining adsorption sites significantly reduced backround control values. So the plate wells were filled with 3% BSA and incubated and washed as above, before applying the sample. Test samples (200 µl) were applied in PBS-Tween and incubated at 5°C overnight. Samples were removed and the wells were washed three times (15 minutes each) with PBS-Tween. 200 µl of enzyme-linked gamma globulin (diluted in PBS) was applied to each well and the plates were incubated at

37°C for 4 hours. The plates were washed five times (10 minutes each) with PBS-Tween and the substrate was added as 300 µl of p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8 (adjusted with HCl). The plates were incubated at 24°C in the dark for 15 to 30 minutes and the reaction was stopped by addition of 50 µl of 3M-NaOH. The solution was then removed and the absorbance at 405 nm was measured. The plates could then be washed and if desired, incubated for the same or a different reaction time with fresh substrate and the absorbance measured again.

In later experiments the technique was standardised. The concentration of gamma globulin used to coat the wells was 10 ng per ml , and the substrate was incubated for 20 minutes.

2.27 Agarose Gel Electrophoresis of Intact Virus

Intact virions were analysed by electrophoreses in horizontal slab gels (20 x 20 x 0.2 cm) of 0.5% agarose (electrophoresis grade, BDH) in TBE-buffer, containing 0.0005% ethidium bromide. The gel mix was prepared by boiling 1g of agarose in about 150 ml water, allowing to cool to 60°C, adding buffer and ethidium bromide making up to 200 ml, pouring into a template and allowing to set for 2 hours. Combs were used so that sample wells had a capacity of 50 µl.

Samples were made dense by the addition of sucrose, and bromophenol blue tracking dye was added. Gels were electrophoresed at 50V (30mA) for 5

to 18 hours, fully submerged in TBE-1 buffer containing ethidium bromide. Gels could be removed at any stage for location of viral nucleic acid by fluorescence on a 254nm ultraviolet lamp. Viral protein was detected by Coomassie Blue staining (0.1% Coomassie Brilliant Blue R, 10% 5-sulphosalicylic acid, 10% TCA: destained in 5% acetic acid).

2.28 Agarose Gel Electrophoresis of DNA

Agarose gels were used to examine DNA samples and in the "Southern Blot" procedure. Samples were made 1% - SDS and after addition of bromophenol blue sucrose heated to 60°C for 10 minutes and after cooling to room temperature electrophoresed in 0.1 - 1.8% agrose horixontal slab gels in TBE-1 buffer (containing 0.0005% ethidium bromide) at 100V for 4 to 18 hours, fully submerged in TBE buffer. Bands were located by fluorescence on an ultraviolet lamp or by auto-radiography.

DNA samples to be sized were sometimes electrophores ed in alkaline gels consisting of 0.6 - 1.8. agarose in 0.03M - sodium hydroxide, 2mM - EDTA. Under these conditions (McDonnel et al, 1977) DNA is completely denatured and migrates as single strands. Restriction digests of λ or ϕX174 DNA were used as size markers.

2.29 Polyacrylamide Gel Electrophoresis (PAGE) of dsRNA

Viral dsRNA was analysed by electrophoreses in 4% polyacrylamide gels (Ratti and Buck, 1972). For preparing 10ml of gel, 1ml acrylamide stock solution (40% acrylamide, 0.4% N, N^1-methylenebisacrylamide, both electrophoresis grade, BDH), 1ml 1% u/v TEMED, 0.4ml 25 x TAE buffer and 6.6ml of water were mixed and de-aerated, 1ml of 1.5% ammonium persulphate was added, and the mix was poured into the gel container. Tube gels were overlaid with water. For maximum resolution, gels were allowed to set overnight.

Gels were prepared as: 10 x 0.25 cm diameter 'microgel' tubes, 11 x 0.6 cm diameter 'semi-micro' tubes or 20 x 20 x 0.15 cm vertical slabs. These were pre-electrophoresed at 100V for 2 hours after which the electrode buffer (TAE) was changed.

For analysis of dsRNA in virus samples, the virus was disrupted by heating at 60°C for 20 minutes with 1% SDS. RNA samples were treated in the same way to remove molecular aggregations and secondary structure. Before loading onto the gel 1 µl of saturated bromophenol blue was added (to 50µl sample) and a few crystals of sucrose. Electrophoresis in microgel tubes was at 120V for 1 to 2 hours, in semi-micro tubes at 40V for 16 hours, and in slab gels at 60V for at least 18 hours.

RNA bands were visualised after electrophoresis by staining in 0.01% ethidium bromide, destaining in water and placing on a 254mm ultraviolet lamp.

For electrophoretic scanning of semi-micro tubes gels, the gels were stained in 0.01% toluidine blue, and scanned at 550nm with a Gilford gel scanner.

For determination of molecular weights of dsRNA a sample was mixed with molecular weight markers, and bands located by having sample and molecular weight markers in adjacent tracks of a slab gel. The mobility of the marker bands were measured and plotted against the logarith of their molecular weight. A standard curve was thus constructed. The mobility of the sample bands was measured and plotted on the graph, so that the logarithm of their molecular weight could be read off. The method is that described by Buck and Ratti, (1977).

The following dsRNA mol. wt. standards were used covering the mol. wt. range 0.9×10^6 to 6.3×10^6: dsRNA from Pencillium stoloniferum viruses s and F (Bozarth et al,1971); Aspergillus foetidus viruses s and F dsRNA (Buck and Ratti, 1977); bacteriophage Ør dsRNA (Semancik et al, 1973); Helminthosporium maydis virus dsRNA (Bozarth, 1977).

2.30 Urea-Poly acrylamide Gel Electrophoresis (urea - Page) of RNA

Single-stranded RNA species were separated in 4% polyacrylamide gels containing 8M-urea and 0.1%-SDS. The gels (20 x 20 x 0.15cm slabs) were prepared from the following mix (for 50ml); 5ml of acrylamide stock solution (4 0/0.4), 40ml 10M-urea -0.125%-SDS, 2ml 25 x TAE, 0.5ml 5% TEMED, catalysed by 2.5ml 1.5% ammonium persulphate. The gel mix was deaerated, ammonium persulphate was added, and the gel was poured and set. The gel was pre-electrophoresed for 4 hours at 50V and electrophoresed at either 30V or 100V for 18 hours with a top electrode buffer of TAE + 0.1% -SDS and a bottom buffer of TAE + 0.1% -SDS + 8M -urea. RNA and virus samples were treated as described for 4% polyacrylamide gels.

After elctrophoresis the gels were washed in water for 2 hours to remove SDS and urea, then stained with 0.01% toluidine blue or 0.01% ethidium bromide and destained in water. Radioactive bands were detected by fluorography or autoradiography.

2.31 SDS-Polyacrylamide Gel Electrophonesis (SDS-page) of Proteins

Proteins were analysed by electrophoresis in 20 x 20 x 0.15cm slab gels of 8% polyacrylamide -0.1% SDS (Buck and Ratti, 1975). For preparation of gel: 10ml acrylamide stock solution (40/0.4), 5ml 1% TEMED,

5ml 1% -SDS, 25ml 0.2M -sodium phosphate 0.02M -EDTA, pH 7.2 and 5ml 1.5% ammonium persulphate was added. The gel was electrophoresed and pre-electrophoresed at 100V with 0.1M -sodium phosphate 0.01M -EDTA, pH 7.2 as electrode buffer.

Virus and protein samples were denatured by heating at 100°C for 3 minutes in 1% -SDS, 1% 2-mecaptoethanol, 0.01M -EDTA before addition of sucrose and bromophenol blue.

After electrophoresis gels were washed in water for 2 to 3 hours to remove SDS, then stained for 1 to 2 hours in Coomassie Blue (2.5% Coomassie Brilliant Blue R, 10% acetic acid, 50% methanol) and destained in 50% methanol - 10% acetic acid solution.

Occasionally, the gel system of Laemli (1970) was used for greater sensitivity and resolution of bands. The gel consists of a 12% polyacrylamide separating gel, overlaid with a 3% stacking gel. The separating gel was made from the following mix: 20ml acrylamide stock solution (40/0.4), 25ml 0.75M-tris-HCl buffer, pH 8.8, 2ml 2.5% SDS, 0.5ml 5%-TEMED, 2.5ml 1.5% ammonium persulphate. The gel was poured to a depth of 13cm, overlaid with 0.1% SDS and allowed to set. The stacking gel was prepared from the following mix: 3ml acrylamide stock solution (40/0.4), 1.6ml 2.5% SDS, 0.4ml 5% TEMED, 33ml 0.15M-tris-HCl buffer pH 6.8, 2ml 1.5% ammonium persulphate. The 0.1%-SDS was removed and the stacking gel was poured over the separating gel. The

comb was placed in position and the gel allowed to set. After loading samples the gel was electrophoresed with 0.025M-tris, 0.192M-glycine, 0.1%-SDS pH 8.3 as electrode buffer, at 100V, and stained as above.

For molecular weight determination, the following internal standards were used (subunit mol. wt. in parenthesis are those given by Weber et al, 1972): phosphorylase A (100000), bovine serum albumin (68,000), glutamate dehydrogenase (55000) and lactate dehydrogenase (36000).

2.32 Location of Radioactive Bands in Gels

(i) Fluorography

Tritium was detected in polyacrylamide gels by fluorography, (Larkey and Mills, 1975).

After electrophoresis and staining the gel was soaked, with shaking, in 20 vols DMSO for 30 minutes, then in fresh DMSO for 30 minutes. The gel was then soaked, with shaking, in 4 vols. 22.2% w/v PPO in DMSO for 3 hours; DMSO was then completely removed by washing in several changes of water for 1 hour. The gel was then placed on Whatman 3MM paper, covered with Saran Wrap polythene film and dried under vacuum with heating for 1 hour. Labelled bands were located by exposure of pre-flashed Fuji RX film at -70°C.

(ii) Autoradiography

^{32}Phosphate was detected in gels and filters by direct autoradiography. Labelled bands were located by exposure of pre-flashed Fuji RX film at -70°C.

2.33 Virion RNA Polymerase Assay

RNA polymerase activity of virus preparations was assayed by measuring the incorporation of (^{3}H)-UTP into TCA insoluble material, after incubation in a reaction mixture containing ATP, CTP, GTP, UTP and Mg^{2+}. The basic assay mix was that used by Buck (1975) for Pencillium stoloniferum virus S (PsV-S), 100µl of reaction mixture contained: 50µl of virus (A_{260}=0.5 to 5 in TNE buffer), 20µl of 0.75mM - ATP, CTP, GTP, and UTP in TNE buffer, 10µl of 20mg/ml bentonite in TNE buffer, 10µl of 1mg/ml actinomycin D (Sigma) in 50% ethanol - TNE buffer, and 10µl of 50mM - $MgCL_2$ in TNE. Bentonite was added as a ribonuclease inhibitor (Frankel - Conrat et al, 1961) and actinomycin D to suppress bacterial growth. 5-(^{3}H) - UTP (ammonium salt, 10Ci/m mol, 1mCi/ml RCC Amersham) was added as a radioactive label, at a final concentration of 83 Ci/mole (1.25µl per 100µl mix). The final Mg^{2+} concentration could be varied from 0 to 10mM by diluting a stock solution of 100mM $MgCl_2$ in TNE, but was usually at 5mM. Incubation was at 30°C, usually overnight and reactions were stopped by placing on ice.

Incorporation of (^{3}H) -UTP label into TCA - insoluble material was measured by filter assay (Lhater and Morgan, 1974). Aliquots, up to 100µl, of reaction mixture were made up to 1.5ml with 10% TCA and left on ice for 30 minutes. The suspensions of TCA - precipitate were then washed onto wetted glass-fibre filter discs (Whatmann GF/A, 2.5cm) with 2% TCA and the filters washed under vacuum with 8 x 10ml of 2% TCA, 3 x 10ml ethanol, and once with 10ml of diethyl ether, then oven dried at 180°C for 5 minutes, placed in 5ml of scintillation fluid and counted for 3hours.

The products of the polymerase reactions were incubated with 1% SDS at 60°C for 20 minutes. The suspension was centrifuged briefly to pellet bentonite and absorbed actinomycin D and the supernatant applied was analysed by urea - Page

When the RNA product was required free of protein the SDS disrupted virus particles were phenol and ether extracted and RNA was ethanol precipitated as described in Section 2.18.

In some cases the virion reaction mix was separated into fractions binding to bentonite and fractions not binding to bentonite. If this was required the bentonite was centrifuged down (before SDS-treatment) and the supernatant was removed and retained. The bentonite was then washed with fresh TNE several times.

2.34 Lithium Chloride Precipitation of ssRNA

Single-stranded RNA was precipitated from solutions containing ssRNA and dsRNA by 2M LiCl. Equal volumes of 4M LiCl and RNA solution were mixed and left overnight at 0°C. The precipitate was collected by centrifugation, washed by re-suspending in 2M-LiCl and centrifuging twice, and taken up in the required buffer. Double-stranded RNA was recovered by dialysis to remove LiCl, and precipitated with ethanol.

2.35 Denaturation and Glyoxalation of dsRNA

Double-stranded RNA was dissociated and kept in a denatured state by glyoxalation, for use as a ssRNA electrophoresis marker. (McMaster and Carmichael, 1977). In a modified method (R. Empson, 1978, 3rd year undergraduate project, Imperial College) dsRNA was heated at 50°C for 1 hour with 1M purified glyoxal, 60% DMSO, and 10mM sodium phosphate pH7.0. The reaction mix contained 60µl DMSO, 16µl 40% glyoxal, 4µl 250mM-sodium phosphate, pH 7.0, and 20µl dsRNA in .1 x TNE.

Glyoxal (technical grade, BDH) was purified before use, by shaking with 0.5 vol. of Amberlite MB-3 resin, (analytical grade, BDH) leaving for 5 minutes, and repeating twice with fresh resin. This treatment removed oxidation products capable of damaging nucleic acids.

To concentrate the denatured RNA, and in order to avoid distortion of polyacryamide gels by glyoxal and DMSO, the RNA was usually precipitated from the reaction mix with 0.1 volume of 5M-sodium acetate, pH5.5 and 2 volumes of ethanol overnight. The precipitate was washed twice with 2 volumes of ethanol containing 0.5M-sodium acetate, pH 5.5.

2.36 RNA-RNA Hybridization Analysis

RNA-RNA hybridization analysis was performed in order to determine the amount of sequence homology between ssRNA polymerase product and viral dsRNA and hence to show that the ssRNA product is a faithful transcript of virion dsRNA. The hybridizations were performed exactly as Vandevelle and Siegel (1976). The reaction is performed with purified tritiated ssRNA probe and an excess of denatured dsRNA. The reaction was allowed to go to completion and the amount of ribonuclease resistant radioactivity was determined as a measure of the amount of hybrid formation.

Between 0.1 and 0.5µg of 2H-ssRNA (1000 to 10000 ct/min) and 0 to 20µg of unlabelled dsRNA, in 150µl of 0.1 x TNE buffer, were heated at 100°C for 5 minutes then plunged in ice. To this denatured RNA was added 50µl of 8 x STE buffer, and the mixture was incubated at 70°C for 5 hours to allow re-annealing. After cooling, 25µl of ribonuclease A (10 g/ml in 2 x SSC buffer) was added, and the solution was incubated at 30°C for 2 hours. Ribonuclease-resistant hybrid dsRNA molecules were then precipitated with TCA, filtered, washed, dried and counted for 3 hours.

Under these conditions of dsRNA excess, complete homology between the ssRNA probe and the dsRNA should give almost complete protection of the ssRNA to ribonuclease digestion in 2 x SSC.

2.37 Electron Microscopy of DNA and RNA

Nucleic acids were visualised for electron microscopy using the Kleinschmidt (1968) cytochrome C technique.

A mixture consisting of 55.5µl of double-distilled water, 35µl of formamide, 2µl of cytochrome C solution (0.1 mg/ml in 2mM-tris - HCl pH 8.5, containing 0.2mM-EDTA) and 10µl of sample (containing about 0.05 g RNA or DNA) were spread over a fresh hypophase of 10% formamide in 10mM pipes buffer (Sigma), pH 7.0. The sample was mounted on parlodion-coated grids, stained with uranyl acetate (5×10^{-5}M) shadowed with platinum in an Edwards E306 shadower at an angle of 4 - 8° and examined in a Hitachi H-600 electron microscope, operating at 50kV. Micrographs were recorded on Kodak technical pan 2415 film and negatives were projected to a final magnification of 75,000 x for contour length measurement with a Hewlett-Packard 9821A calculator coupled to a 9864A digitizer. For calibration a Polaron cross-grating replica with 2160 lines/mm was used.

2.38 Electron Microscopy of Transcribing Virus Particles

For examination of transcribing virus particles, standard RNA polymerase mixtures, without bentonite or actinomycin D were incubated for between 1 and 15 minutes. Samples were then spread using a modification of the Kleinschmidt cytochrome c technique as described by Bartlet et al (1974).

Polymerase reaction mixtures were diluted to a virus concentration of about 10μg/ml, made 1% in formaldehyde and 100 g/ml in cytochrome c, spread on a fresh hypophase of double-distilled water, mounted on parlodion-coated grids (not stabilised with carbon - this modification gave clearer micrographs and increased reproducibility) positively stained with uranyl acetate, shadowed and examined exactly as described for nucleic acids.

2.39 Visualisation of Ggt Nuclei

Preparation of cellular DNA involved retention of the intactness of nuclei. Direct visualisation by oil immersion, phase contrast light microscopy of Ggt mycelia (as reported by P. Blanch, 1977) was not successful; nuclei if seen at all, could not be distinguished from other cellular components. Staining methods using either nile-blue stain or Giemsa stain method and light microscopy were equally unsuccessful. Also, any involved staining

method would clearly be unsuitable for visualization of nuclei in cell homogenates. It was therefore decided to use fluorescent microscopy for visualisation of nuclei in conidia mycelia and cell homogenates as described for Aspergillus nidulans (Gealt et al, 1976).

The procedure consisted of first fixing the cells and making them permeable to stain in a solution of 5% mercuric chloride, 3% potasium dichromate for 10 to 15 minutes at room temperature. 6% of a 3% solution of formaldehyde was added immediately before use and the cells were rinsed several times in 70% ethanol. The cells were stained by incubating in 0.03M-sodium phosphate, 0.15M sodium chloride, 0.5M-sucrose pH 7.2 (homogenisation buffer) containing acridine orange at 50mg/l for 15 minutes at room temperature. The cells were rinsed several times with homogenisation buffer until they no longer appeared orange, soaking in buffer for 10 minutes between washes.

The nuclei were visualised using a Zeiss 1V F1 fluorescent microscope with excitor filter 136-38 and B6-12 with barrier filter 53/54. Nuclei appeared as yellow-green spheres.

When my celial material was used the most convenient method of fixing and staining was with the mycelia suspended in an Ependorf tube; the changes and rinses were performed by spinning down the mycelia and resuspending. For observation of free nuclei (during

DNA extraction) the material was resuspended directly in staining buffer and visualised. Under these conditions free nuclei could be seen but nuclei could not be distinguished within mycelia.

Later in this work, it was discovered that cells could be made permeable to stain simply by freezing the mycelia, or conidia at -70°C, and thawing. The cells could then be directly stained with acridine orange and destained for visualization of nuclei.

2.40 Preparation of Ggt DNA

High-molecular weight host DNA was required for detection of a possible DNA proviral form of viral RNA. Mycelial fungi contain low DNA/biomass ratios, and extraction of high-molecular weight DNA, necessary for restriction endonuclease digestions, is known to be difficult since harsh treatments required to produce sufficient cell breakage tend to shear the DNA and also many fungi are known to contain very potent nuclease activity that can be resistant to SDS (Gealt et al, 1976).

One of the techniques available to overcome the first difficulty is to produce protoplasts (or spheroplasts) from the fung al cells which can be gently lysed to yield either intact nuclei or high-molecular weight DNA. This method has been successfully used to obtain high-molecular weight DNA from yeast, but has not been very successfully applied to mycelial fungi. Attempts to produce protoplasts from Ggt, using the snail enzyme Helicase,

were unsuccessful. However a method of cell disruption which yields intact nuclei has been described for Aspergillus nidulans by Gealt et al, (1976). Since preliminary experiments preparing DNA from Ggt by standard methods (Marmer 1961 and Britten et al, 1973) produced highly sheared DNA (measured by electrophoresis in alkalineagarose gels) this method was attempted. The method consists of homogenising mycelium in liquid nitrogen in osmotically stable media to yield free intact muclei, which can be purified to give a preparation of nuclear DNA. The nuclei are visualised by fluorescent microscopy. It was possible to produce a preparation of nuclei by this method, however extracted DNA was again highly degraded. This was thought to be due to nuclease action during the lengthy procedure required for nuclei purification. It was decided therefore to commence the DNA extraction immediately upon thawing the homogenate in order to minimise nuclease action. This precluded the possibility of preparing DNA from various cell fractions but should give a preparation of total cell DNA. The DNA extraction procedure is basically that of Marmur (1961) modified by Britten (1973) and by Timberlake (1978).

DNA was extracted from fungus grown in 500ml conical flasks containing 100 ml MEB medium, on a rotary shaker at 24°C in the dark for 10 to 14 days.

The fungus was harvested by filtration and washed extensively with water then with 0.03M-sodium phosphate 0.15M-sodium chloride, 0.5M-sucrose pH 7.2, in order to remove any extra cellular nucleases. The mycelium was filter dried and weighed (usually 100-300g) and 50µg of spermidine hydrochloride (Serva) per gram (wet weight) of mycelia, was added in order to stabilise the DNA. The mycelial mass was cut into small pieces (about 1cm cubes) and immersed in liquid nitrogen.

An MSE Atomix homogeniser, which had a punctured lid to enable evaporating gas to escape, was precooled with liquid nitrogen. The frozen mycelium was transferred to this and the tissue was homogenised in liquid nitrogen with ten 30 sec. bursts, replenishing the liquid nitrogen between bursts. The mycelium (which at this stage has an appearance of fine sand) was removed and a small aliquot was warmed to 5°C and examined for cell breakage (light microscopy) and presence and intactness of nuclei (fluorescent microscopy). If the cell breakage was estimated to below 10% then the frozen tissue was homogenised in liquid nitrogen for a further 5 x 30s bursts before proceeding. If the cell breakage was estimated to be about 15-20% and free intact nuclei were present, the frozen tissue was rapidly warmed to 5°C by placing in an 80°C waterbath and the homogenate was adjusted to 2%-SDS, and 15mM-EDTA by addition of 10% SDS and 0.1M-EDTA pH 7.0. Proteinase K (Bochringer) was added to a concentration of 100 g/ml. The homogenate was heated rapidly to 65°C and incubated at this temperature for 10 minutes in order to inactivate nuclease activity before cooling to 37°C.

The homogenate was then shaken in 500ml conical flasks at 37°C on a rotary shaker (200rev/min, 4.5cm throw) for 3 hours, then cleared by centrifuging at 40,000g for 10 minutes and 100 g of proteinase K per ml of cleared homogenate was added to the mixture. This was then incubated at 37°C for 90 minutes. 0.1 volume of 5M-$NaClO_4$ was then added and the homogenate was transferred to 500ml Sorval centrifuge bottles. An equal volume of isoamyl alchohol chloroform (1:24) (IAC) was added and an emulsion was produced by gently inverting the bottle repeatedly for 45 minutes. The emulsion was broken by centrifuging at 9,000g for 30 minutes. The top aqueous layer was removed and two volumes of 95% ethanol were carefully poured as a layer above the aqueous layer. An attempt was made at this stage to 'spool' the precipitated DNA threads onto a glass rod. This was never successful so the layers were mixed and the liquid was kept at -20°C for 18 hours, then centrifuged at 5,000g for 10 minutes. The supernatant was removed and the precipitated material was resuspended in 15ml of 0.1 x SSC, by gently stirring for 24 hours at 5°C. The solution was adjusted to 1 x SSC, 5mM-EDTA and ribonuclease A (100μg/ml) then incubated at 37°C for 2 hours. The solution was adjusted to 1% SDS and proteinase K (500 g/ml) then incubated at 60°C for 30 minutes, then at 37°C for 3 hours. The solution was made 0.5M with respect to sodium chlorate and was extracted with one volume of IAC as before. It was then phenol extracted twice, again by gentely inverting the tube, with an equal volume of phenol, for 30 minutes. The tube was cooled to -5°C and centrifuged at 8,000g for 30 minutes. The upper aqueous layer was

removed and reextracted with an equal volume of phenol. The procedure was repeated until no material could be seen at the phenol-aqueous interface. The DNA solution was then dialysed against 0.03M-sodium phosphate, 10mM-EDTA pH7.4 for 24 hours to remove metal ions, then dialysed for 18 hours against 0.1 x SSC. The DNA was concentrated by freeze drying until the volume had been reduced to 1ml and any precipitated material was re-dissolved. The DNA solution was then dialysed against 0.03M-sodium phosphate, 10mM-EDTA, pH 7.4 for 72 hours against several changes of buffer, then dialysed against 1 x SSC for 24 hours. The DNA was analysed by spectrophotometry, and thermal denaturation in a spectrophotometer. If the DNA was not to be banded in caesium chloride gradients then it was stored at -20°C in 100µl aliquots.

2.41 Purification of DNA in Caesium Chloride Density Gradients

DNA was fractionated and purified free of contaminating protein, RNA and polysaccharide by centrifuging to equilibrium on isopycnic caesium chloride gradients. Ethidium bromide was usually added in order that DNA bands could be easily visualised by fluorescense: and in order that any closed circular (plasmid) DNA would be separated from nuclear DNA (Vinograd, 1963).

The DNA solution was made up to a density of approximately 1.619 g/ml by adding 1g of solid caesium chloride per ml of DNA sample to the DNA solution. 0.1ml of ethidium bromide solution 10mg/ml was then added per ml of DNA solution and the mixture centrifuged (1000g, 10 min) to remove any precipitated material. The sample was pipetted into a 40ml Beckman (self-sealing) centrifuge tube and the tube filled with caesium chloride solution (ρ=1.619) to which had been adjusted to .5mg/ml in ethidium bromide. The tube was heat sealed and centrifuged at 45000 revolutions/min. in a Beckman vertical VTi rotor. The tube was removed, DNA bands located under long-wave u.v. irradiation, and the DNA bands removed using a syringe. The DNA was dialysed against TNE buffer to remove caesium chloride then phenol extracted twice to remove ethidium, and dialysed extensively to remove phenol.

Throughout the operation, whenever DNA was in contact with ethidium, DNA solutions were exposed to a minimum of light, particularly u.v. irradiation.

2.42 Preparation of Ggt Total RNA

RNA was extracted from Ggt. for use as a probe on 'Southern blots' of Ggt DNA.

RNA was extracted exactly as described for extraction of DNA in the previous section except for the following modifications: (i) no precautions were taken to ensure intactness of nuclei, (ii) the homogenate was cleaned and extracted with IAC immediately upon thawing of the frozen tissue and addition of SDS and EDTA, no proteinase K was added at this stage, (iii) no ribonuclease treatment was used.

The preparation containing DNA and RNA was applied to a caesium chloride, ethidium bromide gradient in a 40ml tube and centrifuged in a vertical rotor at 45,000 rev/min overnight. RNA could be seen under u.v. illumination as a broad band at the bottom of the tube and was removed, dialysed against TNE, phenol extracted three times, ethanol precipitated, taken up in TNE and dialysed extensively. The DNA could also be recovered using this procedure.

The yield of RNA from 100g (wet weight) of fungal mycelium was about 51mg. The RNA was examined by urea -Page with wheatgerm total RNA as marker.

2.43 DNA Transfer Hybridizations

DNA samples (usually after digestion with restriction endonuclease) can be subjected to electrophoresis on agarose gels and the DNA (bands) directly transferred, after denaturation, to nitrocellulose filter sheets. The DNA can be permanently bound to the filters and is available for hybridization with homologous DNA. The technique is that described by Southern (1979) and is usually referred to as "Southern blotting".

DNA samples subjected to electrophoresis in agarose slab gels in TBE/ethidium buffer and the DNA was visualised by long-wave uv radiation. The gel was then treated to depurinate the DNA by two 15 minute washes in 0.25M - hydrochloric acid and rinsed with water. The DNA was then alkali-denatured by washing the gel twice in 15 minute washes of 0.5M - sodium hydroxide, 1.0M - sodium chloride and rinsed with water. The gel was then washed twice for 30 minutes each wash in 0.5M - tris - HCl, pH 7.4, 3M - sodium chloride. Transfer of the DNA to nitrocellulose filter was effected by placing the gel on a sheet of Whatman 3MM paper, which had been wetted with 20 x SSC; the ends of the 3MM paper dipped into a bowl of 20 x SSC, so that the paper was continuously wet. A sheet of Scheiler and Schuell BA95 paper is then laid on top of the gel (this nitrocellulose sheet having been boiled in several changes of water). A sheet of Whatman 3MM paper is then placed on top of the filter and a block of sheets of post-slip paper (a blotting paper) was then laid on top of this

(to a thickness of about six inches). The whole ensemble is weighted down with some heavy object. The transfer is allowed to take place over 20 hours; the nitrocellulose sheet is then removed, washed several times with 20 x SSC and baked at 80°C for 2 hours. The gel can be stained again and visualized so that the transfer can be checked.

The filter was then treated to prevent further ssDNA binding, using the technique of Denhardt (1966). The filter can then be hybridized with DNA or RNA probe. If RNA probe is being used then it is usual to perform the hybridization in 50% formamide in order to inactivate any ribonuclease present. In this study, only RNA probes are used so this procedure is described. The method is that described by Burton and Davis (1976). The filter was preincubated for 5 hours in 200 ml of solution consisting of 50% - formamide (Rose chemicals) 5 x SSC, 5 x PM (PM is 0.02% bovine serum albumin, 0.02% polyvinyl pyrollidine 0.02% ficol), 50mM - sodium pyrophosphate, pH 6.5, 250 µg / ml ssDNA (ssDNA is salmon sperm DNA, sonicated for 5 minutes to eliminate viscosity and boiled in water for 5 minutes before use). The incubation was at 42°C.

The filter was removed and placed in a heat sealable plastic bag with 20 ml of a solution consisting of 5 x SSC, 1 x PM, 20mM - sodium pyrophosphate, 50% formimide, 10% dextran sulphate (average mol. wt. 500000), 100 µg / ml ssDNA and the labelled probe. The filter was allowed to hybridize for either 18 or 48 hours at 42°C in the heat sealed bag. The filter was removed, washed in three successive changes of

2 x SSC, 0.1% - SDS, then in three successive changes of 1 x SSC, 0.1% SDS at 50°C. The filter was then blotted and autoradiographed.

2.44 Hybridization of Denatured RNA and DNA Transferred to Nitrocellulose

dsRNA was transferred to nitrocellulose by a modification of the method of Thomas (1980) RNA (or DNA, or RNA and DNA) to be denatured and transferred was dialysed against 0.1 x TNE buffer then added to 60 µl reaction mixes containing 30 µl of formamide, 10 µl of 37% formaldehyde, 3 µl of 10 x MOPES buffer and 17 µl of sample (containing usually 0.5 - 15 µg of RNA or DNA). This was incubated at 60°C for 30 minutes then cooled on ice. Bromophenol blue was added to each sample.

A horizontal agarose slab gel was prepared (20 x 20 x 0.3 cm) of 0.6 - 1.5% agarose in 1 x MOPES buffer and 2.2M formaldehyde. The gel mix was prepared by boiling the agarose in 100 ml of water allowing to cool to 60°C and adding 47 ml of water followed by 20 ml of 10 x MOPES buffer and 33 ml of 37% formaldehyde. The gel was poured and allowed to set in a fume cupboard (as formaldehyde is an irritant and weak mutagen) for 2 hours.

Samples were applied to the gel and electrophoresed at 100V(90mA) for 3 to 8 hours fully submerged in MOPES buffer. The buffer ions were kept circulating by having the gel tank placed on a pair of identical magnetic stirring plates and two magnetic stirrers in the tank. The turbulence produced by these stirrers kept the buffer constantly recirculating.

After electrophoresis the gel was removed. If required, the whole or part of the gel was stained in 0.01% Toluidine blue (denatured RNA or DNA cannot be visualised very well with ethidium bromide) and destained in water. If the gel was required for transfer to nitrocellulose it was immediately laid on a sheet of Whatman 3MM paper and the DNA or RNA transferred to a nitrocellulose filter sheet exactly as described in Section 2.44. When the transfer was over the filter sheet was removed and washed briefly (3 minutes) in 5 x SSC (lengthy washes or washes in low salt remove RNA) and baked at 80°C for 3 hours. The filter was hybridized and washed exactly as described in Section 2.44.

2.45 Polynucleotide Kinase End Labelling of Nucleic Acids

Polynucleotide kinase, extracted from phage T4-infected E. coli cells, was used for in vitro labelling of RNA and DNA. The enzyme was purchased from Boehminger
and stored at -20°C in 50% glycerol. The enzyme labels 'free' (non-phosphorylated) 5' - termini of polynucleotides with ^{32}p - ATP as substrate for labelling of RNA for use as a probe, the RNA is first partially hydrolysed with dilute alkali to produce short (about 100 bases long) polynucleotide 2' and 3' - phosphates. These can be labelled at the 5' - termini with polynucleotide kinase. Restriction endonuclease digestion of DNA produces DNA fragments bearing 5' - terminal phosphate groups and 3' terminal hydroxyl groups. In order to kinase label these, the 5' - terminal phosphate must be removed with phosphatase. The labelling procedures are as follows:

(i) Labelling of RNA

For most efficient labelling, a maximum number of termini must be available for the enzyme. However, if the labelled fragments are too short they may be unstable as hybrids. A length of about 100 bases was therefore aimed for. The RNA (in low ionic strength buffer or water) was base-cleaved in 0.1M-NaOH at 4°C for 1 hour, then ethanol precipitated, washed in 70% ethanol and dissolved in 20 µl of kinase buffer. This consisted of 66mM-tris-HCl pH 7.6, 10mM $MgCl_2$, 15mM dithiothrietol, 200 µg/ml gelatin (autoclaved). Between 20 - 200 µCi of (32p) - ATP (3000 Ci/mole) RCC Amersham, dried in vacuo in an Eppendorf tube) was added, then 1 µl of polynucleotide kinase (1.6 units/µl) and the mixture was incubated at 37°C for 1 hour. The RNA was then passed through Sephadex G-50 (pre-swollen in TNE) and the excluded RNA was collected. Aliquots were counted for total radioactivity and TCA precipitable radioactivity. Generally the percentage incorporation of radioactive nucleotide was between 5 - 20%. The size of the labelled material was checked by urea - Page , using wheatgerm RNA as marker.

(ii) Labelling of DNA

Restriction digests of DNA were labelled using the following procedure: 0.2µl of bacterial alkaline phosphatase (100 units/ml) was added to 50 µl of restriction digest (containing between 5-20 µg DNA) and incubated at 37°C for 45 minutes. The mixture was phenol-extracted once and ether extracted 5 times. The DNA was ethanol precipitated, taken up in 20 µl of kinase buffer and kinase-labelled exactly as for RNA. If the DNA was to be used as an electrophoresis marker, then only 10 to 20 µ Ci of (^{32}p) - ATP was used.

2.46 Assay for Detection of RNA-Directed DNA Polymerase

The basic reaction mixture was as described by Baltimore and Smoler (1971) for Rous sarcoma virus.

The mixture consisted of: 50mM tris-HCl pH 8.3, 15mM Mg-Acetate, 20mM dithiothrietol, 60mM-NaCl, 0.01% Nonidet P-40, dATP, dGTP and dCTP at 5mM and dTTP at 0.2mM. Actinomycin D was not added as it was thought it might inhibit any activity, so all reagents were filter sterilised or autoclaved. Each assay contained 2μCi of -(^{32}P)-dTTP so that a 0.1% incorporation would incorporate 4000 ct/min into the acid insoluble fraction. Virus (filter sterilised) was added and the mixture was incubated at 30°C for a fungal virus enzyme activity would be unlikely. The reactions were analysed by filter assay and/or urea - page and autoradiography.

This standard reaction mix was altered as described in the Results.

2.47 Nuclease Tests for Distinguishing ssRNA, dsRNA and DNA

It was determined whether nucleic acid was ssRNA, dsRNA or DNA by nuclease tests as described by Buck and Ratti (1975). 1μg of nucleic acid sample was incubated with 0.05μg of (i) ribonuclease A

in 0.1 x SSC; (ii) ribonuclease A in 2 x SSC; (iii) deoxyribonuclease in 2 x SSC, for 30 minutes at 37°C. The products were analysed by Page. The sample was shown to be ssRNA if digested by RNase in 0.1 x SSC and 2 x SSC but was not digested by DNase; was shown to be dsRNA, if digested by RNase in 0.1 x SSC but not in 2 x SSC and not digested by RNase in 0.1 x SSC but not in 2 x SSC and not digested by DNase; was shown to be DNA if digested by DNase but not by RNase.

2.48 DNA-DNA Hybridization in Solution

It was required to determine the total genome size of Ggt by DNA renaturation for these experiments and it was decided to monitor the hybridization reaction using S1 nuclease. This enzyme is specific for single-stranded nucleic acids and can be used to monitor the amount of hybrid formation. (Firtel and Bonner, 1972). The method is quicker and more convenient than the use of hydroxyapetite chromatography - the other commonly used method.

The purity of DNA used in reassociation is critical since the presence of contaminating proteins and metal ions can markedly alter the reassociation rates. The purity of DNA preparations to be used was checked by: scanning the DNA between 320 and 220 nm; melting in a spectrophotometer: and checking its susceptibility to digestion with a DNA restriction enzyme. A pure DNA should show a characteristic nucleic acid spectrum with a peak at 260 to 296 nm, a minimum at 230 nm, no shoulder between 260 and 290 nm, a zero absorbance at 300 nm and an A_{260}/A_{280} ratio of about 2. It should also have a hyperchromicity of about 40% and show a steady fall in absorbance until 10°C below the melting transition. It should also be digestible with a DNA restriction enzyme such as EcoR.

In order to obtain controlled rates of reassociation the fragment size must be controlled. In order to ensure independent reassociation of different repetitive sequence elements, which may

be linked in long fragments of DNA, it is usual to use short DNA fragments. The DNA in these experiments was sheared using a MSE ultrasonicator to a size of 450 nucleotides, an optional size for these experiments (Britten et al, 1974). The size of the DNA was checked by electrophoresis in alkaline-agarose gels with restriction digests of phage DNA as molecular weight markers.

To monitor the DNA with S_1 nuclease it is required that the DNA be labelled. It was decided to label Ggt DNA with (^{32}p) by nick translation - (described elsewhere) E. coli DNA, to be used as an internal standard in order to determine the rate constant was labelled with tritium by nick translation. Since the digestion with deoxyribonuclease I in the nick translation reaction is calculated to give a fragment size of 500 nucleotides native DNA was used, and the products size checked by alkali agarose gels electrophoresis. The product was also checked for its sensitivity to nuclease S_1, digestion. Native DNA should be 95% resistant to digestion and denatured DNA (by boiling in low ionic strength buffer and cooling rapidly on ice) should be 98% sensitive. This last point is particularly important as DNA polymerase, particularly old preparations of the enzyme, can produce hairpin-like DNA molecules in the nick translation reaction that form double-stranded (snap-back) molecules spontaneously on cooling. If this occurs then the product may show only 70 to 80% sensitivity to S_1 on denaturing. This effect can mimic snap-back DNA repetative sequences in native DNA which do occur in several organisms.

If this result is obtained then it may be necessary to use *in vivo* labelled DNA in order to eliminate this effect; however since the same enzyme would be used to label *E. coli* DNA which is known not to contain any snap-back sequences then it was felt that this would be an adequate check on the enzyme activity. In the event the products from nick-translation all showed 94 to 98% S_1 sensitivity of denatured DNA so this was not thought to be a problem. Denaturation and reassociation were performed essentially as Britter *et al*, (1974) except that they were performed in tris buffer, as phosphate poisons the S_1 nuclease enzyme.

DNA (with labelled DNA) in 10mM-tris-HCl pH 7.6, 2mM-EDTA was placed in a siliconised glass teflon-stoppered reaction tube and denatured by boiling in a water bath for 5 minutes. The tube was removed and rapidly cooled to 0°C by swirling in an ice-water bath. A zero time sample was taken and salt was added to a final concentration of 0.15 M-NaCl (by addition of 1.5 M-NaCl). The DNA solution was overlaid with a layer of liquid parafin in order to prevent evaporation and incubated at 25°C below the Tm for the DNA, with the cap tightly sealed. Samples (25 μl) were withdrawn at time intervals and diluted into siliconised Eppendorf reaction tubes containing 250 μl of S_1 buffer. The reaction mix was digested with S_1 enzyme and filter assayed, as described elsewhere in the next section.

For the experiments described here, each of the S_1 digestion products was frozen after digestion and assayed together for acid precipitable radioactivity

2.49 Nuclease S_1 Digestions

Nuclease S_1 (Sigma), an enzyme prepared from Aspergillus oryzae (U. Ando, 1961) is relatively specific for ssDNA (Sutton, 1971) and is used to determine the amount of hybrid formed in a DNA-DNA reassociation experiment. The enzyme assay was conducted in a 250 µl reaction mixture consisting of 0.03M - sodium acetate pH 4.5, 4.5 mM - zinc sulphate and 25 µg/ml of sonicated ssDNA. (DNA was added because the enzyme is inhibited at low substrate concentrations). The DNA to be analysed was added (25µl) enzyme was added and the mixture was incubated at 37°C for 2 hours.

The amount of enzyme to be used to digest the DNA was determined by titrating increasing amounts of the enzyme in a reaction mix containing native DNA and in a reaction mix containing rapidly quenched denatured DNA. The concentration of enzyme giving the greatest differentation between native and denatured DNA is used for further experiments. This was found to be 1.5 units (.1µl) in these experiments. The amount of dsDNA is determined as acid-precipitable radioactivity.

2.50 Nick Translation

DNA was *in vitro* labelled using DNA polymerase I by nick translation according to the method of Rigby *et al*, (1977).

Deoxyribonuclease I was diluted to 133 ng/ml and activated by incubating at 0°C for 2 hours. DNA to be labelled was in 0.5M-tris-HCl pH 7.5, 0.05M-$MgCl_2$, 0.5 mg/ml bovine serum albumin, 5m M-dATP, 5mM-dGT 5mM-dCTF and 5mMdTTP containing 20-200 Ci of either (^{32}p) or (^{3}H) labelled dATP and dCTP and containing 0.5µg of DNA in 20µl . 1µl of deoxyriboniclease I was added and the mixture was incubated at 37°C for 1 minute. 0.5µl of DNA polymerase I was added (1 unit/µl - a gift from P.W.J. Rigby) and the mixture was incubated at 14°C for 90 minutes. The reaction was stopped by addition of 10µl of 0.25M-EDTA, pH 7.4 and was incubated at 68°C for 10 minutes. Unincorporated nucleotides were removed by passing through a column of Sephadex G-50 (fine equilibrated with 10mM-tris-HCl pH8.0), 10mM - NaCl, 2mM-EDTA.

The deoxribonuclease digestion in the reaction was calculated to give a fragment length of about 500 nucleotides, an optimum size for DNA reassociation experiments.

2.43 Thermal Analysis of DNA Determination of T_m

The thermal denaturation of DNA in solution was followed spectrophotometrically by measuring the absorption of the solution at 260 nm. The procedure is that described by Mandel and Marmur (1967).

A Varian Cary 219 uv/vis Spectrophotometer was used with a Temperature Readout Accessory, Thermostattable Sample Base Accessory, Thermal Isolator Accessory and Cell Programmer Accessory. An external circulating temperature bath system was used. The cells used were rectangular silica glass of 1 cm path length and fitted with a Teflon cap to prevent evaporation. The cell for the temperature probe was constructed by filling a standard 1 cm cell with buffer solution, cutting and fitting a rubber Pasteur pipette bulb over the top of the cell, puncturing the bulb with a razor blade to form a tiny slit, and inserting the probe through the slit into the solution. The probe was then plugged into the Temperature Readout Accessory socket so that the measured temperature appeared on the Temperature Readout Accessory display and the recorder x-axis was controlled by the accessory. The Thermal Isolator Accessory was installed and the unit was continuously insulated with running cold tap water. The Temperature Readout Accessory converts the chart recorder to provide profiles of absorbance (y-axis) as a function of temperature (x-axis) where the temperature scale was $1^{o}C/cm$.

DNA solutions (1.8 ml, A_{260} = 0.5 - 1.5) were inserted into the Thermostattable Sample Base Accessory and a cell containing the solvent buffer

was inserted into the reference base. The cell containing the temperature probe was inserted into another compartment of the Sample Base Accessory. The initial absorbence at 260nm was recorded and the DNA was scanned between 320 and 220 nm. The pen was aligned on the chart paper. The temperature was quickly raised to 50°C by connecting to the temperature bath circulator, previously equilibrated to 50°C. The cuvettes were removed, inverted and tapped to remove any gas bubbles adhering to the walls of the cuvette. The cuvettes were returned to the chamber and the temperature was brought to 60°C. The temperature was then raised 5°C at a time, with 10 minute intervals to equilibrate. When the absorbence began to rise, or the temperature became near the expected rise the temperature was raised in 2°C increments. The temperature was increased until no further absorbance increase was observed. It was found that the maximum temperature attainable using a water filled temperature bath was 98°C. Both the sample and reference cells, containing sample and solvent were accurately weighed before and after the determination. If the difference was more than 3% the run was discarded.

The hyperchromicity was calculated as:

$$\frac{\text{the final absorbance - initial absorbance}^{(25^{\circ}C)}}{\text{initial absorbance } (25^{\circ}C)} \times 100$$

The final absorbance was corrected for the concentration dilution caused by solvent expansion at higher temperatures, according to Mandel and Marmur (1967). The temperature corresponding to half the final increase in absorbance (corrected for volume expansion) is designated the Tm.

3. RESULTS

3.1 Isolation and Characterisation of Viruses in Single Conidial Isolates of Ggt

3.1.1 Production of Germinable Conidia and Isolation of Single Conidia

The method of Deacon (1977) was used to attempt to induce isolate F6 to produce germinable conidia. Two isolates that had previously been shown to produce germinable conidia under these conditions, 45/10 and 3b1a, were used as controls.

Three flasks of each isolate were inoculated on media that had been prepared as described, but the pH of the media was not adjusted. Under these conditions it was found that isolates 3b1a and 45/10 produced polymorphic hyaline bodies measuring about 7.5 m x 2.4 m x 2.4 m, some of which could be seen to be germinating. These were identified as germinable conidia. However, the flasks of isolate F6 produced another type of spore: spherical, thick walled and much larger, measuring about 12µm diameter (Fig. 2). These bodies appeared to be budding from the ends of hyphal strands made up of enlarged cells, (Fig. 2a). Many of these spores could be seen to be germinating. These spores were recognised as 'chlamy dospores', noted by Sivasithamparam and Parker (1976) to be produced by Ggt under acidic conditions. It was thought unlikely that these would be uninucleate and so were not used. As chlamydospores of this type are normally produced under acidic conditions the pH of medium was measured before autoclaving, and was found to be pH 5.5. It was

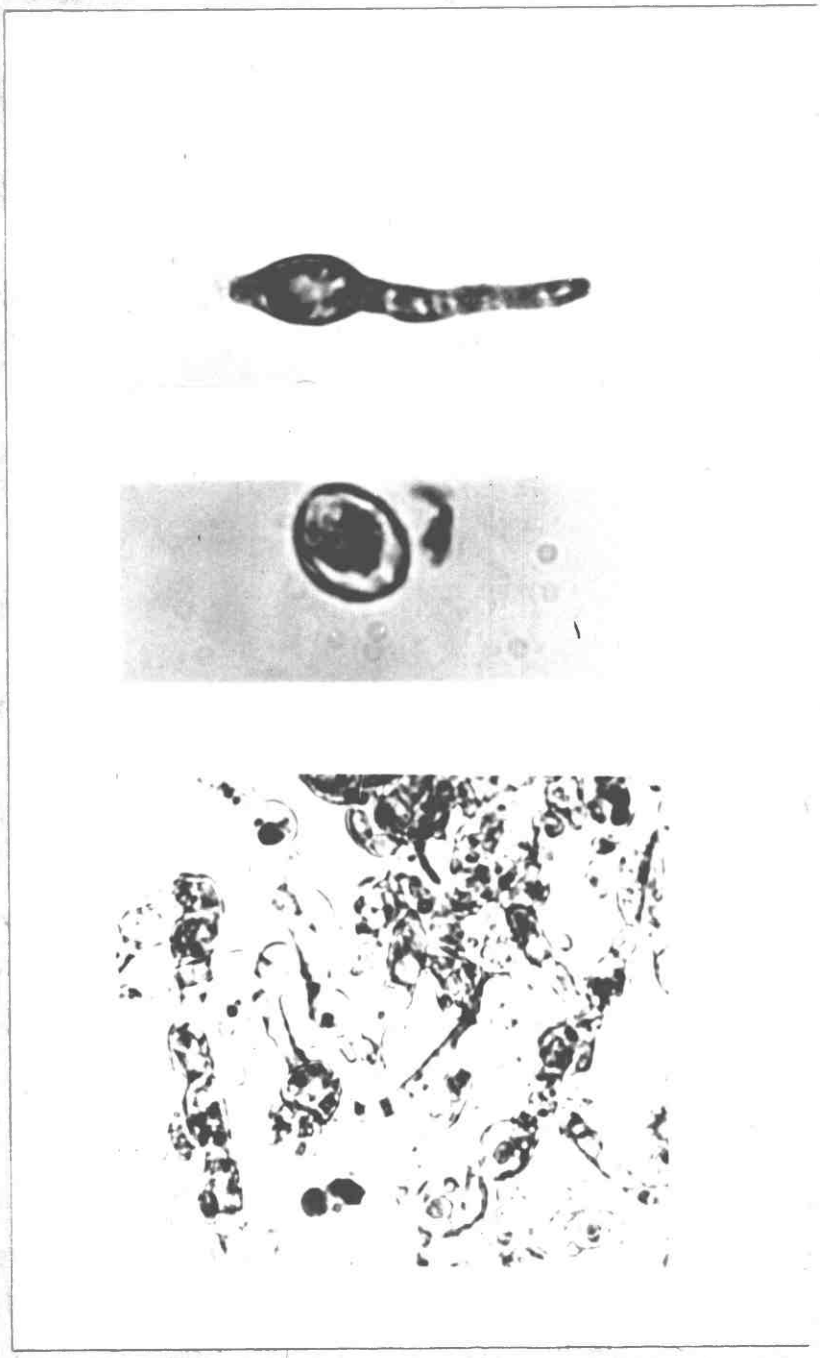


FIG. 2 Germinable Chlamydospores Produced by Ggt Isolate F6. (a) Germinatinating Chlomydospore; (b) Chlamydospore; (c) Mycelia Producing Chlamydospores

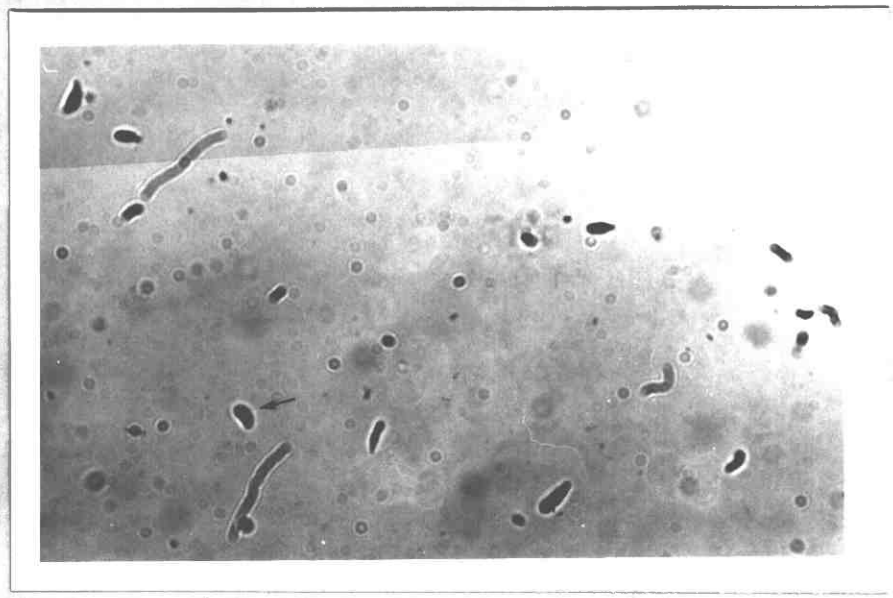


Fig. 3 Germinable conidia produced by Ggt isolate F6.
A typical conidium is shown arrowed (magnification x 1000)

therefore decided to correct the pH of the medi um to three values, pH 6.2, pH 6.6 and pH 7.0 prior to autoclaving and to inoculate three flasks of media at each pH value with isolate F6.

It was found that under these conditions, the flasks of F6 at pH 6.2 produced a mixture of chlyamydospores and germinable conidia. The flasks corrected to pH 6.6 contained only germinable conidia (Fig. 3) produced at six days after flooding, but by twelve days were no longer seen, presumably because they were no longer being produced and conidia that had been produced earlier had, by now, germinated. Seven days after flooding, one of the flasks of isolate F6 on medium corrected to pH 7.0 was harvested for conidia and eighteen single conidia were isolated. Ten of these germinated to give ten single conidial colonies, designated F6 con 1 - F6 con 10. (These are referred to by Buck et al (1981) as F6 SM1 - F6 SM10).

3.1.2. Visualization of Nuclei in Conidia and Mycelia

Conidia from isolates F6 and 3b1a were collected by centrifugation. The conidia were stained with acridine orange and their nuclei were examined by fluorescent microscopy.

The conidia were seen to be almost invariably uninucleate, occasionally (less than 1% spores were seen that seemed to contain

two nuclei. These occasional binucleate spores may be an artefact of staining or may represent cells in the process of division. This result is in agreement with that obtained by Deacon (1973) using other Ggt isolates.

Ggt mycelia were also examined for the presence of nucleiby fluorescent staining. Nuclei were clearly seen and individual cells were seen to be predominantly uninucleate. This agrees with the finding of Blanch (1977).

3.1.3. Extraction and Characterisation of Virus Particles from F6 Conidial Isolates by Electron Microscopy

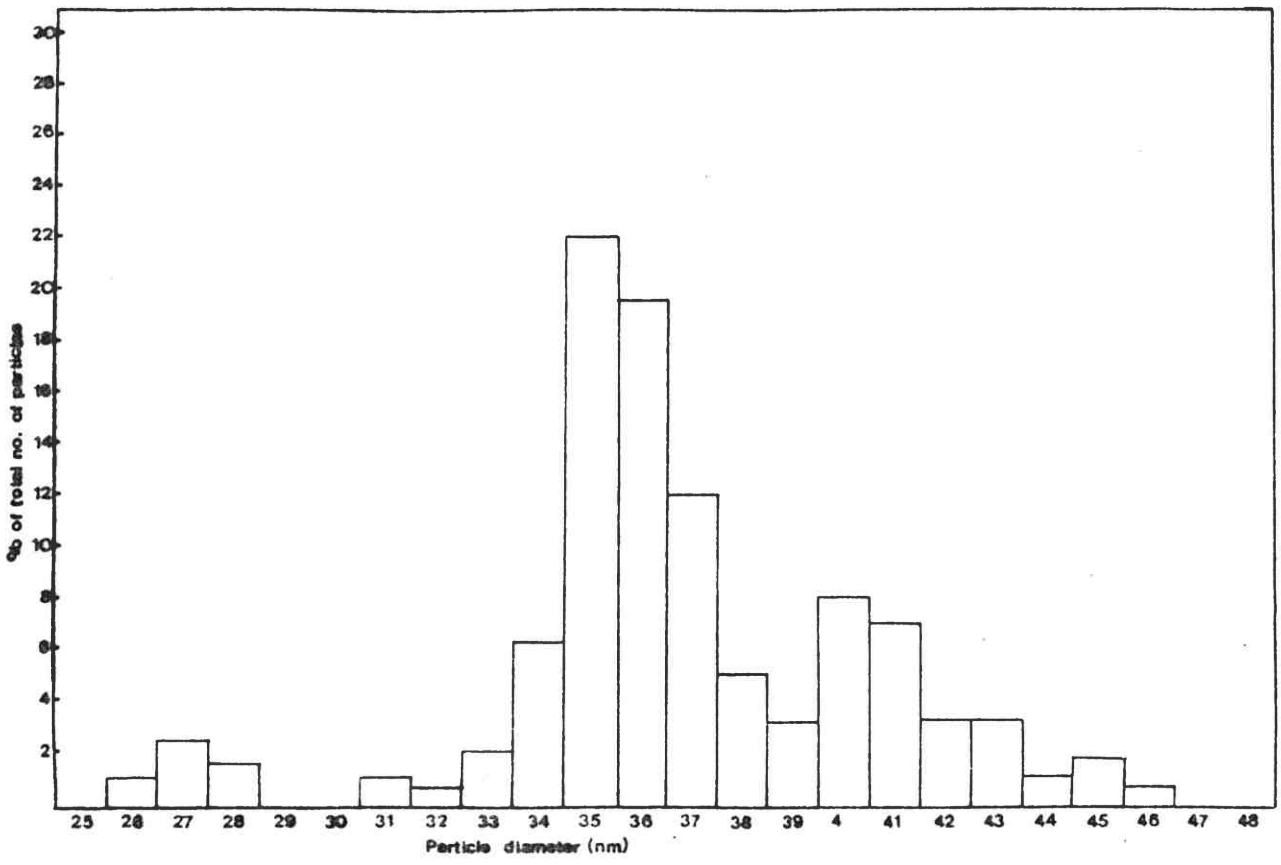
Mycelium from each of the 10 conidial isolates and the parent F6 was grown in liquid culture and small scale virus extractions were performed on each. The second virus pellet was not however passed through a sucrose gradient but was dialysed against dilute phosphate buffer and then examined by electron microscopy. The diameters of virus particles were measured and histograms were constructed of diameter of virus particles in size classes of 0.1nm between 25nm and 48nm.

The yields of virus were about 5µg/g wet weight of mycelium. All of the conidial isolates and the parent F6, contained virus particles. The histograms of particle sizes for each of the conidial isolates and

the parent F6 showed the same characteristics; that is, three modes of particle size: 26-28nm (approx.30% of total particles) 34-37nm (approx. 57% of total particles) and 40-42nm (approx. 18% of total particles). "Empty" particles in which the stain had penetrated were seen in all samples. Since all of the histograms are very similar, only one, that for F6, is shown here (Fig. 4). A typical electron micrograph of F6 virus particles is also shown (Fig. 4).

3.1.4. Extraction and Characterisation of dsRNA from F6 Connidial Isolates

Mycelia from each of the ten single conidial isolates were grown and dsRNA was extracted. The dsRNA was passed through a column of Whatman CF11 cellulose and fractions containing nucleic acid were collected and examined by electrophoresis on 4% polyacrylamide gels. It was found that dsRNA was present in the 35% ethanol (DNA) wash. This was shown to be dsRNA by nuclease tests and probably represents a fraction of the dsRNA that does not bind to CF11 cellulose. Other minor bands also present in the 35% ethanol wash, were shown by nuclease tests to be DNA. DsRNA was also present, as expected, in the 0% ethanol wash; this gave cleaner bands than the 35% ethanol wash, lacked the minor DNA bands, but contained the same dsRNA banding pattern as nucleic acid from the 35% ethanol wash. The yield of dsRNA was about the same for each isolate, about 1-2μg of dsRNA per gram (wet weight) of mycelium.



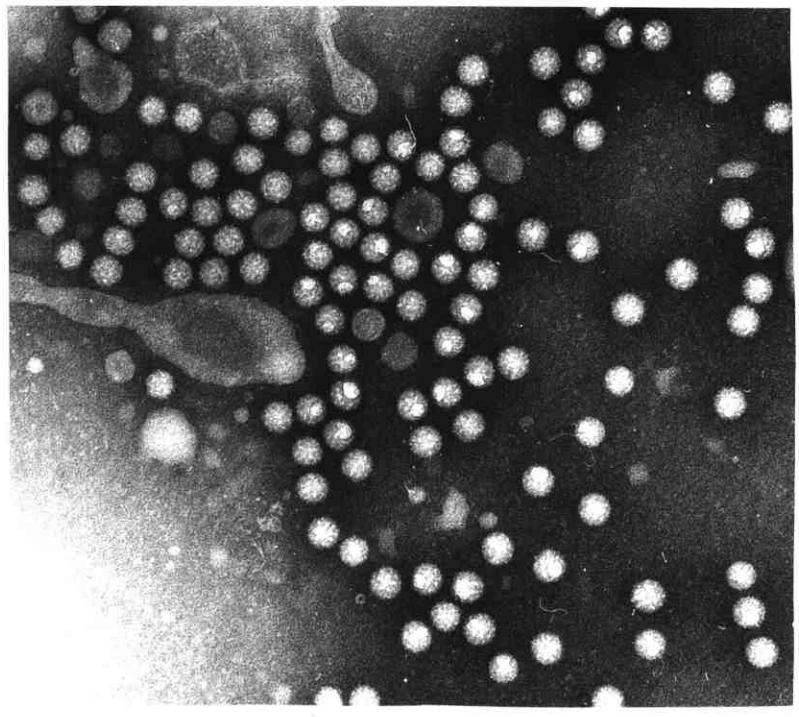


Fig. 4a Histogram of virus particle diameter of total virus isolated from isolate F6. Fig. 4b shows a typical electron micrograph from which the above histogram was derived.

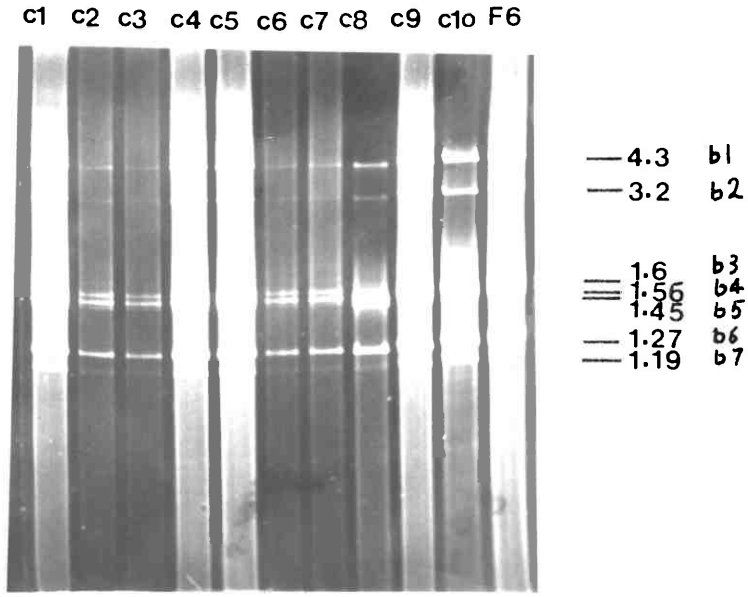


Fig. 5 PAGE of dsRNA (20µl samples) extracted from Ggt isolate and conidial progeny con 1 - con 10. The mol. wt. of dsRNA components (x 10^{-6}) is shown. The gel was electrophoresed at 50V for 24 hours and stained with ethidium bromide.

The dsRNA banding patterns for con 1, con 2, con 3, con 4, con 5, con 6, con 7 and con 9 were identical to that of the parent F6. However, it can be seen (Fig. 5) that con 8 and con 10 have both lost a single dsRNA component, b 3 (molecular wt. 1.60×10^6). The remaining bands co-migrated with the equivalent bands of F6.

3.1.5. Separation and Characterisation of Three Serologically Unrelated Viruses from F6 Single Conidial Isolate con 8.

In order to discover the number of unrelated viruses in F6, and also to assign dsRNA components to distinct viruses, virus prepared from F6 con 8 was purified and individual viruses were separated and characterised. At this stage it was hoped that ascospores would be produced from F6 conidial isolates. F6 con 8 was chosen for further study, as in the attempts to induce F6 conidial isolates to produce perithecia, F6 con 8 was at this time producing what were thought to be immature perithecia (these developed no further and were later assumed to be sclerotia).

F6 con 8 was grown in two 60 litre fermenters and the mycelium (8kg wet weight) homogenised and the virus-containing PEG precipitate was obtained as described in Methods. After resuspension of the PEG precipitate and ultracentrifugation, crude virus preparation of 120 ml. volume in PBK buffer was obtained.

Page of 25µl of the preparation (after SDS treatment) showed the expected pattern of six dsRNA components.

The virus was further purified by rate zonal sucrose density gradient centrifugation in two 60ml portions. Each portion was centrifuged at 40000 rev/min for 3-5 hours in a 20-50% (w/w) sucrose gradient. The gradients were then passed through an ISCO u.v. analyser and collected in 15ml fractions. A typical ISCO trace is shown in Fig. 6.

The dsRNA content of the zonal fractions were examined by Page (Fig. 7). Viral dsRNA bands were found in fractions of the peak, contained only the four lower molecular dsRNA components (b3 to b6). The fractions 19 to 36 contained increasing amounts of dsRNA components b1 and b2 and decreasing amounts of b3 to b6. The dsRNA components b3 to b6 could not be detected in fractions 31-34.

These results suggested that at least two viruses were present in F6 con 8: one virus containing b1 and b2 dsRNA components which will be termed virus A, and a second virus containing dsRNA bands b3, b4, b5 and b6, which will be termed virus BC, for reasons that will become clear. It can be seen that in the early fractions in which virus A appears, e.g. fraction 22, b2 is more intense than b1, however in later fractions b1 becomes more intense than b2. This is consistent with b1 dsRNA and b2 dsRNA being encapsidated separately: b1 particles would be denser than b2 particles and would sediment faster.

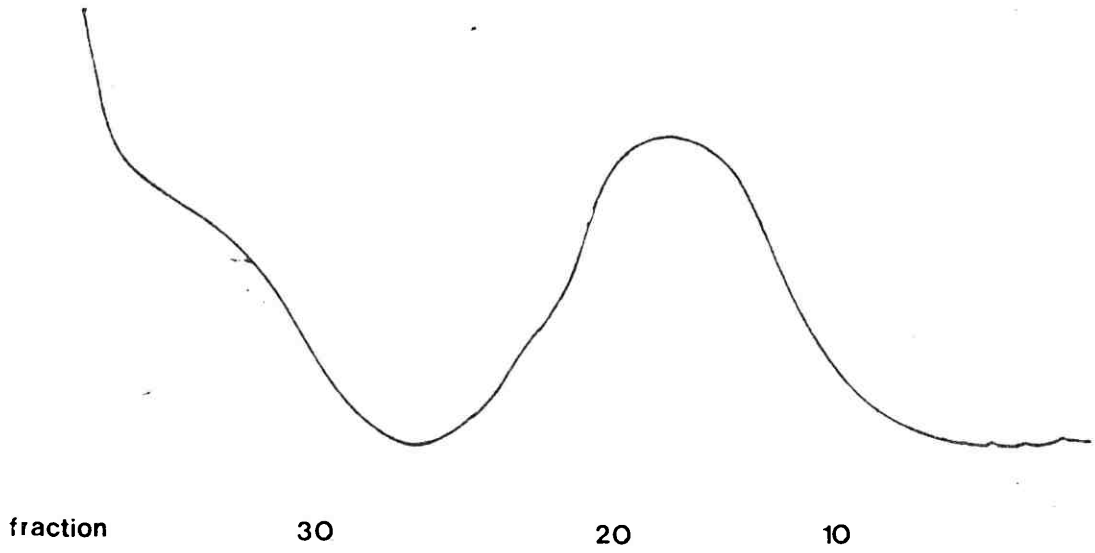


Fig. 6 A_{254} (ISCO) trace of F6 con 8 zonal sucrose density gradient fractions.

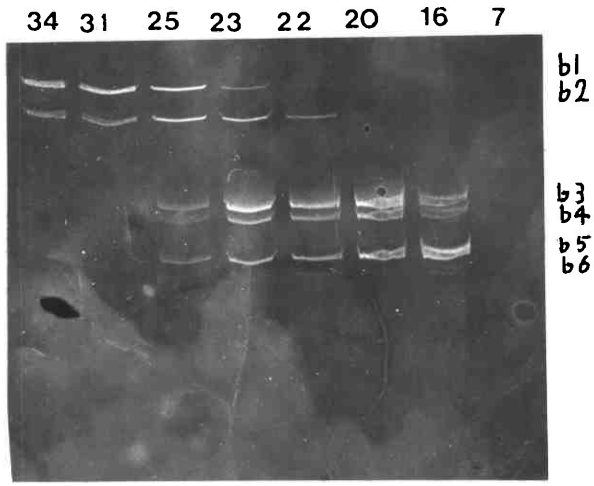


Fig. 7 PAGE of dsRNA (20μl samples) extracted from F6 con 8 zonal sucrose density fractions. The gel was electrophoresed at 50V for 24 hours and stained with ethidium bromide.

Electron micrographs of fraction 16 particles showed predominantly 35nm particles with a small number of possibly empty 40nm particles. Electron micrographs of fraction 33 particles contained predominantly 40nm particles. From this it could be postulated that 40nm particles encapsidate b1 and b2 dsRNA components and 35nm particles encapsidate dsRNA components b3, b4, b5 and b6. 27nm particles were seen in crude preparations of the virus (prior to sucrose gradient fractionation) but were not seen in any of the u.v. absorbing fractions collected. This suggests that 27nm particles are unstable to sucrose gradient fractionation or do not contain u.v. absorbing (at 260nm) nucleic acid ("empty" particles). It would seem unlikely that they contain dsRNA since all the dsRNA components seen in the crude preparation are present in the virus fractions examined.

In order to further purify the viruses present in F6 con 8 it was decided to use DEAE-cellulose chromatography, essentially as described by Buck and Kempson-Jones (1973). DEAE-cellulose (Whatman DE-52) is an anion exchanger, it is therefore necessary to select a pH at which the virus is negatively charged and a buffer of lower ionic strength so that the virus particles are selectively absorbed. Virus particles are later displaced by increasing the concentration of anion in the buffer, this is usually achieved by applying a salt gradient. Test runs were first performed using small aliquots of virus (1ml of fraction 26; $A_{260} = 0.8$) applied

to columns of Whatman DE52 containing 1 ml of DE-52 in a 5ml column. The conditions for absorbtion and elution planned were: absorbtion in PB and elution in a gradient of 0-1, 5M-NaCl in PB; absorbtion in PB/20 and elution in gradients of PB/20 - PB followed by a gradient of 0-1.5M-NaCl in PB; absorbtion in 0.2M-sodium acetate pH 7.0 and elution in a gradient of 0 - 1.0M -NaCl in the acetate buffer; absorbtion in TBE-2 and elution in a gradient of 0-1.0M-NaCl in TBE-2. Fractions containing nucleic acid were located by fluorescence on ethidium bromide plates and dsRNA bands were identified by PAGE after disrupting virus with SDS.

It was found that all virus present in fraction 23 precipitated on dialysis against 0-2M-sodium acetate pH 7.0 and so chromotography was impossible. Virus did not bind to DE52 in PB and so no separation was achieved. Virus bound to DE52 in PB/20 and eluted between 0.0-1.0M sodium chloride in PB/20; however little separation of the individual viruses was achieved. Virus bound to PE52 in TBE-2, and eluted between 0.0-0.7M sodium chloride in the same buffer. Microgels of dsRNA-containing fractions revealed that three virus had eluted separately with little overlap. It was decided therefore to use these conditions to purify larger amounts of each virus.

Fractions 13-16 of the zonal sucrose gradient containing virus B and C were combined and dialysed against TBE. The total volume was 180ml and the absorbance at 260nm was 1.52. A column

was prepared containing 80ml of DE52, in TBE-2, in a 200ml column. The virus solution was passed through the column and eluted in a gradient of 0.0-0.8M-sodium chloride in TBE-2 and 10ml fractions were collected. Virus eluted at 0.2M-NaCl containing dsRNA components b3 and b4 and was designated virus B. Virus eluted at 0.5M-NaCl containing ds components b5 and b6 and was designated virus C. Virus A was purified by salt elution of combined sucrose gradient fractions 20-33 and was eluted at 0.4M-NaCl. A 4% polyacrylamide gel of the dsRNA components of separated viruses is shown in Fig. 8. Electron micrographs of purified viruses A, B and C (Fig. 9) confirmed that virus A consisted of 40nm particles and viruses B and C contained 35nm particles.

In order to examine the polypeptide components of F6 viruses A, B and C the viral polypeptides were analysed by SDS-PAGE in a12% Laemlli gel Virus A contained three polypeptide components, virus C contains a single polypeptide component. The three polypeptides of F6-A were shown to co-migrate with three polypeptides of mol. wt. 87000, 83000 and 78000 respectively of another Ggt virus, 3b1a-A (Buck et al, 1981). The single polypeptide component of F6-C was shown to migrate just below the single polypeptide component of mol. wt. 55000 of 3b1a virus C, its molecular weight was determined (Buck et al, 1981) as 54000.

No bands were visible in the track for F6-B virus, nor was there any component of unfractionated F6 virus that was not accountable

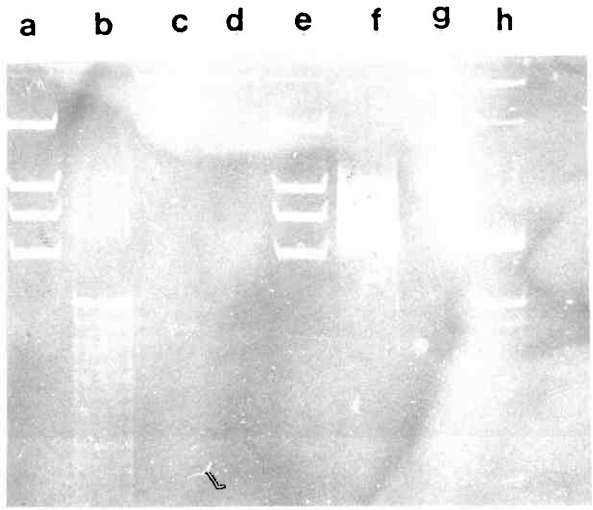


Fig. 8 PAGE of dsRNA (20μl samples) extracted from separated viruses F6-A, F6-B and F6, and mol. wt. standards, (a) Aspergillus foetidus virus F (Asf-F) dsRNA components of mol. wt. 2.7, 1.87, 1.70 and 1.44 x 10^6 (from Buck and Ratti, 1977), (b) virus F6-C, (c) Aspergillus foetidus virus S (Asf-S) dsRNA components of mol. wt. 4.1 x 10^6, (Buck, 1980) (d) virus F6-A, (e) Asf-F dsRNA, (f) virus F6-B, (g) Asf-S dsRNA, (h) dsRNA extracted from F6 con 8, electrophoresed and stained as in Fig. 7.

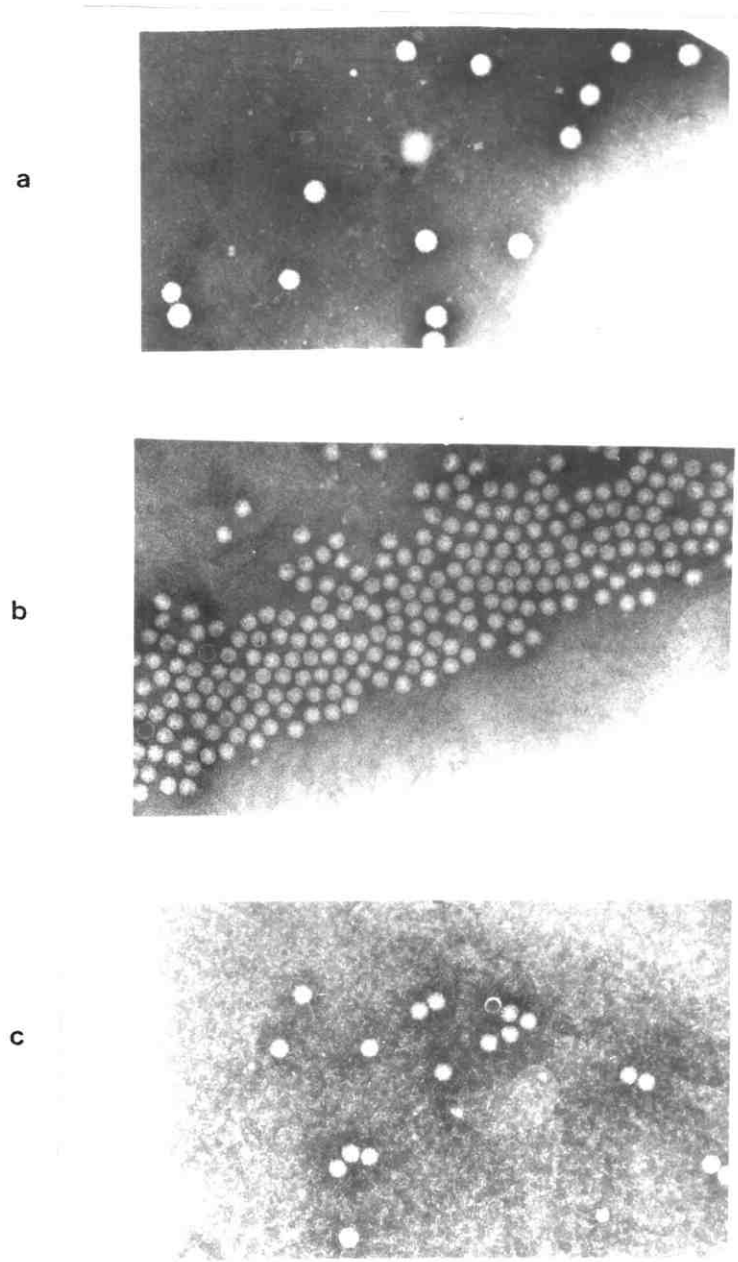


Fig. 9 Electron micrograph of (a) F6-A virus (40nm particles), (b) F6-B virus (35nm particles) and (c) F6-C (35nm particles), all at '75,000 x magnification.

by the polypeptide components of viruses A and C. In the track containing F6-B virus, and also in the total F6 virus track, were seen, high molecular weight polypeptides, presumably consisting of aggregated virus B viral polypeptides.

3.1.6. Attempts to Disaggregate Virus F6-B Capsid Protein

The capsid protein of virus F6-B appeared in SDS-PAGE only as aggregated subunits. The following treatments were tried in order to disaggregate the virus polypeptides: (i) Incubation of virus in 8M-urea or 6M-guanidinium chloride (followed by dialysis into 8M-urea since SDS is not soluble in 6M-quanidinium chloride) at 50°C for 3 minutes and electrophoresis in 8M-urea-SDS polyacrylamide gels. (ii) Oxidation with performic acid (Hirs, 1956) 0.5ml of virus was dialysed against 98% formic acid at 5°C overnight. 5µl of 30% hydrogen peroxide was then added and the mixture incubated at room temperature for 30 minutes before the reaction was stopped by addition of 20ml of water. The mixture was then freeze dried and the residue was disolved in 0.5ml of PBS, containing 2mM-EDTA. (iii) Reduction and carboxymethylation (Etten et al, 1976). To 0.5ml of virus was added 0.05ml of 0.5M-boric acid and the pH of the mixture adjusted to pH9.0 with NaOH. The mixture was boiled for 1 minute, cooled, then made 20mM in iodoacetamide and incubated for 15 minutes at 50°C. (iv) Treatment with alkali (Seghel et al, 1977). Virus was dialysed against 1%-ammonium carbonate for 18 hours at 5°C. After treatments (ii), (iii) and (iv) the product was made 1% in 2-meriaptoethanol, heated to 100°C for 3 minutes, cooled and analysed by SDS-PAGE.

All of these methods failed to disaggregate the F6 polypeptides into monomers. Various combinations of these methods were tried ((i) with (ii), (i) with (iii), (i) with (iv), (ii) with (iv), (ii) with (iii)) but produced similar results, some treatments degrading the proteins so that nothing was visible on the gel.

It is po sible that the polypeptides may have become covalently cross-linked during virus purification as a result of action of polyphenol oxidases (Bencroft et al, 1971).

Viruses related to virus F6-B have polypeptide components in the range 68-73000 and often show a tendency to form stable aggregates (Buck et al, 1981). Almond (1978) mentions no polypeptide of this mol. wt. but re-examining his photographs a polypeptide of mol. wt. 73,000 can be seen in SDS-PAGE of his samples of F6 virus. Buck (Buck et al, 1981) on applying Almond's samples to SDS-PAGE confirmed a polypeptide of mol. wt. 73,000, however much of the material seems to be aggregated. The occurrence of monomers of virus F6-B in Almond's preparations of F6 virus possibly reflects a different degree of action of polyphenol oxidases in the preparation.

3.1.7. Serological Studies with Viruses F6-A, F6-B and F6-C

Virus from isolate F6 was used to produce antisera in rabbits. This was in order to determine the number of unrelated viruses in F6 and also, for use in ELISA tests (Section 3.3.3) for detection of virus in hyphal-tip cultures.

At this time, only virus purified by sucrose gradient was available. It was decided therefore to obtain two antisera, one elicited using sucrose gradient fraction 17 (A_{260}=1.5) containing viruses B and C, and the second elicited using sucrose gradient fraction 33 (A_{260}=0.5) containing virus A plus trace amounts of virus B and C. These viruses were infected into separate rabbits. Test bleeds were made and blood was harvested from the rabbit infected with sucrose gradient fraction 17, four weeks after the first infection antiserum was prepared and termed antiserum L (light virus-directed). Blood was harvested from the rabbit infected with sucrose gradient fraction 33, six weeks after the first infection; antiserum was prepared and termed antiserum H (heavy virus-directed).

The nature of serological relationships between the viruses in F6 was examined by double immunodiffusion (Ouchterlony) tests. Antisera L reacted against homologous virus giving two precipitin lines, one titrating to 3200 and another line titrating to 4096. Antisera H reacted against homologous virus giving two precipitin lines: one titrating to 3200, and a weak line titrating to 800. When these two antisera were mixed in equal proportions and reacted against a mixture (1:1) of sucrose gradient fraction 17 plus sucrose gradient fraction 33, three precipitin lines were seen (Fig. 10). This was one of the first indications that F6 virus may be composed of three distinct viruses.

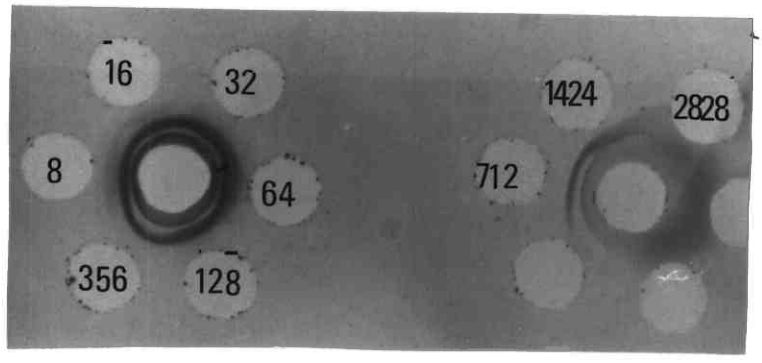


Fig. 10 Gel immunodiffusion tests of F6 virus mixture (sucrose gradient fractions 1:1, Section 3.1.4.) reacted against dilutions of rabbit antiserum to F6 viruses (H antisera Section 2.1.4.2). The gel was stained with coomassie blue.

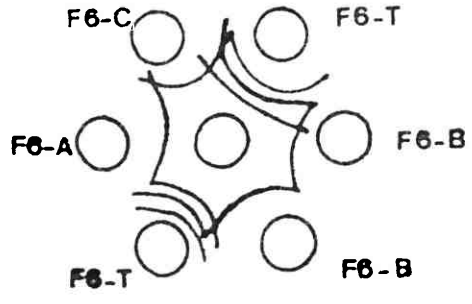


Fig. 11 Gel immunodiffusion tests of viruses from F6, using rabbit antiserum to F6 viruses (H antisera). Diagrammatic representation lines were too faint to be photographed.

As described above, F6 virus was fractionated into virus A, virus B and virus C. In order to investigate any serological relationships between these viruses, antiserum (L + H antisera, mixed 1:1) was reacted against purified separated viruses.

Virus A gave a single band titrating to 800, virus B gave a single band titrating to 3200; virus C gave a single band titrating to 2800. When the three viruses were mixed, three bands were obtained. In order to investigate the serological relationships between the viruses it was necessary to have separated viruses in adjacent wells and at a dilution such that the precipitin lines would cross. This proved to be difficult and involved several immunodiffusion plates. For this reason and the fact that some of the precipitin lines were faint, a diagramatic representation of the result is shown in Fig. 11.

Each combination of viruses produced crossing precipitin lines indicating the absence of common antigenic determinants between viruses F6-A, F6-B and F6-C, detectable using these antisera.

3.1.8. Sedimentation Coefficients of F6 viruses

The sedimentation coefficients of separated viruses of F6 were determined by measurement of boundary sedimentation. The values for sedimentation coefficients (S_{20}) were F6 virus A = 159S,

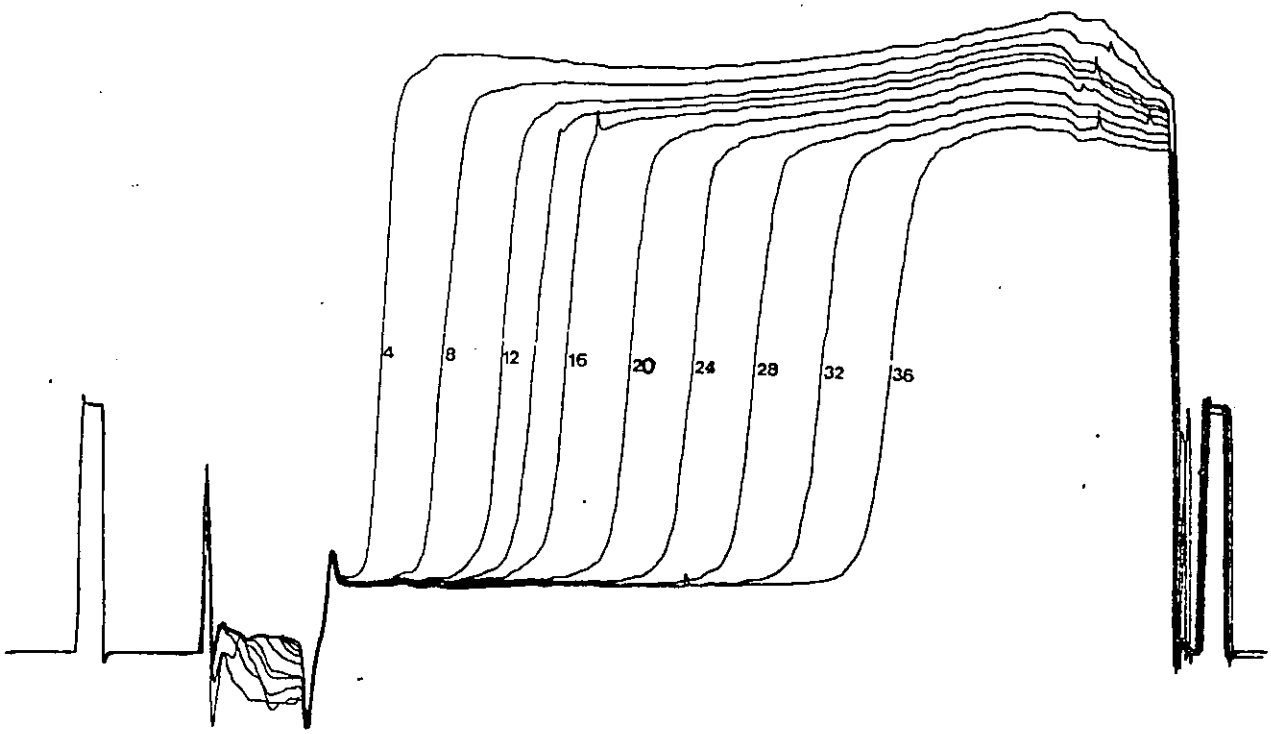


Fig. 12 Sedimentation trace of virus F6-B using the MSE analytical ultracentrifuge The time phase (in mins.) of each trace, from the start of centrigutation, is shown.

F6 virus B = 133S; F6 virus C = 128S. Determinations were in PBK buffer and the values are the means of the three determinations. In no case did any of the three values differ by more than 2 units from the mean value. A typical boundary trace, produced by virus F6-B, is shown in Fig. 12.

The value of the sedimentation coefficients reflects the particle size and dsRNA components. Since each dsRNA component is probably separately encapsidated as in other isometric dsRNA mycoviruses (Hollings, 1978) the values of sedimentation coefficient are therefore averages of the different component particles in each virus. PAGE of sucrose density gradient fractions of F6-con 8 viruses (Section 3.1.5.) showed differing proportions of two dsRNA components of virus F6-A in different fractions. This suggested that separated encapsidation occurred at least in this Ggt virus.

3.1.9. Viruses from Ggt Isolate 3b1a

Work by K.W. Buck and M.R.A. Almond (Buck et al, 1981) showed that Ggt isolate 3b1a contained polyhedral particles of two sizes, 35nm and 40nm diameter, which in agarose gels migrated as three distinct bands and gave three precipitin lines in gel immunodiffusion tests against antiserum prepared against the virus mixture, indicating the presence of three viruses. The 40nm particles (3b1a-A virus were obtained free from the other two viruses by sucrose density gradient centrifugation and shown (Fig. 13) to contain the dsRNA components of

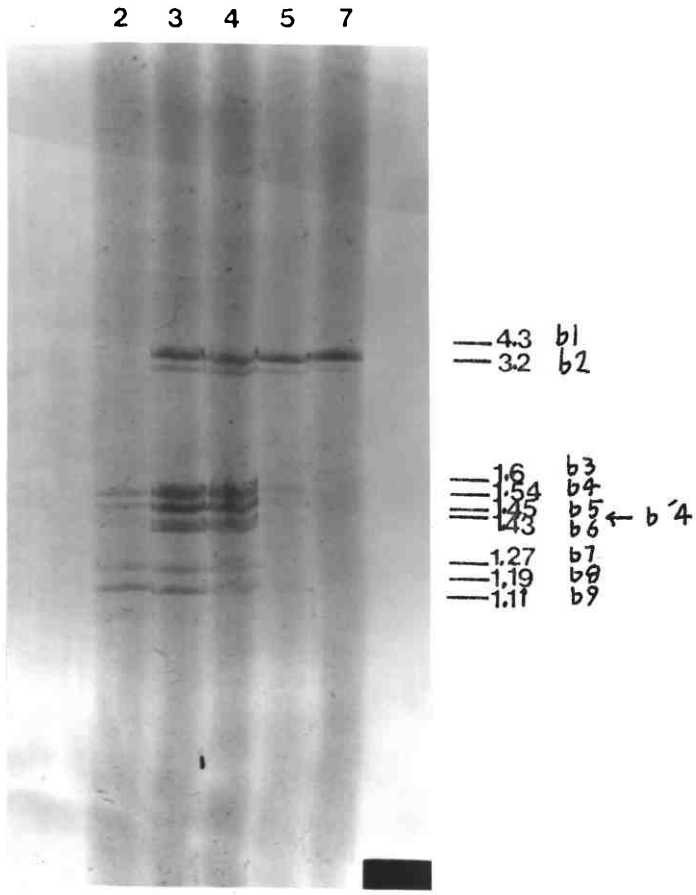


Fig. 13 PAGE of dsRNA extracted from 3b1a sucrose gradient fractions 2, 3, 4, 5 and 7, by K.W. Buck. The mol. wt. of dsRNA components are shown. The gels were stained with 0.01% toluidine blue.

mol. wt. 4.1 and 3.5 x 10^6. The two 35nm particles were separated by salt gradient elution from DEAE-cellulose (as described in Methods).

Virus 3b1a-B was shown to contain four dsRNA components of mol. wt. 1.60, 1.54, 1.45 and 1.43 x 10^6, and virus 3b1a-C was shown to contain three dsRNA components of mol. wt. 1.27, 1.19 and 1.11 x 10^6. The serological relationships between these viruses and the viruses of F6 were investigated: 3b1a-A and F6-A were found to be serologically related; as were 3b1a-B and F6-B, and 3b1a-C and F6-C viruses.

The polypeptide components of the 3b1a viruses were examined: 3b1a-A contain 3 polypeptides (mol. wt. 87, 83 and 78000), 3b1a-B contained two polypeptide components (73000 and 68000), the lower mol. wt. component was thought to be possibly a degradation product or the 73000 mol. wt. component (these were determined by M.R.A. Almond as 68 and 66000 mol. wt. but redetermined by K.W. Buck as 73 and 68000).

Evidence obtained from this work suggested that virus 3b1a-B contained five dsRNA components, the fifth, a minor band behaving somewhat anomolously. On PAGE, this band migrates between the 1.45 and 1.43 mol. wt. dsRNA components, however, it is too faint to be photographed, but is seen on scanning PAGE (tube gels) of 3b1c virus mixture (Fig. 42). On urea-PAGE however, this band migrates between the 1.54 and 1.45 dsRNA components,

presumably because of secondary structure that is denatured in urea. This band was designated (as were the other 3b1a dsRNA bands (Fig. 13) as b'4 and its mol. wt. on urea-PAGE determined as 1.49×10^6.

3.1.10. Evidence for Four Viruses in Ggt Isolate 3b1a

Bentonite was added to 3b1a virus mixture and the mixture separated into fraction binding to bentonite and fraction remaining in the supernatant when the bentonite was centrifuged down, it become clear on analysis of the fractions by PAGE (Fig.14a) that virus 3b1a-B wa separated into a fraction binding to bentonite containing the lower dsRNA components of virus B (b5 and b6) and fraction remaining in the supernatant containing all five dsRNA components of 3b1a-B. When the polypeptides of these fractions were analysed by SDS-PAGE, the result (Fig. 14b) showed that each fraction contains both the 73000 and 68000 polypeptides. The bentonite bound fraction however contained a higher proportion of the 73000 polypeptide than the bentonite supernatant fraction. The interpretation of this is unclear: the bentonite supernatant material contains all 3b1a dsRNA components and so would be expected to contain the polypeptide components of all viruses in 3b1a-B, however the bentonite bound material contains only b5 and b6 dsRNA components and so would be expected to contain only their associated capsid polypeptide.

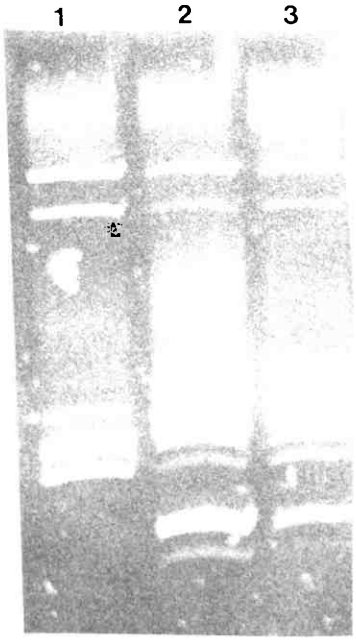


Fig 14a PAGE of 3b1a viruses separated into fraction binding to bentonite and fraction not binding to bentonite, (i) 3b1a bentonite bound, containing viruses 3b1a-A and B2, (ii) 3b1a, B1, C and small amounts of 3b1a-B2, (iii) 3b1a sucrose gradient fraction 4 viruses, electrophoresed at 90V for 20 hours, and stained with ethidium bromide.

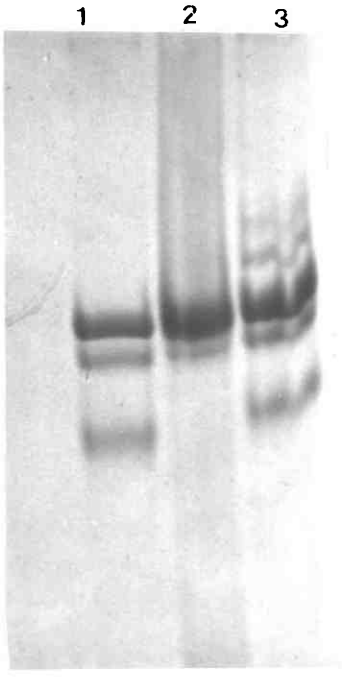


Fig. 14b SDS-PAGE of 3b1a viruses separated into fraction binding to bentonite and bentonite supernatant. (i) bentonite supernatant (from sucrose gradient fraction 2, not containing virus 3b1a-A), (ii) bentonite bound fraction, (iii) 3b1a viruses sucrose gradient fraction 4, electrophoresed in an 8% polyacrylamide gel at 40V for 18 hours.

In order to examine the serological relationship between the bentonite bound and bentonite supernatant material 3b1a (sucrose gradient fraction 4) was separated into bentonite bound and bentonite supernatant material and the bentonite supernatant was titrated against 3b1a mouse antiserum. Two bands were seen. Excision of the bands and analysis by PAGE, as above, showed that the lowest titre band (titre of 4) contained the dsRNA components of virus C, and the higher titre band contained the dsRNA components b3, b4 and b5.

3b1a viruses (sucrose gradient fraction 4, Fig. 13) and bentonite supernatant viruses were placed in adjacent wells and reacted against 3b1a mouse antiserum. The result (Fig. 15) showed that the precipitin line formed by the reaction of viruses in 3b1a virus mixture (titre of 128) formed a spur with the precipitin line formed by virus in the bentonite supernatant (spurring towards the well containing bentonite supernatant.

The results indicated that 3b1a-B is in fact composed of two viruses, 3b1a B1 (containing b3, b4, b4 dsRNA) and B2 (containing b5 and b6 dsRNA) which are serologically distinct but the viruses have the same titre against 3b1a antiserum so that their precipitin lines form at the same position. The interpretation of this result shown in Fig. 15, virus B1 in 3b1a virus mixture was able to dissolve the precipitin line formed by B1 virus in the bentonite supernatant, but the precipitin line formed by B2 in 3b1a virus mixture was not dissolved by the virus in the bentonite supernatant.

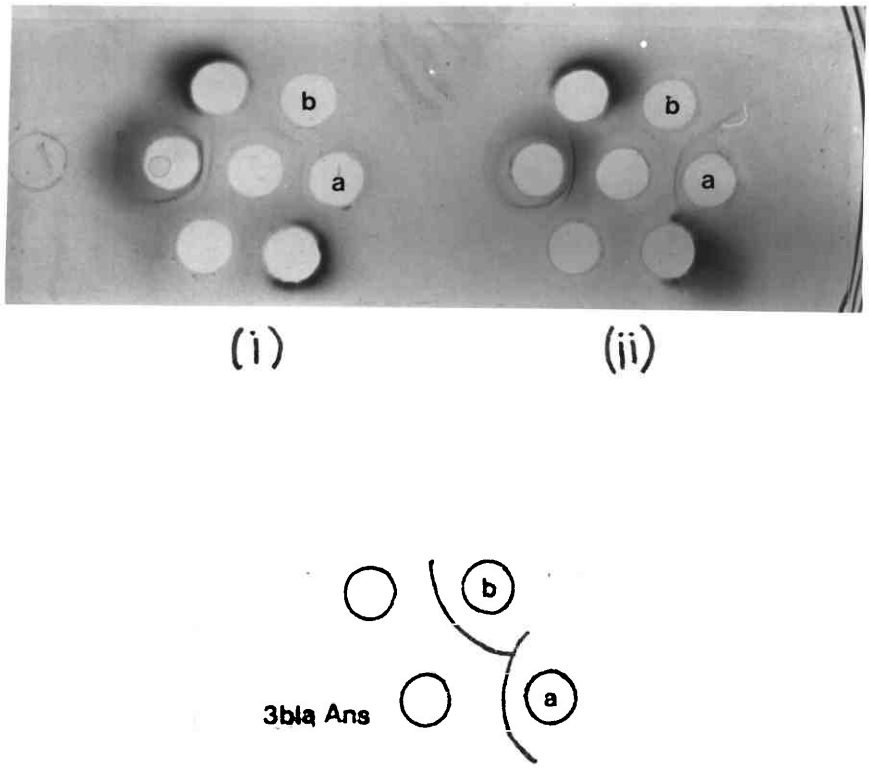


Fig. 15 Gel immunodiffusion tests of 3b1a viruses reacted against mouse antisera against 3b1a virus mixture, stained with coomassie blue, (i) at 1:64 dilution of antisera, (ii) at 1:128 dilution of antisera (in centre wells). (a) contains 3b1a sucrose density gradient fraction 2, (b) contains 3b1a virus (sucrose gradient fraction 2) fraction not binding to bentonite (bentonite supernatant). A diagramatic representation of the result is shown beneath.

The analysis of the polypeptide components of the bentonite bound and bentonite supernatant material suggested B1 contains capsid polypeptide of mol. wt. 68000, and B2 (bentonite bound virus) contains polypeptide component of mol. wt. 73000. This was not however, certain from these results; and would suggest that the virus with the lower mol. wt. dsRNA components encoded the larger capsid polypeptide. This is theoretically feasible since the dsRNA component of lowest mol. wt. in 3b1a-B2 has the coding capacity for a polypeptide of mol. wt. 79000.

3.1.11. dsRNA Content of Single Conidial Isolates Derived from Isolate 3b1a

Single conidial isolates 3b1a con C, 3b1a con D and 3b1a con E, derived from 3b1a were supplied by Deacon. dsRNA extractions were performed on each of these isolates and nucleic acid samples were analysed by PAGE. (Fig. 16)

The result shows all 3b1a conidial isolates to have an identical dsRNA pattern as the parent 3b1a. Ten dsRNA components are present (thought only nine are clearly visible on photography); their molecular weights were determined by co-electrophoresis with F6 dsRNA and dsRNA extracted from Aspergillus foetidus viruses S and F (Buck and Ratti, 1977) and agreed with the values determined by Buck (Buck et al, 1981); thought Buck noted only four dsRNA components

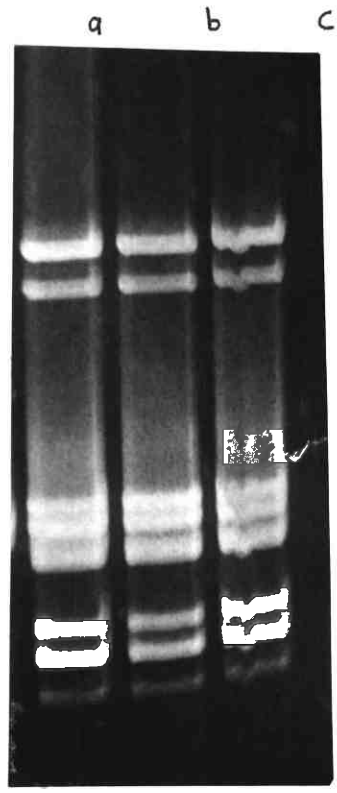


FIG. 16 Page of dsRNA extracted from (a) 3b1a con C, (b) 3b1a con D, (c) 3b1a con E. Electrophoresis was as described in Fig. 14.

in the range 1.43 - 1.60 x 10^6 molecular weight. This can be accounted for by differing gel resolution.

3.1.12 Viruses from Ggt Isolate 45/10

M.R.A. Almond (Almond, 1979) purified virus from Ggt isolate 45/10 and analysed the dsRNA and polypeptide components. Nine dsRNA components were seen of mo. wt. 1.60, 1.56, 1.54, 1.43, 1.37, 1.22, 1.20, 1.16, 1.05 x 10^6 (as redetermined by K.W. Buck personal communications); and four polypeptide components of mol. wt. 72000, 68000, 66000, 55000 were noted. Only partciles of 35nm diameter were seen in electron micrographs. Comparison with the polypeptide and dsRNA components of separated viruses of (Table 6) 3b1a and F6, suggests that isolate 45/10 contains at least two viruses, possibly varients of the viruses 3b1a-B and F6-B, and 3b1a-C and F6-C, found in isolates 3b1a and F6.

dsRNA Content of Single Conidial Isolate Derived from Isolate 45/10

Single conidial isolate 45/10 con B was obtained from Deacon and standard dsRNA extraction performed on the isolate. PAGE of dsRNA from the conidial isolate demonstrated that each of the nine dsRNA components present in the parent 45/10 were present in the conidial progeny.

TABLE 5 Physiochemical and Serological Properties of Ggt Viruses of F0 and 3b1a

Virus	Group[a]	Particle dia.(nm)[b]	S_{20}[c]	Virion dsRNA components (mol.wt. x 10^{-6})[d]	Capsid Polypeptides (mol.wt. x 10^{-3})[e]	Antiserum titre with F6 antisera[f]
F6-A	III	40	159	4.3, 3.2	87, 83, 78	4096
3b1a-A	III	40	163	4.1, 3.5	87, 83, 78	512
F6-B	II	35	133	1.60, 1.56, 1.45	73	4096
3b1a-B	II	35	140	1.60, 1.54, 1.45, 1.43	73, 68[g]	128
F6-C	I	35	128	1.27, 1.19	54	1024
3b1a-C	I	35	115	1.27, 1.19, 1.11	55	128

From Buck et al, 1981

a, b, c, d and E All as described in Table 4

f Maximum reacting dilution of rabbit antiserum, prepared against F6 virus mixture. From Buck et al, 1981

g Amended values, redetermined by K.W. Buck (personal cummunications).

3.1.13. Discussion

Isolate F6 contained three serologically distinct viruses. Single conidial strains derived from F6 each contained all three viruses demonstrating, since single germinable conidia were shown to be uninucleate, that all three viruses were contained in a single cell. Accumulation of two or more viruses by a fungus is a likely consequence of the intracellular mode of transmission of these viruses: mixed virus infections have been found in several other fungi (Hollings, 1978). Viruses F6-A, F6-B and F6-C were separated and characterised.

On the basis of these and other results, Buck et al, (1981), Table 4, demonstrated that Gaeumannomyces graminis viruses fall into three groups, distinguishable in their physiochemical (sedimentation coefficients capsid size, dsRNA and polypeptide mol wt.) and serological properties. Viruses F6-C, F6-B and F6-A are typical members of group I, II, and III respectively. Viruses were found to be serologically related within a group, but no relationships were detected between virus groups. This suggests that the viruses within a group may be all variants of a single virus. Again, the intracellular life cycle of fungal viruses may create opportunity for the survival and selection of virus mutants.

As discussed in the Introduction, Buck (1980) suggests that isometric dsRNA mycoviruses may be of two basic types: the

first type has an undivided genome of polycistromic dsRNA in the range 3 x 10^6 to 6.5 x 10^6. The second group consists of viruses with divided dsRNA genomes with a minimum of two monocistronic dsRNA segments, each with a mol. wt. in the range 1 x 10^6 - 2 x 10^6, one encoding the single capsid polypeptide, and the other the viral RNA-dependent RNA polymerase. G. graminis viruses of groups I and II, including viruses F6-B and F6-C ar probably members of this second group.

G. graminis viruses of group III, including virus F6-A may be of the former group - viruses with undivided genomes,. The dsRNA components are within the range of this group, but two dsRNA components are present in F6-A and 3b1a-A, the only other known member of this group. It is known that with many group III viruses, although only a single dsRNA component is sufficient for virus replication, in some fungal strains infected with this type of virus, there are additional dsRNA segments, which may be satellite dsRNA, encoding specific proteins, eg MdsRNA of yeast which encodes killer protein (Bostian et al, 1980), or may be deletion mutants, e.g. SdsRNA of yeast (Bruen and Kane, 1978). It is not known whether both dsRNA segments of F6-A or 3b1a-A are required for infection, and since no in vitro infection system is available, this would be difficult to determine. A study of the nucleic acid homology and polypeptide products (by in vitro translation of mRNA) of the separated dsRNA components may resolve this question.

This study demonstrated that each of the viruses of F6 were present in conidial isolates. However, in two conidial isolates, (F6 con 8 and con 10) a single dsRNA component of virus F6-B had been lost. This dsRNA component was of higher mol. wt. than the dsRNA components that remain in virus B in this isolate; suggesting that the dsRNA component that was lost is a satellite dsRNA of unknown function. As well as encoding killer protein in yeast and Ustilago, satellite RNA components may reduce infectivity of standard virus RNAs (Fritsch et al, 1978) and in cucumber mosaic virus (CMV) the presence of a satellite RNA component produces a striking change in disease symptoms on tobacco plants (Takanami, 1981). Determination of the nature of the RNA component that is lost would require analysis of the genome by either nucleic acid hybridization, RNA fingerprinting or sequencing. Romanos et al (1982) using such techniques obtained evidence for the presence of a satellite dsRNA component in virus 38-4-A - another Ggt virus.

The mechanism of exclusion of a single dsRNA component in two F6 conidia probably involves the low levels of virus present in fungal hyphal tip cells, from which conidia are produced, leading to the occasional exclusion of non-essential dsRNA components (since they are probably separately encapsidated).

The nature of the 27nm particles seen in crude preparations of F6 viruses is unknown, since the 27nm particles were not seen in preparations after sucrose density gradient centrifugation. This

indicates that these particles were either unstable in sucrose gradients or were empty particles containing no nucleic acid (and therefore not detected spectrophotometrically at 260nm).

The studies with conidial isolates derived from 45/10 and 3b1a demonstrated that all viruses and dsRNA components of the parent were inherited by the conidial progeny. It is probably, by anology with F6, that if enough conidial progeny were examined, then some would be found with fewer dsRNA components than the parent isolate. The physiochemical properties of viruses of 3b1a and F6 are compared in Table 6.

3.2 RNA Polymerases of Ggt Viruses

Many isolated mycoviruses show virion-associated RNA polymerase activity *in vitro*, demonstrated by the ability to incorporate radioactive nucleotides into high molecular weight acid-insoluble material (Buck, 1980).

The RNA polymerase activity of Ggt viruses was investigated in order to prepare radioactive ssRNA probe for detection of provirus and viral dsRNA.

In this study the dsRNA dependent RNA polymerase activity of Ggt viruses was examined in reaction mixtures similar to that used for *Penicillium stoloniferum* virus S (Buck, 1975) and *Aspergillus foetidus* virus S (Ratti and Buck, 1978) and consisted of ATP, GTP, CTP AND UTP all at 0.15mM, ^{3}H-UTP was added at a specific activity of 83mCi/mol. Actinomycin is added to inhibit bacterial growth and bentonite is added as nucleas inhibitor. The products of the polymerase reaction were examined. (i) By acid precipitation of radioactivity incorporated into high molecular weight material. (ii) By PAGE, usually urea-PAGE in which ssRNA is denatured and electrophoreses at about half the rate of dsRNA of the same ssRNA length (since dsRNA has twice the charge/mass ratio. The products were visualised by staining (usually with toluidine blue which is more sensitive for ssRNA) or by fluorography whereby scintillant was impregnated into the gels and the tritiated products visualised by exposure to photographic film. (iii) By nucleic acid hybridization for determining the homology between nucleic acid species.

F6 viruses used for study of RNA polymerase activity were either sucrose gradient fractions or purified F6-A, F6-B and F6-C (Section 3.1.5). The polymerase activity of 3b1a viruses was investigated using sucrose gradient fractions 2, 4 or 7, prepared by K.W. Buck as described in Section 3.1.9. The dsRNA components of virus from these fractions is shown in Fig. 13.

3.2.1. Virion RNA Polymerase of F6 Viruses

The RNA polymerase activities of F6 viruses A, B and C purified as described in Section 3.1.5 were examined.

3.2.1.1 Magnesium Optimum

The incorporation of (H)-UTP into acid insoluble product by F6 virus mixture (sucrose gradient fraction 17 plus 32) and viruses F6-A, F6-B and F6-C at various $MgCl_2$ concentrations was measured. The result showed F6 virus mixture and virus F6-B to have RNA polymerase activity with magnesium optima at 5mM-$MgCl_2$. Viruses F6-A and F6-C showed only very low incorporation with no optimum.

3.2.2.2 Gel Electrophoresis of Products

RNA was extracted from polymerase reaction mixtures containing F6 virus mixture (as above) and viruses F6-A, F6-B and F6, analysed

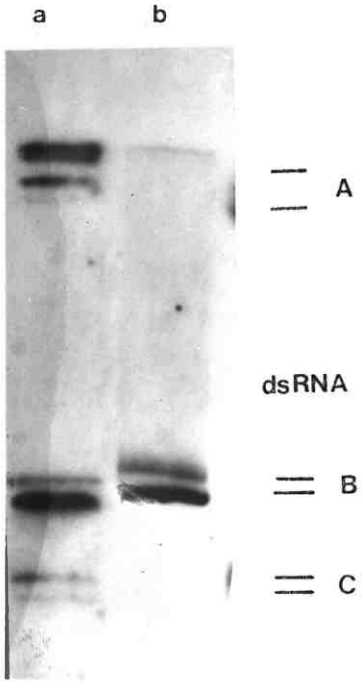


Fig. 17 Fluorograph of urea-PAGE of RNA polymerase reaction products of F6 virus mixture (a) and virus F6-B, (b) 1000 ct/min of RNA extracted from a reaction mixture was electrophoresed for 18 hours at 100V. The dsRNA components of F6 viruses are indicated (F6-A dsRNA components are present, but are not visible on fluorography, the position of the dsRNA components are indicated). 3 day exposure.

by urea-PAGE and fluorographed (Fig. 17). Virus F6-B dsRNA components were tritiated in the reaction and two ssRNA products (confirmed by nuclease tests) were produced. F6 virus mixture showed incorporation of radioactivity into F6-B and F6-C dsRNA and four ssRNA products were produced, two of faster mobility (and therefore lower mol. wt) than the products of virus F6-B and presumably the products of virus F6-C. Purified virus F6-C however, did not show any detectable polymerase activity; presumably the activity present in F6 virus mixture (sucrose gradient fractions) was inactivated on subsequent separation of virus F6-C. No incorporation into any radioactive bands was detected for virus F6-A; nor did the dsRNA components of virus F6-A become tritiated in the reaction using virus mixture.

3.2.2. Virion RNA Polymerases of 3b1a Viruses

The dsRNA-dependent RNA polymerase activities of 3b1a viruses was examined.

3.2.2.1 Magnesium Optimum

The incorporation of 3H-UMP into TCA-insoluble material by 3b1a sucrose gradient fraction 4 was measured in overnight reactions at $MgCl_2$ concentrations of 0, 2, 4, 5, 6, 7, 8 and 1-mM. The results showed that the reaction had a sharp dependence on magnesium concentration with optimum activity at 5mM (Fig. 18).

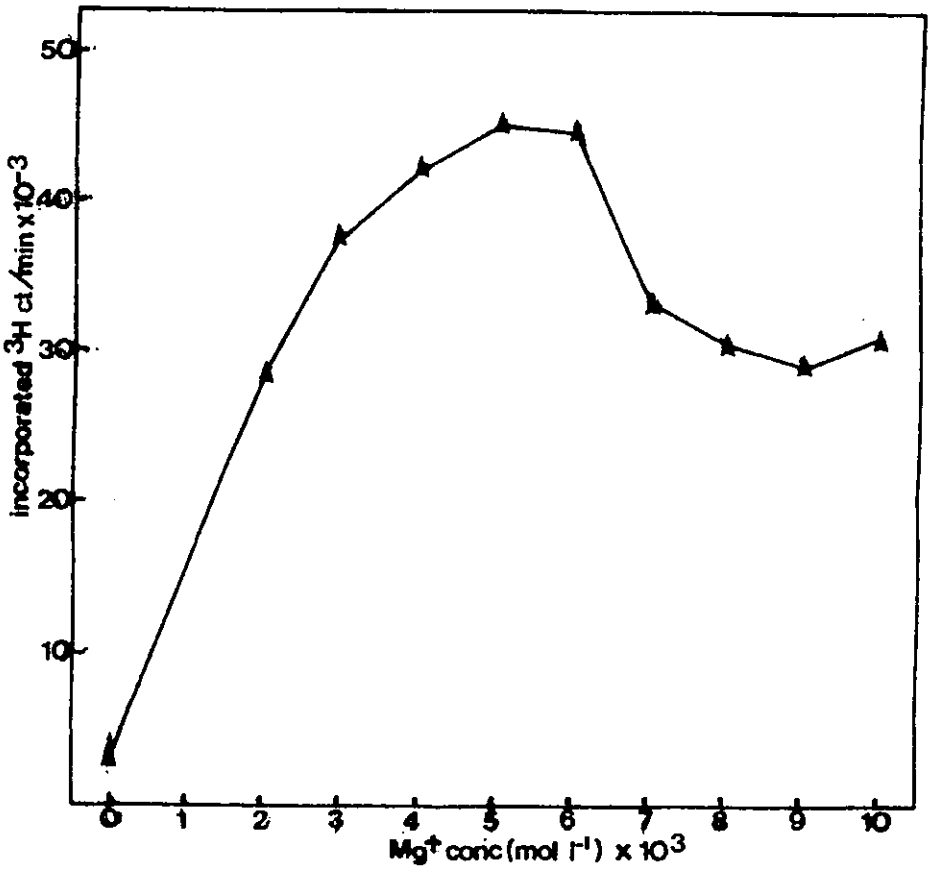


Fig. 18 Incorporation of radioactivity into acid-insoluble fraction by the RNA polymerase activity of 3b1a virus mixture (sucrose density gradient fraction 4) in an 18 hour reaction (30°C) at various magnesium chloride concentrations.

3.2.2.2 Time-course of Reaction

The incorporation of radioactivity into TCA-insoluble material was measured as a function of time. The results showed the highest rate of incorporation in the first ten hours, then a linear increase in incorporated material continuing for 5 days, the longest incubation time measured.

3.2.2.3 Gel Electrophoresis of Products

RNA was extracted from SDS-disrupted polymerase reaction mixtures. This was sometimes done after the reaction mixture had been separated into the material bound to bentonite and that remaining in the supernatant after spinning down the bentonite. This was in order to obtain information on the release of transcripts as it was thought since bentonite binds proteins that intact virus would remain bound to bentonite whereas transcripts would be released into the supernatant. Aliquots were electrophoresed in urea gels alongside viral dsRNA. The gels were stained with ethidium bromide or toluidine blue photographed then fluorographed.

3.2.2.3. (i) Products at Different Magnesium Concentrations

Products of the polymerase reaction at four magnesium concentrations were analysed by urea-PAGE. Also, the reaction

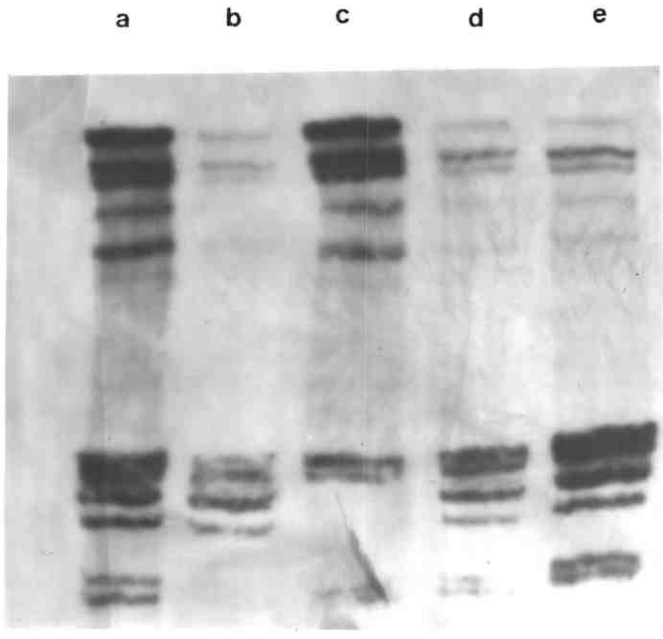


Fig. 19 Fluorograph of urea-PAGE of RNA polymerase reaction products of 3b1a virus mixture (sucrose gradient fraction 4) at (a) 3mM-$MgCl_2$, (b) 5mM-$MgCl_2$, reaction mixture separated, after polymerase incubation into fraction binding to bentonite, shown in this track, and (c) 5mM-$MgCl_2$, fraction not binding to bentonite, (d) 7mM-$MgCl_2$, (e) 9mM-$MgCl_2$. Electrophoresis was as in Fig. 17 (3 days exposure).

mixture at 5mM magnesium was separated into that bound to bentonite and that remaining in the supernatant. The result (Fig. 19) shows the same products at each magnesium products consisting of 16RNA bands, eight of these could be seen to co-migrate with the dsRNA bands of viruses B and C.

(ii) Time-course of Reaction

Products of the polymerase reaction were examined by urea-PAGE after various incubation times. Before SDS-disruption the products were separated into material binding to bentonite and the bentonite supernatant and applied to a gel. The result (Fig. 20) showed initial incorporation of radioactivity into the faster migrating (dsRNA) bands.

(iii) Polymerase Reaction using different Sucrose Gradient Fractions

The polymerase reaction products were examined for reaction (Section 3.1.9) mixtures containing sucrose gradient fractions 2, 4 and 7. The dsRNA profile of each fraction is shown in Fig. 13. The products using different sucrose gradient fractions were qualitively similar; fraction 7 however showed very little incorporation of radioactivity.

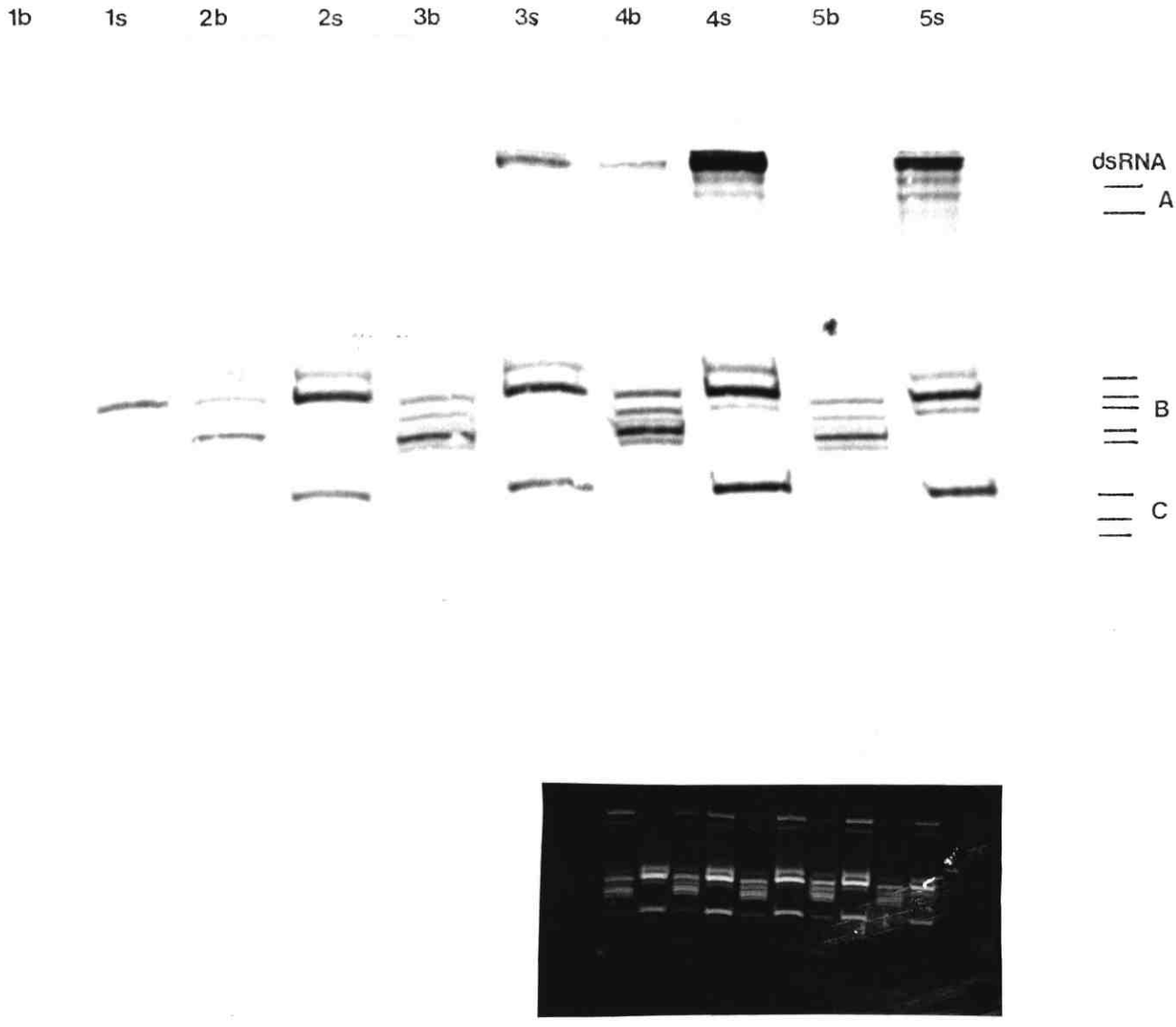


Fig. 20 Fluorograph of urea-PAGE of RNA polymerase reaction products of 3b1a viruses (sucrose gradient fraction 4) at various incubation times. 50μl of reaction mix were incubated for the times shown and the products separated into fraction binding to bentonite and fraction remaining in the supernatant after removal of bentonite by brief centrifugation. The bentonite fraction was resuspended in 20μl of TNE and both fractions denatured in SDS (60°C) and electrophoresed at 100V for 18 hours. The reaction incubation times were (i) 1 min, (ii) 2.5 hours, (iii) 8 hours, (iv) 24 hours, (v) 72 hours. The bentonite bound fractions are labelled b, the bentonite supernatant fractions are labelled s. The position of the dsRNA components of virus 3b1a-B and c are shown, the dsRNA components of virus 3b1a-A were not visible on the fluorograph but their position on the gel is indicated, (3 day exposure). The ethidium stained gel is shown beneath.

(iv) Ribonuclease action on the Polymerase Products

RNA was extracted from polymerase reaction products (using sucrose gradient fraction 4). The RNA was digested with ribonuclease in 2 x SSC (in which dsRNA but not ssRNA is stable to RNAase and 0.1 x SSC (in which ds and ssRNA is digested by RNAase The result (Fig.21) demonstrated that the faster migrating eight bands seen on the fluorograph were the dsRNA components of viruses B and C and the eight slower migrating bands were ssRNA products. Virus F6-A dsRNA components were present on ethidium staining, but had not incorporated ^{3}H-UMP and were therefore not visible on fluorography.

(v) Polymerase of 3b1a and Polymerase Products of F6 Viruses

The polymerase products of 3b1a were co-electrophoresed with polymerase products of F6 viruses (Section 3.2.1). The results (Fig. 22) showed two of the slower migrating ssRNA products of the 3b1a reaction were seen to migrate with the ssRNA products of virus F6-B, the faster mobility ssRNA products were seen to migrate with virus F6-C products.

(vi) Separation of dsRNA and ssRNA

Total RNA was extracted from a (1 week) polymerase reaction was precipitated with lithium chloride in conditions which precipitate

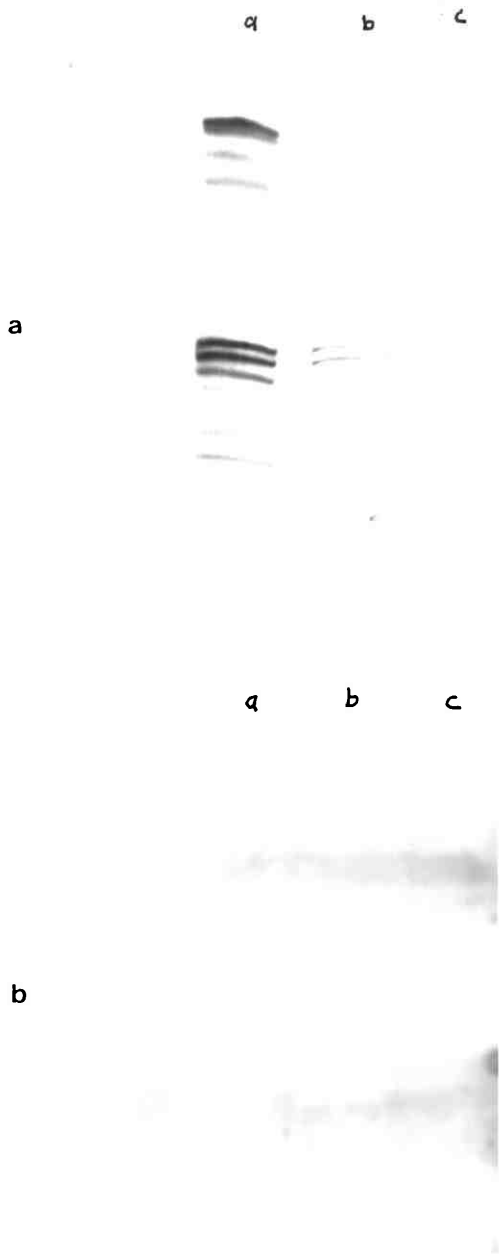


Fig. 21a Fluorograph of urea-PAGE of (a) RNA polymerase reaction products (50µl) of 3b1a viruses (sucrose gradient fraction 4), (b) RNA polymerase reaction products after treatment with ribonuclease A in 2 x SSC buffer, (c) RNA polymerase reaction products after treatment with ribonuclease A in 0.1 x SSC buffer. The ethidium stained gel is shown in Fig. 21b. The gel was electrophoresed as in Fig. 19.

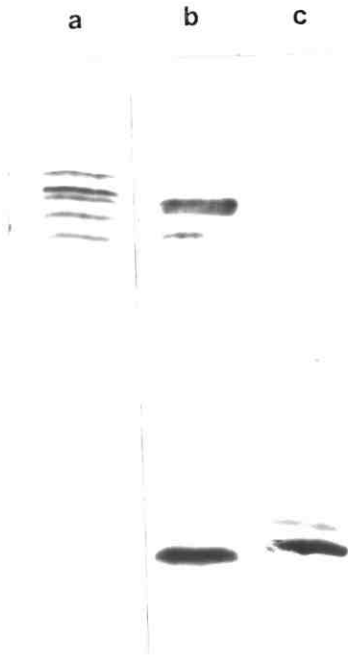


Fig. 22 Fluorograph of urea-PAGE of RNA polymerase reaction (50μl) products of (a) 3b1a virus mixture (sucrose gradient fraction4) (b) F6 virus mixture, (c) virus F6-B. The gel was electrophoresed as in Fig. 19.

ssRNA but not dsRNA.

The separated products were analysed by urea-PAGE (Fig. 23). As expected, eight dsRNA products were seen and eight ssRNA products.

(vii) Glyoxylation of dsRNA

Tritiated dsRNA was prepared from polymerase reaction mixture (as above). The RNA was glyoxylated, ethanol precipitated and analysed by urea-PAGE, alongside unglyoxalated polymerase products. The result (Fig. 24) shows that glyoxylated dsRNA co-migrated approximately with the ssRNA products of the polymerase reaction, showing that the single-strand length of the dsRNA products of the polymerase reaction is the same as the length of the ssRNA products (for each ssRNA and dsRNA template).

3.2.2.4 Hybridization of 3b1a ssRNA with Viral dsRNA

Single-stranded RNA was isolated from a polymerase reaction mixture by phenol extraction and lithium chloride precipitation and shown to be free of dsRNA by gel electrophoresis and fluorography. The concentration of ssRNA was determined by spectrophotometry and an aliquot containing 0.2μg (2000 ct/min) was annealed with 4μg of cold denatured viral dsRNA. The products were incubated with



Fig. 23 Fluorograph of urea-PAGE of RNA polymerase reaction products (50µl) of (a) 3b1a virus mixture, (b) ssRNA isolated from polymerase reaction products by precipitation in 2M-lithium chloride, (c) dsRNA isolated from polymerase reaction products by precipitation of ssRNA in 2M-niCL. The gel was electrophoresed as in Fig. 19.

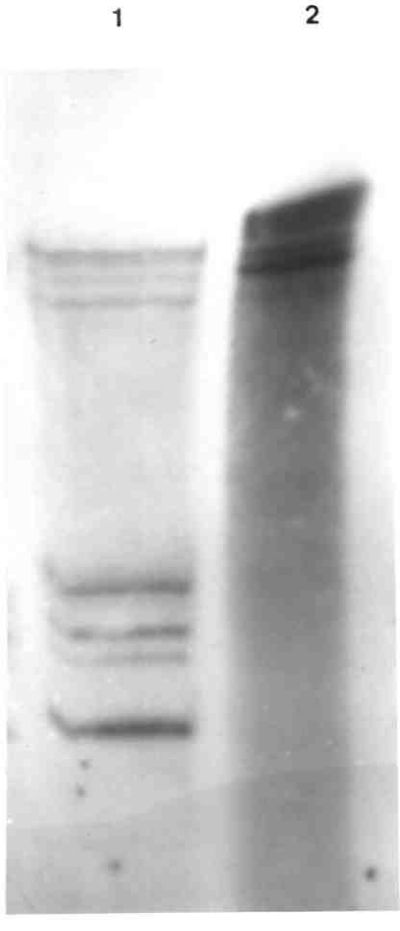


Fig. 24 Fluorograph of urea-PAGE of (a) RNA polymerase reaction products, (b) reaction products glyoxylated, electrophoresed as in Fig. 19.

ribonuclease A and filter assayed, and the ribonuclease resistant, TCA-precipitable radioactivity was determined as a measure of the amount of hybrid formed. The experiment was performed in triplicate with the following controls: (a) annealing in the absence of dsRNA to determine the amount of self-annealing or contamination with dsRNA; (b) annealing in the absence of dsRNA with no ribonuclease treatment, this would give a measure of any thermal degradation during denaturation and annealing; and (c) annealing in the presence of undenatured dsRNA in order to determine whether the dsRNA contains any ssRNA that could anneal to the probe.

The results (Table 6) show that the (^{3}H)-ssRNA transcription product hybridizes with virion dsRNA making it 93% resistant to ribonuclease treatment. The ssRNA polymerase product is therefore a faithful transcript of viral dsRNA.

It was thought that it may be possible to identify those dsRNA components that are forming hybrids to the ssRNA transcripts and are, presumably, the templates for the transcripts, by applying the products of the hybridization reaction to a polyacrylamide gel. Those 'dsRNA' components capable of reannealing with a tritiated ssRNA transcript would be detectable by fluorography whereas any dsRNA component that did not produce a transcript in the polymerase reaction would have only 'cold' dsRNA-derived ssRNA to reanneal to.

TABLE 6 Hybridization of 3b1a ssRNA with an Excess of 3b1a dsRNA

Conditions	% Hybridization
Twenty-fold excess of dsRNA	93
No dsRNA	8
No dsRNA, no RNase treatment	98
Undenatured dsRNA	10

(^{3}H) - ssRNA was annealed with a twenty-fold concentration of unlabelled dsRNA and nuclease-resistant hybrids were assayed (as described in Section 2.36). Percentage hybridization values are shown.

However, when the products were analysed by urea-PAGE, no bands were seen although a smear of tritiated material was seen at the bottom of the gel on fluorography. Omission of ribonuclease treatment gave a similar result but tritated material was seen to have remained at the top of the gel. Presumably the RNA was degraded in the hybridization reaction and the fragments were forming large interstrand molecular aggregates that did not enter the gel, unless subjected to ribonuclease treatment, when breakage of ssRNA loops produced inhomogenous dsRNA fragments of low molecular weight. Performing the denaturation and annealing in 50% formamide (McConaughy et al, 1969) or dimethyl sulphoxide at lower temperatures gave the same result.

3.2.2.5 Discussion

The results so far indicated that the polymerase reaction produced eight ssRNA products and during the reaction eight dsRNA components of the 3b1a viruses became radioactive during the reaction. The dsRNA components that became labelled were identified (by careful measurements of fluorograph and stained gel) as the 3 components of virus C (1.27, 1.19 and 1.11 x 10^6 mol. wt) and the five dsRNA components of virus B (1.60, 1.54, 1.49, 1.45 and 1.43 x 10^6 mol. wt.). After ribonuclease treatment in high salt (2 x SSC) only the dsRNA components of viruses B and C were visible on the fluorograph; also, removal of ssRNA by LiCl precipitation left

only dsRNA components of viruses B and C on the fluorograph. The evidence suggests that the eight ssRNA products are transcripts of the dsRNA components of viruses B and C. This is confirmed by glyoxylation of labelled dsRNA components of viruses B and C, which were seen to co-migrate on electrophoreses with the ssRNA products of the reaction. Further evidence supporting this conclusion was obtained by the co-migration of the dsRNA components and ssRNA products of virus F6-B and virus 3b1a-B (dsRNA components 1.56 and 1.45 x 10^6 of F6-B and 1.54 and 1.45 x 10^6 of 3b1a-B) and virus 38-4-A dsRNA components (1.27, 1.19 and 1.09 x 10^6) and ssRNA components with virus 3b1a-C dsRNA components (1.27, 1.19, 1.11 x 10^6) and ssRNA components.

It was clear that radioactive nucleotide was not incorporated into virus 3b1a-A dsRNA components during the polymerase reaction; also no ssRNA products that could be transcripts of virus 3b1a-A dsRNA were visible on fluorographs. If it is concluded that virus 3b1a-A does not have an active RNA polymerase reaction under these conditions then this would account for the low levels of tritium incorporation using sucrose gradient fraction 7, which contained predominantly virus 3b1a-A.

The reaction products at various incubation times (Fig. 20) showed many interesting features. Firstly, it can be seen from the ethidium stained gel that virus 3b1a-B is fractionated by bentonite

into two distinct fractions, as noted in Section 3.1.10. If this fractionation is performed before the incubation and the fractions incubated separately then it was confirmed (Fig. 25) that virus 3b1a-B was composed of two viruses each with RNA polymerase activity producing distinct products. The virus binding to bentonite (designated virus 3b1a-B2) contained the lower dsRNA components of mol. wt. 1.45 and 1.43 x 10^6. Virus not binding to bentonite (designated 3b1a-B1) contained the higher mol. wt. dsRNAs, 1.60, 1.54 and component b'4; this fraction also contained the dsRNA components of virus 3b1a-B2, presumably representing a fraction of virus 3b1a-B2, that does not bind to bentonite. Virus 3b1a-B2 produced two ssRNA products designated ss4 and ss5 (transcripts of 3b1a dsRNA components ds6 and ds7 respectively); 3b1a-B produced three ssRNA products designated ss1, ss2 and ss3 (transcripts of 3b1a dsRNA components b3, b4, b4 respectively).

Virus 3b1a-C was seen to not bind to bentonite (dsRNA in bentonite supernatant) in the incubation. Virus 3b1a-A was seen to be bound to bentonite in the early stages of the reaction but after 2-5 hours the dsRNA was seen to be released into the bentonite supernatant. This suggested that virus 3b1a-A may be disrupting during the polymerase incubation.

As may be expected, ssRNA transcripts remain in the bentonite supernatant even the transcripts of 3b1a-B2 which binds to bentonite. This suggests that transcripts are released from the virus particles.

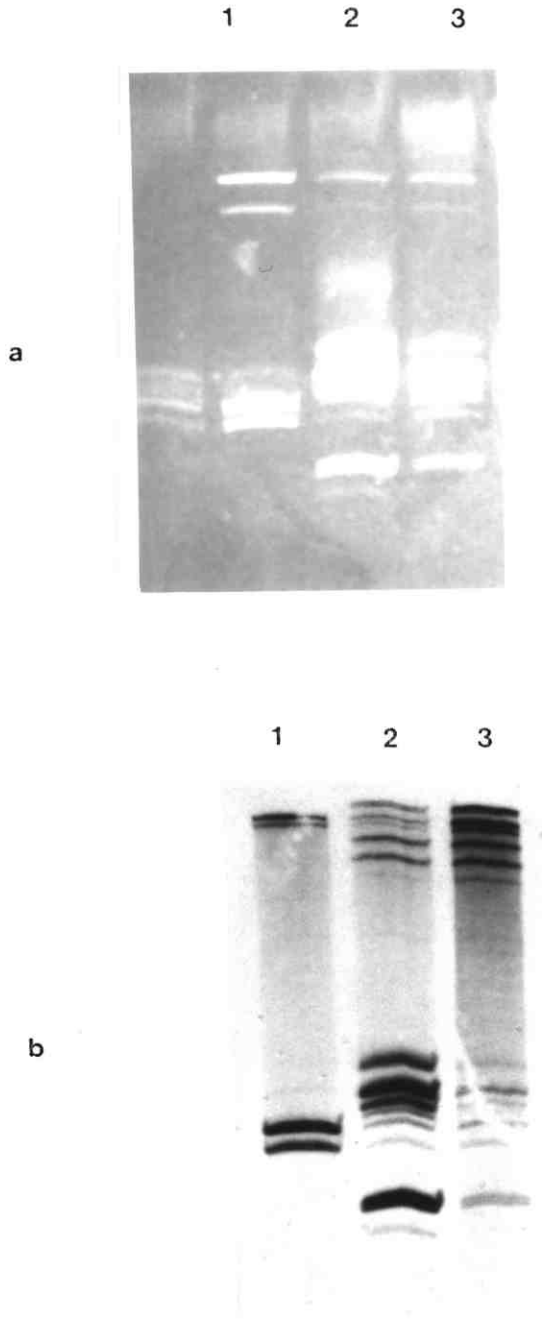


Fig. 25 Fluorograph of urea-PAGE of 3b1a viruses separated into bentonite bound, and bentonite supernatant fraction prior to polymerase incubation. 5µl of stock bentonite suspension was added to 50µl of 3b1a virus mixture (sucrose gradient fraction 4), and the mixture was centrifuged and separated into the supernatant and the bentonite bound fraction. The bentonite bound material was washed twice in TNE and resuspended in 50µl of TNE. The bentonite bound and supernatant fractions were incubated separately in standard RNA polymerase reaction mixtures. The products were denatured and electrophoresed at 100V for 18 hours, the gel was ethidium stained, (a) and fluorographed, (b) (i) bentonite bound fraction products, (ii) bentonite supernatant products, (iii) normal 3b1a virus mixture reaction products (3 day exposure).

It can be seen from the fluorograph of the time course (Fig. 20) that incorporation of (^{3}H)-UMP could be detected in the dsRNA components of virus B and C after only 30 sec; the incorporation into dsRNA was maximal after 8 hours showing no further increase in the 24 hours, 72 hours and 144 hours samples. On the other hand, (^{3}H)-UMP incorporation being greatest in the 144 hour sample for both viruses. This result is consistant with a semi-conservative mechanism of transcription. In such a mechanism (Buck, 1980) dsRNA would initially become labelled since the unlabelled parent strand would be displaced by a daughter strand being synthesised using the homolous strand as template. ssRNA would become labelled in the next round of transcription when the newly synthesised labelled strand would be displaced by the synthesis of a new daughter strand.

3.2.2.6 Electron Microscopy of Transcribing Virus Particles

Nothing is known concerning the mode of release of ssRNA transcripts from virions of any dsRNA mycovirus. However, the release of transcripts by reovirus core particles has been examined by electron microscopy (Bartlett et al, 1974). Polymerase reaction of 3b1a viruses were examined by this method.

Polymerase reactions that had been incubated for 1 minute, 5 minutes and 15 minutes were spread, mounted, stained and shadowed and examined by electron microscope. A control consisting of virus

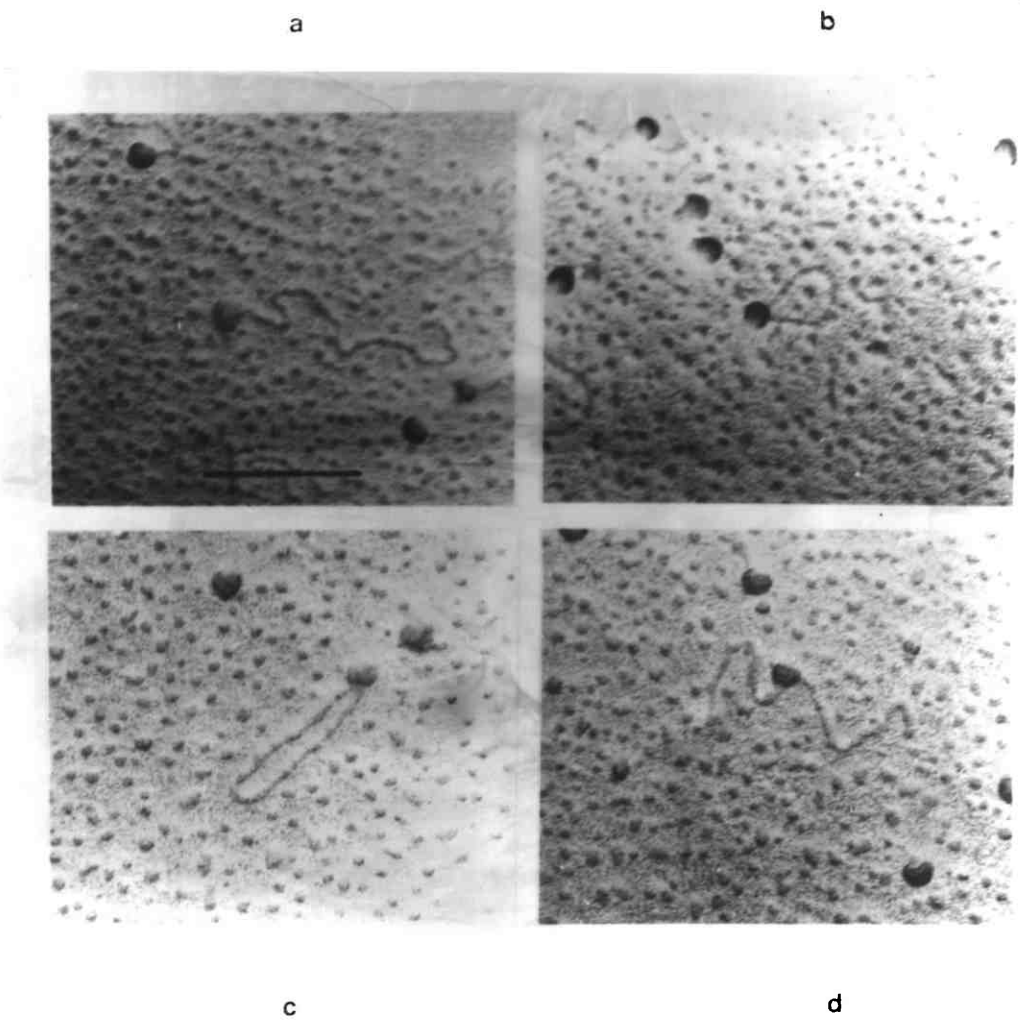


Fig. 26 Electron microscopy of transcribing particles of 3b1a virus mixture (sucrose gradient fraction 4). Particles showed (a) one linear strand; (b) a short RNA loop, (c) a long RNA loop, (d) two linear strands. The bar represents 200nm in all cases.

incubated in TNE buffer alone for 15 minutes was also examined. The control showed only virus particles with no associated RNA strands; however in the 1 minute incubation, already RNA strands were seen to be associated with a few of the particles. By 5 minutes 101 particles (9%) of 1106 examined appeared to be releasing transcripts. At 15 minutes the presence of many released transcripts mixed with virions made any interpretation difficult, except to note that there was an increase in amount of released transcript.. Three types of particles releasing transcripts were seen: particles with a single ssRNA strand attached, particles with two ssRNA strands attached and particles with a loop of ssRNA attached. Examples of each type of particle are shown in Fig. 26.

The ssRNA nature of the transcripts being released was confirmed by their absence if the reaction mixture was made 2 x SSC and treated with ribonuclease A in conditions in which dsRNA is stable (2 x SSC) prior to electron microscopy.

Of the 101 particles showing release of transcripts after a 5 minute polymerase reaction, 35 particles showed the release of a single linear ssRNA molecule, which varied in length between 20nm and 720nm, but with a distinct mode around 560nm. A significant number (28) of particles showed the release of looped ssRNA molecules both ends of which remained attached to the particle; the loops varied in length from 20nm to 500nm with a

fairly even distribution and no obvious modes. Additionally 38 particles showed two ssRNA molecules attached, the lengths of which were similar in some cases and different in others; the combined lengths ranged from 30nm to 720nm with a continuous distribution of lengths.

The length of complete transcripts from template dsRNA molecules of 3b1a viruses B and C (mol. wt. 1.0×10^6 to 1.6×10^6) would be expected to be in the range 490nm to 780nm, assuming the value of 1.05×10^6 daltons ssRNA/nm found for reovirus transcripts spread under the same conditions (Bartlet et al, 1974). The model length of the single linear strands indicates that these correspond to complete or nearly complete transcripts.

The detection of transcribing particles with looped RNA molecules, which had a continuous range of lengths from very short to near full length transcripts suggests that a loop of ssRNA, rather than the 5' end, may initiate the extrusion of the transcript from the virion. Similar loops have been observed by electron microscopy of transcribing cores of reovirus and it has been suggested that the 5' end of the transcript may remain associated with a site in the internal region of the spike, through which it is released, and that the first end to be set free outside the particles might be the 3' OH end (Bartlett et al, 1974). A similar mechanism could operate with G. graminis viruses.

The nature of the particles with two linear RNA molecules is less clear. G. graminis virus particles each contain only a single molecule of dsRNA since each RNA component is separately encapsidated; the two ssRNA molecules cannot therefore be transcripts of different RNA segments, as occurs in reovirus. Initiation of a second round of transcription before the first one has been completed is a possibility but is considered unlikely since in many cases the lengths of the two strands were similar and both shorter than complete transcripts. The fact that the combined lengths of the two molecules never exceed the length of a complete transcript suggests that the two molecules are produced by a single break in one of the looped forms, probably due to ribonuclease action. Unfortunately tribonuclease inhibitors such as bentonite or proteinase K could not be added to the incubations since their addition led to large aggregates of virus particles being formed.

3.2.2.7 Attempts to Induce a Polymerase Activity in 3b1a Virus A

It is clear from the preceding results that 3b1a virus A did not produce any ssRNA transcripts under normal polymerase reaction conditions. The results for F6-A virus indicated that this virus also lacked detectable polymerase activity. Since a probe hybridizing with all three viruses was required for detection

of provirus, attempts were made to find reaction conditions in which a putative polymerase activity of virus A was active. Sucrose gradient fraction 7, (Section 3.1.9.) contained virus A and trace amounts of virus B. When samples of this were incubated in polymerase reaction mixtures at 30°C and analysed, low levels of ^{3}H-UMP incorporation into ssRNA transcripts and dsRNA components of 3b1a virus B were detected, but no labelled transcripts or tritiated dsRNA components of 3b1a virus A could be detected. Various attempts to activate the 3b1a virus A were made by addition or subtraction of substances from the reaction mixtures. Table 7 shows the effects of these alterations on the incorporation of (^{3}H)-UMP into acid insoluble product. Many of these alterations reduced the (^{3}H)-UMP incorporation, presumably by inhibiting the low level of reaction due to 3b1a virus B. In the few cases where a small increase in (^{3}H) incorporation was observed (plus chymotrypsin at 200 g/ml; minus actinomycin D; 10 x normal NTP concentration), the RNA products were examined by gel electrophoresis and fluorography, but no labelled RNA bands, other than those produced by 3b1a virus B could be detected. In another experiment, total 3b1a viruses (sucrose gradient fraction 4) were incubated for 6 hours in RNA polymerase reactions to which had been added 1μM, 10μM, 100μM and 1mM S-adenosyl methionine. No change in (^{3}H) incorporation in any of these incubations, compared to controls without S-adenosyl methionine, and no new products on electrophoresis and fluorography were observed.

TABLE 7 Attempts to Activate the Putative RNA Polymerase of Virus 3b1a-A by Alterations to the Standard Reaction Mixture

Alteration	^{3}H-Incorporation[a] (ct/min)
+ 0.5% Nonidet	128
+ 0.5% Nonidet + 2mM-DTT	75
+ 2mM-DTT	173
+ Proteinase K (50 g/ml)	759
+ Proteinase K (200 g/ml)	870
+ Chymotrypsin (50 g/ml)	950
+ Chymotrypsin (200 g/ml)	1320
+ 10% (V/V) Glycerol	120
+ 10% "W/V) Vovine Serum Albumin	550
- Actinomycin D	1530
- Bentonite	639
10 x NTP conen[b]	1580
2mM-Magnesium Acetate[c]	1044
8mM-Magnesium Acetate[c]	830
12mM-Magnesium Acetate[c]	178
Standard Reaction Mixture[d]	1126

[a] = Acid-insoluble product after a 72h incubation (sp. act. of (^{3}H) UTP in Ci/mol) as described in Section 2.3.3
[b] = Final specific activity of (^{3}H) UTP was the same as in all the other incubations
[c] = Final concn.
[d] = Mg^{2+} concn. was 5mM

The time course experiment showed that virus A dsRNA components initially bound to bentonite but were later found in the supernatant; this suggests that virus A disrupted during the polymerase incubation. This was confirmed by PAGE of 3b1a viruses after normal SDS disruption and without disrupting with SDS. Initially nothing could be seen on the gels when the virus was not disrupted with SDS, but after a 2 hour incubation at 30°C the virus A dsRNA bands were visible in the gel track without SDS disruption, though none of the other virus bands could be seen. This result was the same when the virus mixture was incubated in a polymerase mix, in TNE buffer alone or in PBK buffer, indicating that it was not some component of the polymerase mixture that was causing the disruption.

It was therefore decided to incubate fraction 7 under conditions that it was thought might stabilise the virus. The following additions to the normal polymerase mixture were: (i) +10% glycerol, (ii) +20% glycerol, (iii) +2% bovine serum albumin, (iv) +10% bovine serum albumin. The reactions were analysed as above but no increase in (^{3}H)-UMP incorporation was observed. It was thought that the virus might be either heat labile, or destabilised by actinomyun D. It is known that some fungal virus polymerase reactions will proceed at 5°C, at a much reduced rate (Chater & Morgan, 1974). Omission of actinomycin D from the

reaction mixtures might allow bacterial growth so it was thought necessary to filter-sterilise the virus preparation before incubation. Filter-sterilising fraction 7 virus resulted in removal of virus A, presumably because this fraction, being at the bottom of the sucrose gradient, contained aggregated virus, that did not pass through the filter. It was decided therefore that fraction 4 would be used, as this contained virus A which passed through a millipore filter. The conditions tried were: (i) 30°C, no actinomycin D, (ii) 30°C, no actinomycin D with proteinase (10 g/ml) as ribonuclease inhibitor, (iii) 30°C no actinomycin D in 20% glycerol, (iv) 5°C, normal mixture (72 hours reaction), (vi) 5°C with proteinase K as ribonuclease inhibitor (72 hours reaction).

The products were analysed by urea-PAGE and examined for any new bands. No new bands were detected in any of the reaction products except in the 5°C reaction products. In this track on fluorography could be seen a broad single band appearing where the ssRNA products are normally seen. The band was very faint and so a longer incubation reaction was set up. Two conditions were tried: (i) 5°C, normal mixture, (ii) 5°C, no actinomycin D present. The mixtures were incubated at 5°C for 21 days and the products were analysed by urea-PAGE. In the fluorograph (Fig. 27) a broad band was seen where ssRNA is seen in a normal reaction. This band was not sensitive to ribonuclease in 2 x SSC but was sensitive

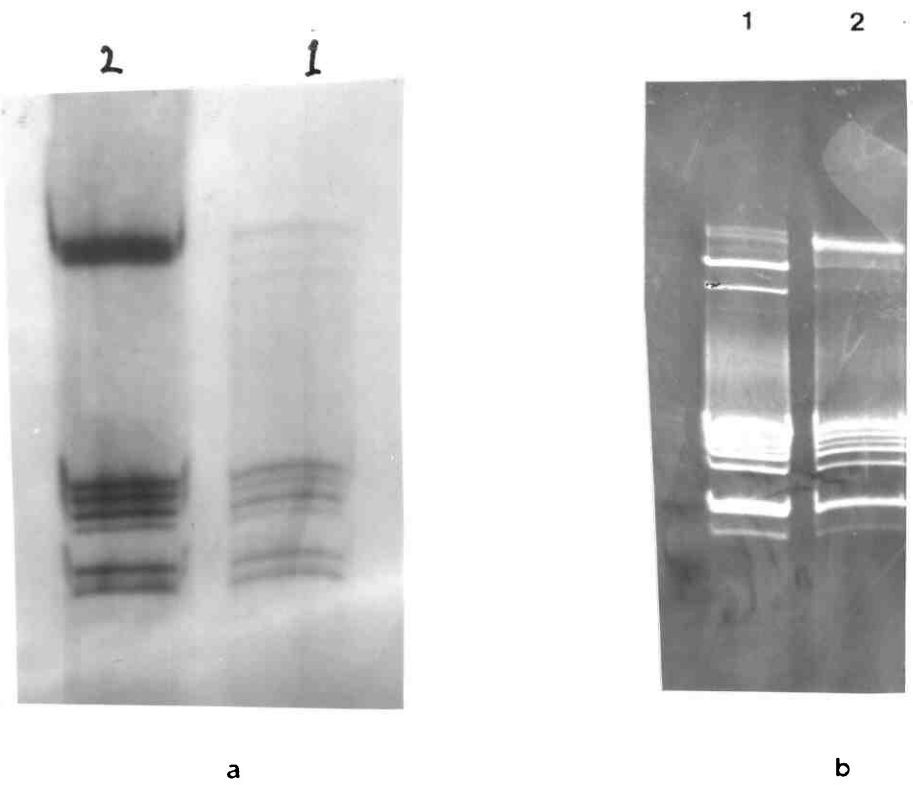


Fig. 27a Fluorograph of urea-PAGE of reaction products extracted from (i) normal polymerase reaction (48 hours at 30°C) and (ii) reaction products from a polymerase reaction mix incubated for 14 days at 5°C. The ethidium stained gel is shown in Fig. 27b.



Fig. 28 Ethidium stained urea-PAGE of reaction products from a polymerase mixture incubated for 4 weeks at 5°C, showing the complete loss of virus 3b1a-A dsRNA bands, (i) normal RNA polymerase reaction products, (ii) 5°C reaction products separated after polymerase incubation into bentonite bound fraction, shown here and (iii) 5°C bentonite supernatant fraction. The electrophoresis and fractionation was as in Fig. 19. (3 days exposure).

in 0.1 x SSC showing the band to be dsRNA. If the fluorograph was compared with the ethidium stained gel, it was seen that this new band was visible on ethidium staining, and migrated above the virus A dsRNA components (confirmed by careful measurement). A striking feature was that when the intensity of the virus A (ethium stained) dsRNA components in the 5°C reaction were compared with their intensity in a normal polymerase reaction (adjacent track) the dsRNA components of virus A were found to have decreased in intensity relative to those of viruses B and C. When other reaction times for the 5°C reaction were examined, it was discovered that the decrease in intensity of the A virus dsRNA components (on ethidium staining) was concomitant with an increase in the amount of this new band (Fig. 28). Glyoxalation of the 5°C reaction confirmed the double-stranded nature of this new band, designated band C_1; the glyoxalated products (Fig. 29) show the normal glyoxalated dsRNA components of virus B and C, co-electrophoresing with the normal ssRNA transcripts; but above these, a band could be seen that must be glyoxalated band c1. It is clear from these experiments that (^{3}H)-UMP was incorporated into a dsRNA product probably of virus A dsRNA components. (^{3}H)-UMP was not incorporated into unaltered virus A dsRNA.

The products of this reaction at 5°C were examined by electron microscopy of RNA extracted from reaction mixtures at 5°C (21 days incubation). Only linear molecules were seen (Fig. 30) in RNA

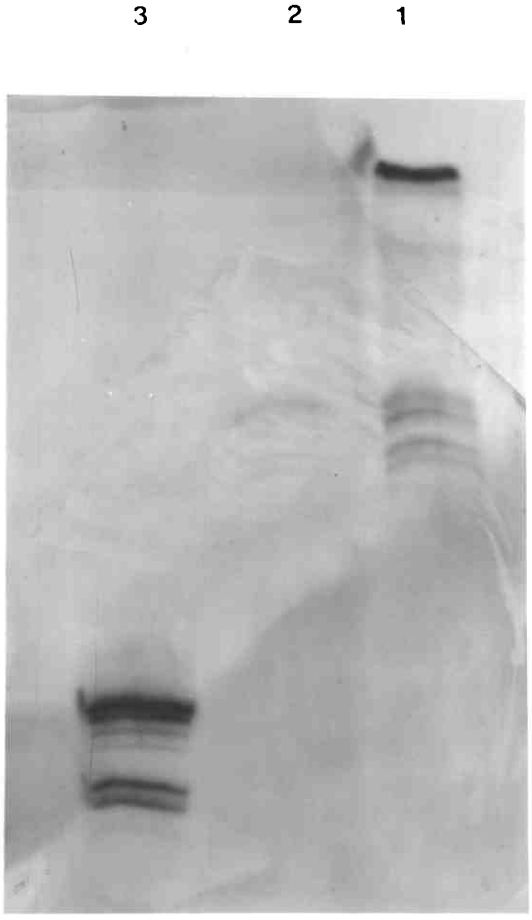


Fig. 29 Fluorograph of urea-PAGE of reaction products and glyoxylated reaction products from normal polymerase incubation and 5°C polymerase incubation, (i) 5°C polymerase incubation (4 weeks) products glyoxylated, (ii) 30°C polymerase incubation (48 hours) products glyoxylated, (iii) 30°C polymerase incubation product dsRNA (separated by precipitation of ssRNA in 2M-LiCl). The gel was electrophoresed at 100V for 18 hours. (3 day exposure).

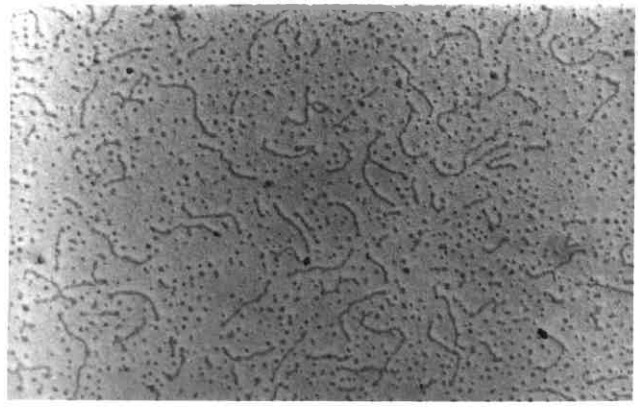


Fig. 30 Electron micrograph of RNA extracted from reaction products of 3b1a viruses polymerase reaction (sucrose gradient fraction 4) incubated at 5°C for 4 weeks. The RNA was extracted, spread and shadowed, as described in Methods (magnification x 100,000).

extracted from the 5°C reaction mixture and the normal reaction mixture. The strand lengths, and distribution of strand length was the same in 5°C products and the normal products.

3.2.2.8 Attempts to Detect RNA-directed DNA Polymerase Activity

It was thought worthwhile to determine whether 3b1a viruses contained an RNA-directed DNA polymerase activity similar to that which occurs in the RNA tumour viruses of mammals and birds (Baltimore & Smoler, 1971). If the viruses did exist in DNA proviral forms then this activity might be needed to transfer the viral genetic information to the host genome. The basic reaction mix as described in methods, was used but the following variations were also tried.: (i) no Nonidet P-40, (ii) no dithiothmetol, (iii) 2mM Mg-Acetate, (iv) 5mM Mg-acetate, (v) 10mM Mg-acetate (vi) addition of KCl at 5mM (vii) addition of ATP at 0.5mM, (viii) addition of proteinase K at 100μg/ml. All combinations of these reaction conditions were incubated with 20μl of 3b1a viruses sucrose gradient fraction 4, and filter assayed.

None of these reaction mixtures incorporated more than 150 ct/min of acid precipitable radioactivity. This represents a 0.003% incorporation, an amount accountable by retention of some label to precipitated virus and was not thought significant. The counts

for each of the assays varied randomly but three of the conditions giving highest counts were repeated with overnight incubations and the products applied to a 4% urea gel and autoradiographed. Nothing could be seen on a 7 day film exposure of the gel.

It was concluded that 3b1a viruses did not contain any RNA-directed DNA polymerase activity that was active under the conditions used.

3.2.3. Discussion

In summary, it thus appeared that 3b1a contained four viruses: virus A, virus B1, virus B2 and virus C. Virus B1, virus B2 and virus C each contained an RNA polymerase activity that produced full size ssRNA transcripts of each dsRNA component. The allocation of ss and dsRNA bands is indicated in Fig. 31. Viruses F6-B and F6-C contained similar polymerase activities and produced similar products. Virus 3b1a-A did not possess an RNA polymerase activity active under normal reaction conditions but at $5^{o}C$ in a reaction mixture lacking actinomycim D (^{3}H)-UMP was incorporated into the dsRNA components of virus A. It was later found that this reaction was sensitive but not completely inhibited by actinomycin D. In the presence of actinomycin D, the product formed more slowly. It was also discovered that F6 virus A contained a similar activity. When F6 virus mixture was incubated at $5^{o}C$ a similar ribonuclease resistant product, to that obtained with 3b1a virus, was seen.

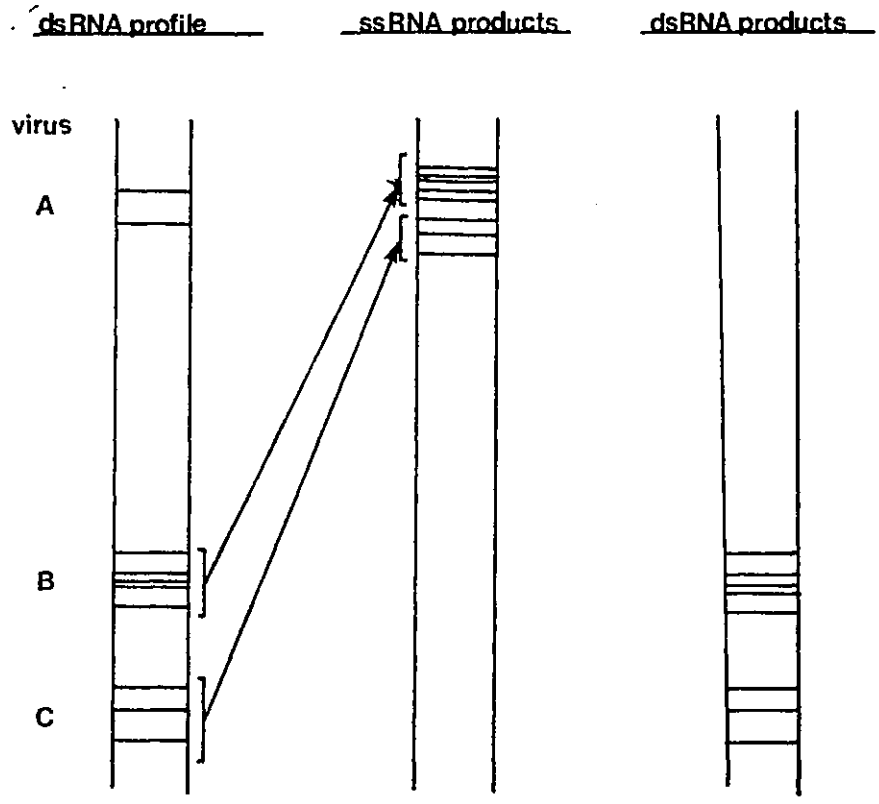


Fig. 31 dsRNA Components and ssRNA polymerase products of 3b1a.

The nature of this product is unclear, but it may be an artifact of a 'faulty' polymerase activity. Since, it seems both 3b1a virus A and F6 virus A are unstable (studies with F6-A virus similar to the above studies using 3b1a virus confirmed this), it may be that the virus particles were damaged in the virus purification procedure. No ssRNA product of viruses 3b1a A or F6-A was ever detected.

Results from other workers in this lab (Buck et al, 1981) demonstrated similar RNA polymerase activity to that obtained for viruses F6-B, F6-C, 3b1a-B, 3b1a-C, in other Ggt viruses 38-4-A and 019/6-A. Both these viruses (group 1 Ggt viruses, Buck et al, 1981) show, like viruses 3b1a-B and 3b1a-C, incorporation of radioactivity into the template dsRNA components. In the case of 38-4-A virus this (^{3}H)-UMP incorporation corresponded to about 40% of one strand per dsRNA molecule (or one complete strand in 40% of the dsRNA molecules). It is clear that this level of (^{3}H)-UMP incorporation cannot be accounted for by 'filling up' of ssRNA tails on predominantly dsRNA molecules by a ss dsRNA polymerase. This suggests that transcription occurs by a semi-conservative mechanism, a proposal that is supported by the initial incorporation of radioactivity into dsRNA of viruses 3b1a-B and C. Such a mechanism has been shown to operate in the case of RNA polymerases associated with virions of Aspergillus foetidus virus S (Ratti & Buck, 1978, 1979) and of bacteriophage ϕ6 (Van Etten et al,

1980). This mechanism does not exclude the possibility of semi-conservative replication of dsRNA occurring within a proportion of the virions, giving rise to diploid viruses, as has been shown to occur Penicillium stoloniferum virus S (Buck, 1975, 1978). The mechanism is however clearly distinct from the virion-associated RNA polymerase activity of reo-virus, which catalyses conservative transcription of dsRNA, with no incorporation of radioactivity into dsRNA (Banerjee & Shatkin, 1970) and probably distinct from the RNA polymerase activity associated with Saccharomyces cerevisiae virus ScV, during which little or no incorporation of radioactivity into template dsRNA occurs (Bruenn et al, 1980).

3.3 Analysis of Virus and dsRNA Content of Hyphal Tip and Ascospore Cultures of Ggt

The presence of virus in conidial isolates of Ggt had been confirmed in Section 3.1; the viruses present were characterised. This section describes the attempts to free Ggt isolates from virus particles, by ascospore production and hyphal tip isolation. Techniques were developed for detection of low levels of virus, using antiserum prepared against virus (Section 3.1.7), and low levels of viral dsRNA using ssRNA probes produced by the RNA polymerase reaction of Ggt viruses (Section 3.2).

3.3.1. Production of Perithecia and Isolation of Single Ascospores

3.3.1.1 F6 Isolates

All of the methods described in Section 2.13 were used in attempts to induce F6 isolates to produce perithecia. The methods were tried, in triplicate, on the parent F6 isolate, each of the ten single conidial isolates, both hyphal tip cultures and Ggt isolate F911-1 as a control.

All methods wre unsuccessful, none of the F6 isolates produced any perithecia. Isolate F911-1 produced abundant perithecia

with most of the techniques. In order to be sure that this was not due to personal unfamiliarity with the techniques, M. Holden of Rothamsted Experimental Station agreed to attempt to induce F6 to produce perithecia using each of the plate and flask methods. Holden's attempts were unsuccessful, despite her experience in producing perithecia (Holden, 1981). This was the first isolate of Ggt that she had found unable to produce perithecia. It was for this reason that the momentum of this work was concentrated on isolate 3b1a.

3.3.1.2 3b1a Isolates

The following media were used in attempts to induce 3b1a to produce perithecia: 1% PDA containing 0.2% yeast extract, 1% agar containing 1% malt extract broth and 1% agar containing 0.5% yeast extract. Two single conidial strains were used, 3ba1 con D and 3b1a con E. Perithecia were produced on all of these media and four single ascospore isolates were obtained from a single perithecium from 3b1a con D and were designated 3b1a con D/A1, 3b1a con D/A2, 3b1a con D/A3, 3b1a con D/A4. Four single ascospore isolates were obtained from 3b1a con E/A1, 3b1a con E/A2, 3b1a con E/A3, 3b1a con E/A4. A single perithecium extruding asci and ascospores is shown in Fig. 32.

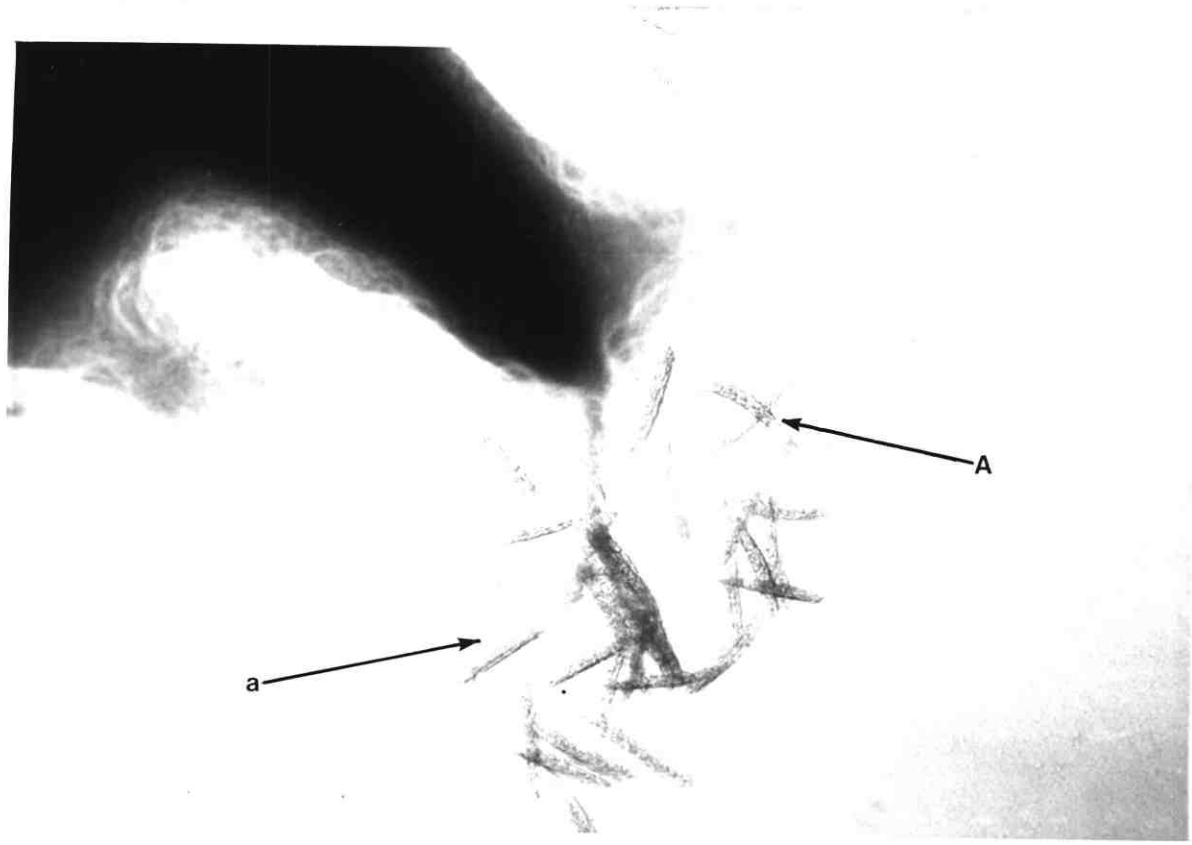


Fig. 32 Perithecium of isolate 3b1a, showing asci (labelled A) and ascospores (labelled a) being extruded from the perithecial ascocarp pore. (magnification 1000 x).

3.3.1.3 45/10 Isolates

Since isolate F6 could not be induced to produce perithecia, it was decided that another Ggt isolate capable of producing perithecia and ascospores was required. (Isolate 45/10 contained virus particles, and dsRNA components. (Almond, 1979). Single conidial isolates 45/10 con B was supplied by Rawlinson (Deacon and Rawlinson, 1977) and shown to contain virus particles. Perithecia were produced on 1% PDA containing 0.2% yeast extract. Three single ascospore isolates were obtained from a single perithecium and designated 45/10 con B/A1, 45/10 con B/A2, and 45/10 con B/A3.

3.3.1.4 Production of Hyphal-Tip Cultures from Isolate F6

Since isolate F6 could not be induced to produce perithecia, it was attempted to free F6 from virus by isolation of successive hyphal tips. When F6 had been subjected to ten successive hyphal tip isolations two of the last isolated germinated hyphal tips were designated F6-HTI and F6-HT2.

Standard virus extractions were performed on F6-HT1 and F6-HT2 and analysed by electron microscopy. No virus particles were detected in either isolate.

3.3.2. Level of Viruses in Ggt Isolates 3b1a and F6

Using data obtained from this study, an estimate of the level of virus and viral dsRNA in Ggt isolates 3b1a and F6, expressed as the viral (or dsRNA) mass per gram of mycelium; and the number of virus particles (or dsRNA component copies) per cell, may be obtained.

Firstly, the level of viral dsRNA in these isolates may be estimated from the yields of dsRNA in dsRNA extractions. The yield of dsRNA in dsRNA extractions of eleven F6 isolates was found to be 2μg of dsRNA/g (wet wt.) of mycelium (Section 3.1.4). The yield of dsRNA from 3b1a conidial isolates (Section 3.1.9) was found to be similar. In the extraction procedure, the cell breakage using the Pascal roll mill (Methods) was estimated as 80%; and the recovery of dsRNA in standard dsRNA extractions was estimated at 70%. The level of dsRNA in isolates 3b1a and F6 was therefore estimated as 3.2μg dsRNA/g (wet wt.) of mycelium. The nucleic acid content of Ggt viruses has been estimated (from the absorbance at 260nm absorbance at 280nm, ratio) as 20% (Almond, 1979); and therefore the weight of virus particles in isolates 3b1a and F6 was estimated as 16μg/g (wet wt.) of mycelium. The yield of virus in large-scale virus preparations of F6 (Section 3.1.5) was 7.2μg/g (wet wt.); and the yield of 3b1a virus in a similar extraction was 15μg/g (wet wt.) of mycelium. (Buck, personal communications). The recovery of virus in large-scale virus extractions in this laboratory has been

estimated (K.W. Buck, personal communications) to be approaching 100%, and so the estimates of level of virus using these values is in good agreement with the estimates obtained from the yields of dsRNA.

If the level of virus in a Ggt isolate is constant then the mean mass of viral dsRNA per cell will be at a constant ratio to the mean mass of DNA per cell, such that the mean mass of dsRNA per cell/the mean mass of DNA per cell = mass of dsRNA per gram of mycelium/mass of DNA per gram of mycelium.

The yield of DNA from 3b1a ascospore isolates was found (Section 3.4.2) to be 8μg/g (wet wt.) of mycelium; the level of cell breakage in DNA extractions was estimated as 15% (Section 2.40) and the recovery of DNA was estimated at 50%; therefore the level of DNA in 3b1a ascospore isolates was estimated as 107μg/g (wet wt.) of mycelium. This value is likely to be similar in all 3b1a isolates and isolate F6.

The mass of DNA per cell is a product of the total genome size (mol. wt.) and the number of genome copies. The genome size of Ggt was determined later in this work (Section 3.4.3.1) as 29.15×10^6 base-pairs, or a molecular weight of 1.93×10^{10} (taking the mol. wt. of one nucleotide base-pair to be 660).

Since Ggt is a haploid organism the number of genome copies will be equal to the number of nuclei per cell (mitochondrial DNA will be only a small proportion by mass).

Fluorescent staining of Ggt mycelia (Section 3.1.2) revealed that at least 90% of cells in Ggt mycelium were uninucleate, agreeing with the findings of Blanch (1977); so the number of genome copies per cell is approximately one. The mass of dsRNA per cell is a product of the molecular weight of dsRNA and the number of copies of dsRNA. The mean molecular weight of a single viral dsRNA component of 3b1a and F6 viruses is 1.83×10^6 and therefore the number of copies of dsRNA components in F6 or 3b1a may be estimated as: (mol. wt. of dsRNA component x number of copies per cell)/(mol. wt. of DNA x number of copies per cell) = mass of dsRNA per gram (wet wt.) of mycelium/mass of DNA per gram (wt. wt.) of mycelium.

Therefore,

(1.83×10^6) x number of dsRNA copies cell $/1.93 \times 10^{10}$ x 1 = 3.2/107.

Therefore, the average number of dsRNA copies cell in isolates 3b1a and F6 is estimated as 315. Most viruses in 3b1a and F6 contain two dsRNA components and so the number of virus particles per cell is approximately 160.

3.3.3. Detection of Viruses using the ELISA Technique

In a study by Adler et al (1980) various immunochemical techniques were used to detect low levels of virus Agaricus bisporus. Of these, enzyme-linked immunosorbant assay (ELISA) was found to be the most sensitive and had advantages of economy and was capable of quantitation. ELISA was therefore adopted for use in detecting Ggt viruses.

3.3.3.1 Preparation of Gamma-Globulin and Enzyme-Linked Gamma-Globulin

Purified gamma globulin (Ig-6) was prepared from F6-L antisera and F6-H antisera (Section 3.1.7). The yield of Ig-6 was 1.2 A_{278} units from F6-L antisera and 0.8 A_{278} units from F6-H antisera.

2 ml of antisera Ig-6 was covalently linked to 1mg of alkaline phosphatase; 2ml of H antisera Ig-6 was covalently linked to 1mg of alkaline phosphatase.

3.3.3.2 Standardisation of ELISA technique

The procedure was optimised for detection of Ggt viruses in isolate F6 using a preparation of F6 viruses, prepared by the standard virus extraction, diluted twenty times.

The procedure was performed, using varying amounts of gamma globulin to coat the wells, plus or minus post-coating the wells with bovine serum albumin and varying the incubation time for substrate hydrolysis. The maximum difference in absorbance between the virus solution tests and a buffer-only control was 2.4 absorbance units (absorbance of virus test = 2.6, absorbance of blank = 0.2). This was achieved by coating the plates with gamma-globulin at 10ng/ml, using 3% bovine serum albumin to coat vacant sites and incubating the substrate for 30 minutes at room temperature in darkness.

3.3.3.3 Measurement of Sensitivity of Technique

The minimum amount of virus detectable in purified virus preparations was determined using a mixture of F6 sucrose gradient I (Section 3.1.5.) fractions 17 and 30, containing viruses F6-A, F6-B and F6-C. The fractions were diluted and the maximum dilution that gave a significant difference in absorbance between the virus solution and a buffer control was determined. All tests were repeated six times in randomised wells. The maximum dilution detectable was 5000 which represents a virus concentration of 20ng/ml.

3.3.3.4 Detection of Virus in Hyphal Tip Cultures

Hyphal tip isolates HT1 and HT2 were subjected to standard

virus extraction, but with only one virus pelleting step. Controls of F911-1 mycelium and F6 mycelium were similarly treated. The final pellets were taken up in 0.5ml of buffer and tested for virus. (6 repeats, randomised) using plates coated with L-gamma-globulin.

The results, Table 8 , show that hyphal tip cultures have a much lower level of virus than F6. However it is not clear whether the isolates are free of virus as they gave a reading which was 6% of the value for F6 (as F6 is diluted 10x) but significantly higher than that obtained for F911-1.

The interpretation of this result is unclear. The reading for the hyphal tip culture was above that for F911-1 and this suggests the presence of virus at a lower level than in F6, but by comparison with the preceding experiment the concentration of virus in the hyphal tip culture could be 10ng per gram of mycelium (a reduction to 0.1% of the value for F6 mycelia). However, the high absorbance reading obtained for F911-1 mycelia may represent cross-reactivity, between antibodies directed against F6 host proteins (due to the infection of these with virus preparation) and similar F911-1 proteins. The higher reading obtained for hyphal tip isolates compared to F911-1 could represent a higher titre against hyphal tip host proteins (since the host is identical) than F911-1 host proteins; such that the hyphal tip cultures could in fact be free of virus.

TABLE 8 Use of ELISA Technique for Detection of Virus in Hyphal Tip-derived Isolates of F6, con 8: HT1 and HT2, and Ggt Isolate F911-1

Sample	Absorbance (405nm)
F6 (diluted 1:10)	2.20
HT1	1.31
HT2	1.37
F911-1	1.04

Microtitre plates were coated with F6L gamma-globulin (Sections 2.75 and 3.1.7) and samples (200µl) of virus preparations were applied. The plates were then incubated with enzyme-linked gamma-globulin (prepared using F6 antiserum), substrate applied and the absorbance at 405nm measured as a measure of product formed, all as described in Section 2.25 and 2.26.

The experiment was repeated, coating the wells with H gamma-globulin. This gave a higher value for F911-1 than that previously obtained using L antisera; possibly because F6H virus being taken from the bottom of the sucrose gradient, contained higher levels of contaminating host proteins and therefore gave rise to higher levels of host-directed antibodies.

It was concluded from these experiments that the ELISA technique gave too high 'background noise' readings with F6 virus, using F6 antisera, to be of use for detection of low levels of virus. This is probably due to contamination of the virus preparations, that were injected for preparation of antisera, with host cell proteins. An alternative explanation for the above results would be that F911-1 contains small amounts of virus related to F6 virus. This technique does not distinguish between these possibilities.

The technique was not investigated further as other techniques for detecting virus (Section 3.3.4.) gave clearer results.

3.3.4. Detection of Viruses by Immune Electron Microscopy (IEM)

The use of serum-activated grids (SAGs) in immune electron microscopy has been discussed in the Introduction. This rapid method was chosen because, unlike ELISA, it gives a clear visual result in detecting low amounts of virus; contamination of antiserum

antibodies directed against host proteins does not effect the technique. The method was tested and its sensitivity for detecting a mixture of 3b1a viruses (sucrose gradient fraction 4, Section 3.1.9.) using 3b1a mouse antiserum (which has a titre of 512, 128 and 128 against 3b1a viruses 3b1a-A, 3b1a-B and 3b1a-C respectively. (Buck et al, 1981). Virus was diluted in PBK and sprayed onto serum-activated grids using these antisera, and also onto normal grids.

The lowest concentration at which virus was visible on normal grids was 1μg virus/ml (which gave an average of one particle per field. Virus could be detected using serum-activated grids at concentrations of 40ng virus/ml. Both antisera gave similar results.

This level of detection is lower than that obtained using the ELISA technique for detecting F6 viruses. However, unlike ELISA, the technique gives a clear visual positive or negative result.

The technique was found to be most useful for examining crude virus preparations, since large amounts of contaminating material (which makes the detection of virus with conventional electron microscopy much less sensitive than with purified virus

preparations) could be easily removed from the grids. The virus particles were then 'decorated' with antibody molecules to further aid identification.

3.3.4.1 Detection of Virus in Hyphal Tip Isolates

Having established that immune electron microscopy could be used to detect low levels of virus, the technique was used to investigate whether the F6 hyphal-tip isolates contained virus. Standard virus extractions were performed on 100g (wet wt.) of both F6 hyphal tip isolate (Section 3.3.1.4) mycelia. Serum activated grids were prepared using a 1:1 mixture of F6-H antiserum and F6-L antiserum (diluted 1:100), and used to detect virus in crude virus pellets.

In both cases (using either hyphal tip isolate) virus particles were detected on the grids. Standard electron microscopy gave an ambiguous result. With the knowledge that virus particles must be present it was possible to identify plausible particles. However, it is doubtful that without *a priori* knowledge, a definitive positive result could have been recorded. The concentration of virus particles was estimated as 1µg/ml giving a yield of 5ng of virus per gram (wet wt.) of mycelium.

Later in this work, 18 months after the hyphal tip isolates had been produced, during which time they had been subcultured approximately nine times, standard virus and dsRNA extraction was again performed on the hyphal tip isolates. Virus particles were clearly visible by standard electron microscopy and dsRNA extraction showed 8 dsRNA bands identical to those present in the parental F6 con 8, the virus concentration in the isolate, had therefore increased from the low level when first isolated.

3.3.5. Extraction and Detection of Viruses in Ascospore Isolates

The first ascospore isolate from which virus was extracted was 3b1a con E/A2. The isolate was grown for one month on PDA, after germination. This included 10 days 'stationary phase' growth when the mycelium had completely colonised the petri dish, as it was thought that this might maximise the virus content in the fungal cells. The isolate was inoculated into a single 60 litre fermenter and standard large-scale virus extractions were performed with the following modifications to maximise concentration of any virus present, and to ensure pelleting of low amounts of virus: (i) PEG precipitates were suspended in 5 x their weight of PBK - 2; (ii) virus pelleting was at 30,000 rev/min for 16 hours in a Beckman type 45 rotor; (iii) virus pellets were resuspended in 20 - 30 ml of PBK (and cleared); (iv) putative virus was not passed through a sucrose gradient but was repelleted at 25,000 rev/min

in a Beckman type 65 rotor for 16 hours and the pellet was taken up in 1-2ml PBK and examined for its virus content.

The first ascospore isolate extraction was performed on 3b1a con E/A2. In order to monitor virus losses at any stage of the extraction procedure, each of the pellets from cleaning spins was phenol extracted twice, and nucleic acid was ethanol precipitated, and then taken up in 0.1 x SSC. Supernatants from virus pelletings were first examined for virus, and the remainder phenol-extracted twice, ethanol-precipitated and taken up in 0.1 x SSC. Nucleic acids were then analysed by PAGE.

Virus was detected in the resuspended second virus pellet by direct electron microscopy and with SAG's. The use of SAG's gave much cleaner electron micrographs (Fig. 33) than electron microscopy alone. The concentration of virus was estimated by diluting the preparation until only one particle could be seen per field by direct electron microscopy. The concentration of virus at this dilution was estimated as 1μg/ml from previous work (Section 3.3.4.). The dilution necessary for this number of particles on the grid was 100 x, so the concentration of virus is estimated as 100μg/ml.

Virus particles could be detected in both of the virus extraction supernatants, using SAG's, but it was present at low

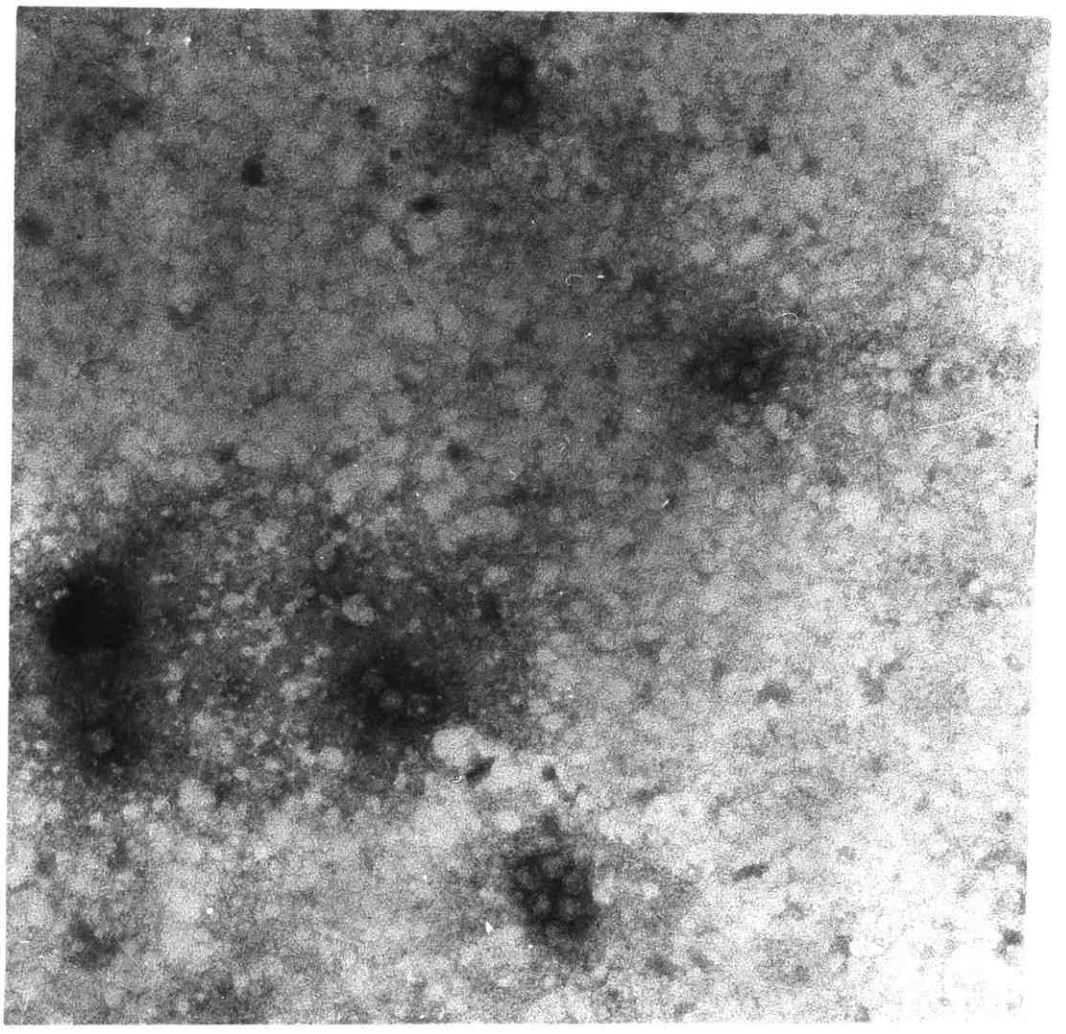


Fig. 33 Electron micrograph of ascospore virus (from 3b1a con E/A2) detected using serum activated grids coated with 3b1a mouse antiserum (magnification 100,000 x).

concentrations in both cases (5-20 particles per field). This was probably because small amounts of pelleted virus became resuspended before or during removal of supernatant.

Viral RNA was examined by standard dsRNA extraction and applying to 4% gels. The phenol extracted material from cleaning pellets and supernatants was also applied to the gel. The result (Fig. 34) showed dsRNA bands in all samples. The bending pattern was identical for each sample. It can be seen that most dsRNA is in the resuspended second virus pellet and that although some loss of virus occurred in the extraction procedure, the loss was not great, compared to the virus yield; and most importantly, no selective loss of any virus, not seen in the final preparation occurred.

It is clear from these results that the virus extraction procedure did not lead to any significant loss of virus and resulted in a concentrated virus preparation that could be used for highly sensitive methods for detection of low amounts of virus.

Virus extractions were therefore performed on the following ascospore isolates: 3b1a con E/A1, 3b1a con E/A2, 3b1a con E/A3, 3ba1 con E/A4, 3b1a con D/A1, 3b1a con D/A2, 3b1a con D/A2, 3b1a con D/A4, 45/10 con B/A1, 45/10 con B/A2, 45/10 con B/A3 (all generated in this

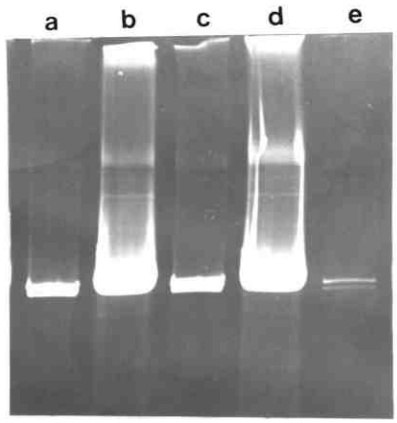


FIG. 34 Page of Virus Fractions from Ascospore Isolate (a) first virus pelleting supernatant, (b) first cleaning centrifugation pellet, (c) second virus pelleting supernatant, (d) second virus pellet (diluted 20x), (e) second cleaning centrifugation pellet. 20ul samples were applied and electrophoreses was at 50V for 24 hrs. The gel was stained with ethidium bromide.

work, Section 3.3) and ascospore isolates derived from 3b1a by Deacon: 3b1a/A49, 3b1a/A51, 3b1a/A52 and 3b1a/A55. Virus extractions were also performed on isolate F911.1 since this (field ascospore isolate) had been reported to be initially free of virus (Rawlinson et al, 1973) but Tivali et al, (1979) reported that after storage in culture for several years this isolate contained virus particles. Each of the isolates were grown for at least one month after germination including 10 days 'stationary phase' growth on a completely colonised petri dish. The isolates were in into single 60 litre fermenters and virus extractions performed as described above. It was expedient to pool discarded supernatants, extract with phenol and examine for dsRNA by PAGE. All discarded pellets were similarly treated. The yield of mycelium obtained from each fermenter was between 1 and 6kg (wet wt.).

Fractions were examined for virus by: (i) direct electron microscopy, (ii) by IEM, using SAG's activated using 3b1a antisera (diluted 1:64) for detection of 3b1a virus, or F6H antisera for detection of virus in isolates 45/10 and F911.1 (since this antisera reacted against a number of Ggt viruses, Buck et al, 1981), (iii) examination of phenol extracts for dsRNA by PAGE.

Virus was found in one other ascospore isolate, 3b1a con D/A1. In the case of this isolate and 3b1a con E/A2, dsRNA or virus

was detected by each of the three methods. However, IEM gave a much clearer result since large amounts of host material obscured the result with conventional electron microscopy.

The yield of virus was estimated by direct electron miscroscopy , using the value obtained in Section 3.3.4. as: a virus concentration of 1μg/ml gave one particle per field. The yield was also estimated from estimates of the amount of dsRNA present in PAGE of fraction aliquots compared to standards. The virus concentration in isolate 3b1a con E/A2 was approximately 2μg of virus/g (wet wt.) of mycelium, estimated by electron microscopy, or .5μg of dsRNA/g (wet wt.) of mycelium, estimated by PAGE. The virus concentration in 3b1a con D/A1 was similarly estimated as .5μg of virus/g mycelium and .2μg dsRNA/g (wet wt.) of mycelium. Virus particles or dsRNA were not detected in any other ascospore isolate.

Later in this work, fermenters became unavailable and the blotting and hybridization method used for detection of dsRNA (Section 3.3.6.3) was used for detection of viral dsRNA in a few remaining ascospore isolates. The sensitivity of this technique is compared with IEM and ELISA in Section 3.3.7.

3.3.6. Detection of dsRNA by Blotting and Hybridization Techniques

In order to detect viral dsRNA in ascospore isolates, possibly without virus particles, a technique was developed for transfer of dsRNA to nitrocellulose and hybridization with viral ssRNA. Such a technique could be used to determine the sensitivity of the method (Section 3.4.4.) for detecting viral DNA. It could also be used to detect dsRNA in fungal isolates (ascospores) and for determining to which dsRNA components ssRNA products are transcripts. The technique could also be valuable in determining relatedness of dsRNAs. A technique is available for transfer of ssRNA to activated paper ('Northern blotting') but the method is expensive. A simpler technique was recently described by Thomas (1980) for transfer (and hybridization) of ssRNA to nitrocellulose filter, where the RNA is denatured with dimethyl sulphoxide and glyoxalated; and then electrophoresed in agarose gels and transferred to nitrocellulose filter. It was investigated whether this technique would also be capable of transferring dsRNA to nitrocellulose.

Using (^{32}p)-labelled dsRNA prepared using the in vitro RNA polymerase reaction of virus particles, the dsRNA was denatured and electrophoresed in a 1% agarose gel as described. The gel was treated as described in Methods, for transferring the RNA onto a nitrocellulose filter, the filter and the gel were auto-radiographed.

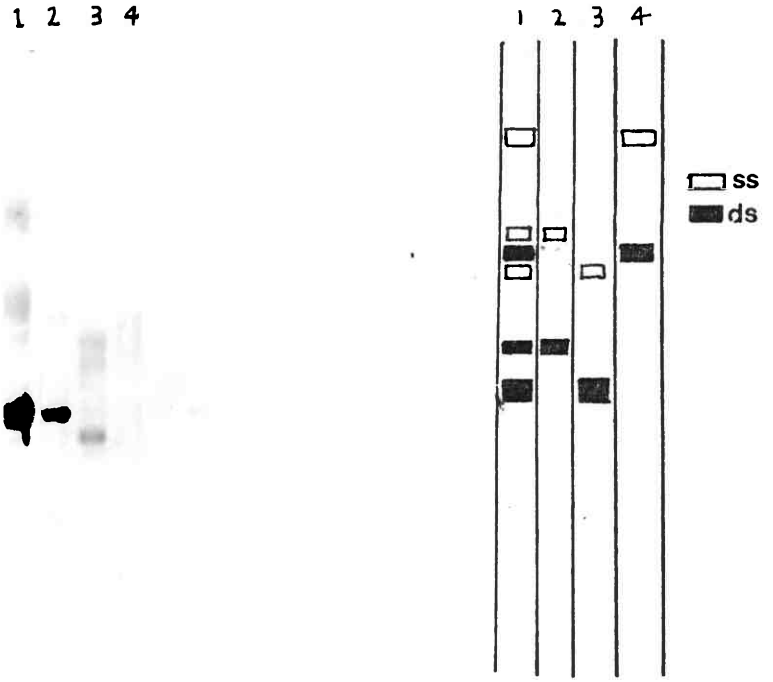
The result showed strong bands of RNA in the gel; denatured RNA had about half the mobility of native dsRNA, however nothing could be seen on the autoradiograph of the nitrocellulose filter, so presumably no dsRNA had transferred to the filter.

The reason for this failure was not investigated further as another method of transfer produced successful results. This method was recommended by Dr. S. Kidd (Imperial College, London) and had been successfully used to transfer ssRNA onto nitrocellulose filters. The method uses formamide to denature the RNA and formaldehyde to maintain the RNA in a denatured state (Section 2.45).

When denatured (^{32}p) labelled dsRNA was transferred by this technique and the gel and filter afterwards autoradiographed, no bands were visible on the autoradiograph of the gel after transfer; bands could be seen however on the filter autoradiograph (Fig. 35). When unlabelled dsRNA was transferred and hybridized with kinase labelled viral ssRNA probe, (1μg, 10^6 ct/min) a pattern of bands identical to (^{32}p) labelled dsRNA was seen. The pattern of bands obtained is however difficult to interpret particularly as the resolution of RNA by agarose gel electrophoresis is not as good as that achieved on polyacrylamide gels. To investigate this further 3b1a native dsRNA was electrophoresed in 0.8% agarose/ formaldehyde gel alongside denatured dsRNA and various dsRNA markers: F6 virus A dsRNA, F6 virus B dsRNA and F6 virus C dsRNA, in order to



Fig. 35 Autoradiograph of 3b1a virus mixture (sucrose gradient fraction 4) dsRNA, transferred to nitrocellulose filter (from 1% agarose/formaldehyde gel electrophoresed for 6 hours at 100V) and probed with 3b1a ssRNA kinase labelled with (^{32}p). (24 hour exposure).



b

Fig. 36a Toluidine-blue stained 1% agarose/formaldehyde gel of electrophoresed native and denature dsRNA samples (i) 3b1a dsRNA denatured in formaldehyde/formamide (50°C) prior to loading, (ii) virus F6-BdsRNA denatured as in (ii), (iii) virus F6-C dsRNA, denatured as in (ii), (iv) virus F6-A dsRNA denatured as in (ii). A diagramatic representation of the ss and dsRNA products is shown in Fig. 36b.

locate the dsRNA and (denatured) ssRNA bands. The result (Fig. 36) (toluidine blue stained) shows clearly that the reaction has not denatured dsRNA completely, some of the dsRNA in the denatured tracks is migrating as dsRNA. Some of the dsRNA is denatured and migrates as ssRNA. This makes interpreting the pattern of bands more difficult but if the banding pattern is compared with that obrained with F6 separated virus dsRNAs, then the identity of each band becomes clear, and is illustrated diagramatically in Fig. 36b.

The pattern of bands obtained on transferring (^{32}p)-labelled dsRNA to nitrocellulose (Fig. 35) can now be seen as a combination of ds and ssRNA bands of virus B and virus C, and the allocation of bands is shown diagramatically alongside. Virus A dsRNA did not become labelled during the normal polymerase reaction, either dsRNA or ssRNA derived from this virus are seen. The bands that appeared on hybridizing ssRNA probe to unlabelled dsRNA, were as expected, only from those viruses that produced an ssRNA transcript during the polymerase reaction.

A puzzling feature is that the ssRNA probe picked out the bands on the filter that have been identified as dsRNA. If the RNA in these bands were in a double-stranded form, it would not be expected to hybridize with ssRNA; however, it does. Possibly, in the denatured dsRNA samples, the RNA that has the mobility of

dsRNA is composed of molecules that have reannealed after denaturation.

Some of these molecules may be hybrids between partially homologous strands, or shorter strands-produced by scission of full length strands during the denaturation. Such hybrids may contain ssRNA loops or tails that may be available for hybridization with other ssRNA (probe). Alternatively the reassociation and denaturation reactions could be reversible during the hybridization incubation conditions such that labelled ssRNA could exchange with a like strand from a filter bound dsRNA molecule. This is unlikely as the reaction dsRNA $\rightleftharpoons$ 2 ssRNA, is reversible only in the melting region of the dsRNA. The hybridization conditions (42°C, 50% formamide, 5 x SSC) would be well below the temperature needed to melt dsRNA.

As separation of ss and dsRNA from polymerase reaction products is a lengthy procedure with variable yield it was decided to investigate whether unfractionated ds and ssRNA or dsRNA alone could be used as probe. For this, 3b1a dsRNA samples were denatured, electrophoresed and transferred to nitrocellulose filter. Adjacent lanes containing dsRNA were cut out of the filter and one of the filter strips was incubated with kinase labelled probe prepared from 3b1a polymerase products (1μg, 10 ct/min per μg). The result

showed an identical banding pattern to that obtained with ssRNA probe. A second strip was incubated with kinase labelled probe prepared from unlabelled 3b1a dsRNA alone. In this case the probe was boiled in water for 3 minutes before use. Again bands were seen, however, the intensity of these bands was low and a long film exposure (7 days) was required. The resolution of this gel was poor; it is possible that 3b1a-A ssRNA was present in the autoradiograph.

The use of dsRNA probe to detect (ds)RNA on a nitrocellulose filter is exactly analogous to the use of (ds)DNA to detect DNA on nitrocellulose in Southern blotting. In both cases RNA or DNA probe in solution will reanneal so that not all the probe is available for hybridization. It has been estimated (P. Rigby, personal communications) for Southern blotting using double-stranded DNA probe, that only 10% of probe will be available for hybridization, it is likely that a similar percentage of denatured dsRNA would be available for hybridization with denatured dsRNA bound to nitrocellulose.

The use of unfractionated ds and ssRNA would probably be more effective for detection of ds or ssRNA than ssRNA (of the same amount as ss and dsRNA) alone, if the RNA is labelled (with polynucleotide kinase) such that dsRNA is labelled to an equal

extent as ssRNA. Although reannealing of dsRNA will occur approximately 10% of the dsRNA will remain available for hybridization (as ssRNA); this will be in addition to the amount of ssRNA in the mixture.

Inclusion of dsRNA in a probe for detecting DNA on a filter would reduce the sensitivity of the probe, as any temperature (or criterion) dsRNA would be formed preferentially to DNA-RNA hybrids, since dsRNA is more thermostable than DNA-RNA hybrid. It is therefore likely that dsRNA alone would not be useful for detection of proviral DNA.

3.3.6.1 Hybridization of 3b1a ssRNA Transcripts of dsRNA components to Ggt Viral dsRNA

dsRNA from various Ggt viruses, and an unrelated fungal virus dsRNA preparation (Aspergillus foetidus viruses S and F dsRNA) were transferred to nitrocellulose filter and hybridized with 3b1a ssRNA. dsRNA was extracted from the following Ggt viruses: 38.4.A, 01-1-4-A, T1-A, 019/6A, (Buck et al, 1981) 45/10 (Section 3.3.3.) F6-A and B and 3b1a con E/A2 virus (Section 3.3.5). Approximately 50ng of dsRNA were denatured, electrophoresed transferred to nitrocellulose and hybridized with kinase labelled 3b1a ssRNA (10^6 ct/min/μg). dsRNA from 3b1a viruses A, B and C was also transferred and hybridized.

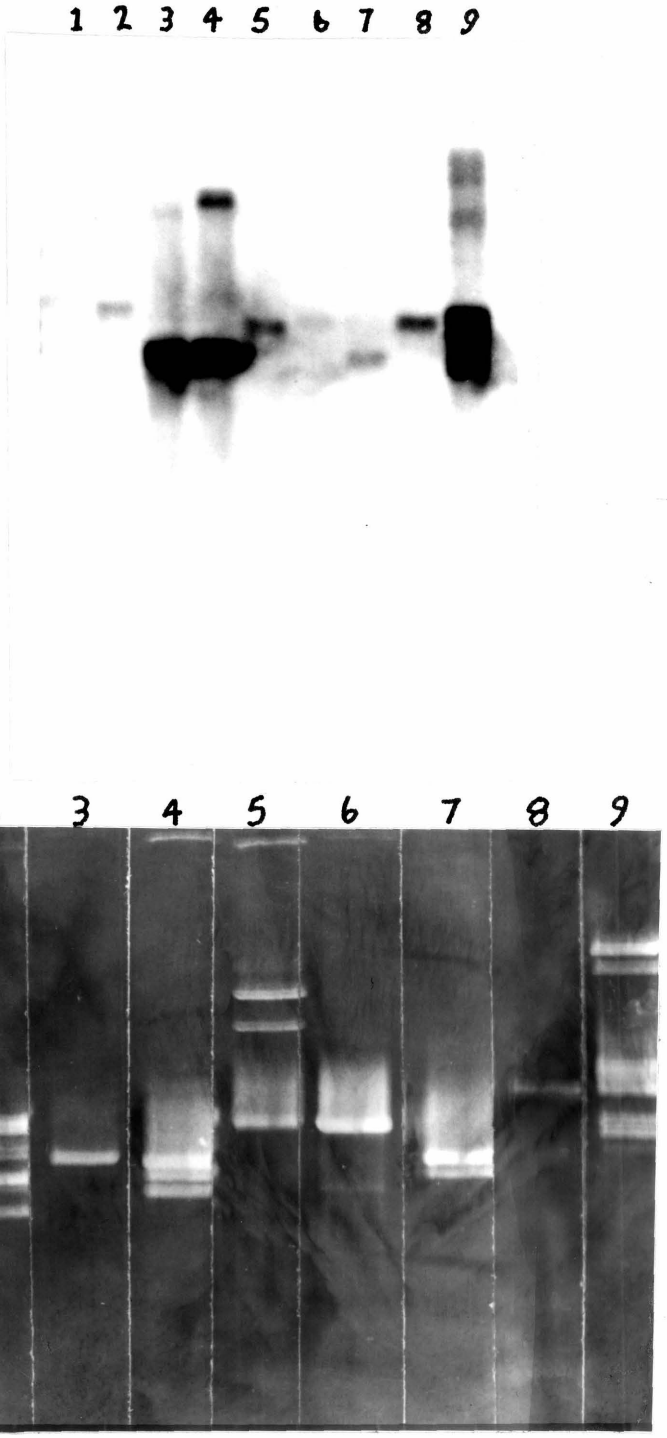


Fig. 37a Autoradiograph of various Ggt viral dsRNAs transferred to nitrocellulose filter (from 1% agarose/formaldehyde gel electrophoresed for 6 hours at 100V) and probed with 3b1a ssRNA (viruses B and C) kinase labelled with (^{32}p) (72 hours exposure). (i) Aspergillus foetidus virus F dsRNA (as control); (ii) 45/10 dsRNA, (iii) virus 019/6-A dsRNA, (iv) virus 38-4-A dsRNA, (v) virus F6-A and F6-B dsRNA, (vi) virus T1-A dsRNA, (vii) virus 01-1-4-A dsRNA (Buck et al, 1981) (viii) 3b1a ascospore virus (from 3b1a con E/A2) dsRNA (viii) 3b1a viruses A, B and C dsRNA. The quantities applied to the gel were approximately 0.05μg of each dsRNA component. Fig. 37b shows PAGE of the same Ggt viral dsRNA samples as in Fig. 37a applying in each case 50 x the quantity of total dsRNA that was applied to the agarose/formaldehyde gel described in Fig. 37a (ethidium bromide stained).

The result (Fig. 37) showed that 3b1a ssRNA hybridized to all of the Ggt viral RNAs but not to Aspergillus foetidus viral RNA. In order to evaluate the relative extent of hybridization, each of dsRNA samples was analysed by PAGE (Fig. 37b). To evaluate the relative extent of hybridization it was necessary to compare the intensity of any particular band on PAGE with the intensity of hybridization observed, i.e. the intensity of the band in the autoradiograph.

As expected, 3b1a dsRNA gave the strongest bands. By comparing the intensity of virus 3b1a - B dsRNA bands with the intensity of ascospore virus 3b1a con E/A2 bands it was clear that the ascospore dsRNA bands gave approximately the same intensity of signal as virus 3b1a-B dsRNA bands. Viruses F6-B, 38-4-A and 019/6-A also gave approximately the same intensity of signal per μg of dsRNA as the corresponding 3b1a viruses.

Virus 45/10 gave a low intensity signal and only one band was visible (probably highest mol. wt. band, 1.6×10^6). Viruses T1-A (from an Australian isolate) and 01-1-4-A also gave quite low signals.

These results confirmed that Ggt viruses show RNA sequence homology. The homology indicated here, agree with the results of

Buck et al (1981 on the serological relationships, i.e. that 3b1a viruses B and C are most closely related to viruses F6, 38-4-A and 019/6-A, and less closely related to the viruses of TI-A and 01-1-4-A. The results also indicate a low level of homology between 3b1a viruses B or C (probably B) and a virus of isolate 45/10.

3.3.6.2 Detection of dsRNA in Ascospore Isolates by Transfer and Hybridization with Radiolabelled ssRNA Probe

In order to investigate the possibility that ascospore isolates contained dsRNA (possibly without virus particles) standard dsRNA extractions were performed on the following ascospore isolates: 3b1a con D/A1, 3b1a con D/A2, 3b1a con D/A3, 3b1a con D/A4, 3b1a con E/A1, 3b1a con E/A2, 3b1a con E/A3 and 3b1a con E/A4, 45/10 con B/A1, 45/10 con B/A2, 45/10 con B/A3 (Section 3.3), 3b1a/A49, 3b1a/A51, 3b1a/A52, 3b1a/A55, 3b1a/A56 and 3b1a/A59 (supplied by J.W. Deacon) and isolate F911.1. ssRNA was precipitated in 2M-lithium chloride, the dsRNA dialysed against TNE, ethanol precipitated and taken up in 100µl of 0.01 x TNE buffer. The dsRNA was denatured, electrophoresed and transferred as described. The nitrocellulose filter was probed with 1µg of 3b1a ssRNA (virus B and C transcripts) kinase-labelled with (^{32}p) to a specific activity of 0.8 x 10^7 ct/min/µg.

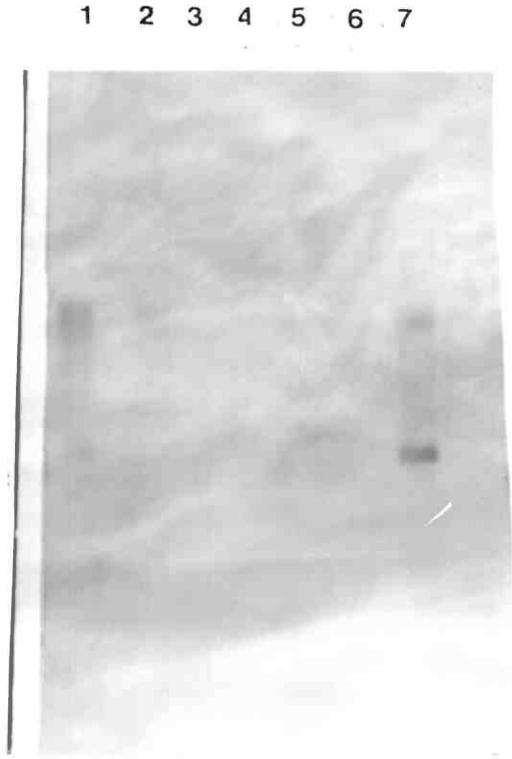


Fig. 38 Autoradiograph of dsRNA extracted from (i) 3b1a con D/A1 (0.1g wet wt. of mycelium), (ii) 3b1a con D/A2, (iii) 3b1a con D/A3, (iv) 3b1a con D/A4, (v) 3b1a con E/A4, (vi) 3b1a con E/A3, all from 10g (wet wt.)of mycelium, (vii) 3b1a con E/A2 (0.1g wet wt. of mycelium). The extracted RNA was denatured in formamide formaldehyde, electrophoresed in 1% agarose/formaldehyde gel, transferred to nitrocellulose and probed with 3b1a ssRNA (polymerase products of viruses B and C), kinase labelled to a specific activity of 0.8×10^7 ct/min/ g with (^{32}p) (5 day exposure).

The result (Fig. 38) showed that dsRNA was detectable only in those ascospore isolates (3b1a con E/A2 and 3b1a con D/A1) in which virus particles were previously detected (the dsRNA from these isolates has been diluted so as not to overexpose the autoradiograph). Only dsRNA previously detected in virus particles were seen.

The sensitivity of the technique for detection of 3b1a dsRNA components was determined by applying known amounts of viruses 3b1a A, B and C dsRNA to gels, transferring and hybridizing with the same probe as above. The minimum quantity of dsRNA detectable (Fig. 39) was 0.15ng of 3b1a dsRNA.

3.3.7. Sensitivity of Techniques for Detection of Viruses and dsRNA in Ascospore and Hyphal Tip Isolates

From Section 3.3.2. the level of virus in Ggt isolates F6 and 3b1a was estimated as 3μg dsRNA/g (wet wt.) of mycelium or 7-16 g virus, say 12μg virus/g (wet wt.) of mycelium. This was found to be equivalent to a mycelial concentration of about 160 virus particles (or dsRNA genomes) per cell. In order to compare the sensitivites of the various methods used here for detection of virus, the sensitivity of each method, expressed as the minimum number of virus particles or dsRNA genomes per cell detectable, was evaluated.



Fig. 39 Autoradiograph of decreasing quantities of dsRNA applied to agarose formaldehyde gel, transferred to nitrocellulose and probed with 3b1a virus B and C ssRNA kinase labelled to a specific activity of 0.8×10^7 ct/min/μg (5 day exposure). (i) .5μg dsRNA (ii) 50ng dsRNA (iii) 5ng dsRNA (iv) 0.5ng dsRNA (v) 0.05ng dsRNA.

(1) ELISA Technique

The ELISA technique (Section 3.3.3.) was found capable of detecting purified F6 virus at a level of 20ng/ml. The smallest volume needed for the test was 1.0ml, containing therefore 20ng, (at 20ng/ml). The recovery of virus in small scale virus extractions (from 10g, wet wt. of mycelium) has been estimated (Section 3.1.3) as gr ater than 20%, and therefore the minimum level of virus detectable by this technique is theoretically 10ng/g mycelium (in 10g) which is equivalent to a mycelial concentration of about one particle per 7 cells. However, when aliquots of F6 viruses were added to F911.1 mycelium, the level of virus detectable was 50ng/g (wet wt.) of mycelium, equivalent to about 1.5 particles per mycelial cell. This loss of sensitivity was presumably due to the high readings obtained using F911.1 alone obscuring the low levels of virus.

(2) Immune Electron Microscopy

IEM was found to be capable of detecting purified 3b1a viruses at a concentration of 40ng/ml. The smallest volume needed for the tests was 1.0ml containing therefore 40ng and therefore the level of virus detectable in a small-scale virus extraction (of 10g, wet wt. mycelium) would be about one particle per 3 cells.

However, in large-scale virus extractions used for detection of virus in ascospores, then a yield of mycelium of at least 1 kg (wet wt.) is obtained. The recovery of virus was estimated from PAGE of SDS-disrupted discarded pellets a d supernatants in the virus extraction of 3b1a con E/A2 (Section 3.3.5) as greater than 10%. The level of virus, detectable in mycelium is therefore less than one virus particle per 150 cells.

3. Transfer of dsRNA to Nitrocellulose of Hybridization with Radiolabelled ssRNA

The sensitivity of this technique for detection of dsRNA components of viruses 3b1a B and C was determined (Section 3.3.6.2) as capable of detecting 0.5ng 3b1a dsRNA. The recovery of dsRNA in a small scale dsRNA extraction has been estimated (Section 3.1.4) to be greater than 10%, therefore from 10g (wet wt.) of mycelium a dsRNA concentration of 0.5ng/g (wet wt.) mycelium. This is equivalent to about one virus particle per 35 cells.

It should be remembered however, that the dsRNA components of virus 3b1a-A are not detectable in this test. The dsRNA components of at least one virus of isolate 45/10 was detectable using 3b1a ssRNA. The sensitivity for detecting this virus was estimated by comparing the signal for 3b1a dsRNA and 45/10 dsRNA (Fig. 37) as

greater than 1/100 of the sensitivity for detecting a 3b1a dsRNA component, i.e. less than three particle per cells detectable.

3.3.8. Characterisation of Virus from Ascospore Isolates

Virus particles and viral dsRNA was detected in two ascospore isolates 3b1a con D/A1 and 3b1a con E/A2. For convenience these will be referred to as virus A1 and A2 respectively.

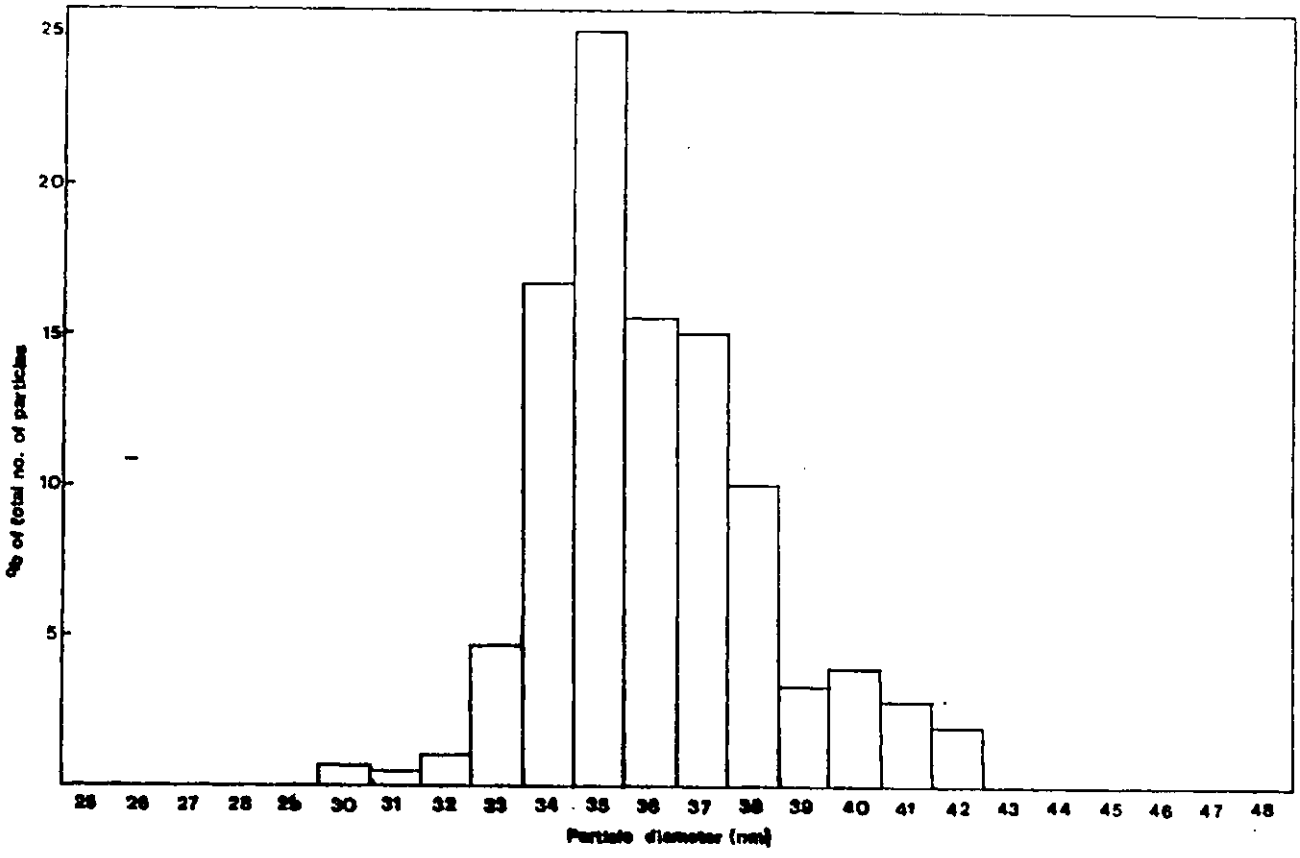
To recapitulate, 3b1a contains four viruses 3b1a-A (dsRNA components 4.1 and 3.5) virus B1 (dsRNA components 1.60, 1.54, 1.49) virus B2 (dsRNA components 1.45 and 1.43) and virus C (dsRNA components 1.27, 1.19 and 1.11). Virus A has 40nm particles and capsid polypeptides of mol. wt. 87, 83 and 78,000; virus B1 and B2 contain 35nm particles with capsid polypeptides of 73 and 68,000 mol. wt.; virus C contains 35nm particles and a capsid polypeptide of mol. wt. 55,000. In order to investigate whether the two capsid polypeptides each represent a single polypeptide of 3b1aB1 or B2, 3b1a virus (sucrose gradient 2, Section 3.2) was separated into fraction binding to bentonite and fraction not binding to bentonite, and analysed by SDS-PAGE. (Section 3.1.10, Fig. 14). From the results, it was postulated that virus B1 contains the 68,000 mol. wt. polypeptide and virus B1 contains the 73,000 mol. wt. polypeptide. The serological relationship between virus B1 and B2 were investigated (Section 3.1.10) and the results suggested that virus B1 and B2 were serologically distinct.

3.3.8.1 Particle Size

Particles were sized by direct electron microscopy and histograms of particle size were plotted (Fig. 40). Virus from 3b1a (sucrose gradient fraction 4, containing viruses A, B1, B2 and C, Section 3.2) was similarly analysed (Fig. 40b). Viruses A1 and A2 (from 3b1a con D/A1 and 3b1a con E/A2 respectively) gave similar histograms so only the latter is shown here. 3b1a virus had two particle modes, one at 34-37nm (containing viruses B1, B2 and C) and the second at 39-42nm (containing virus A). Both A1 and A2 viruses had one mode, at 34-36nm.

3.3.8.2 dsRNA Components

dsRNA components of A1 and A2 were examined by gel electrophoresis. In both cases (Fig. 41) only two dsRNA components were present; and the dsRNA components of A1 and A2 were shown to co-migrate on PAGE. On co-electrophoresis of ascospore and parental 3b1a dsRNA on slab gels (Fig. 41) it was seen that the higher mol. wt. ascospore dsRNA band co-migrated with the 3b1a B1 dsRNA component of mol. wt. 1.54×10^6, but that the lower mol. wt. ascospore dsRNA component migrated between the 3b1a dsRNA components of mol. wt. 1.54 and 1.43×10^6 (minor band of 3b1a B1-b'4, is not visible on the photograph but was seen to be between the 1.45 and 1.43×10^6 3b1a B2 virus dsRNA components).



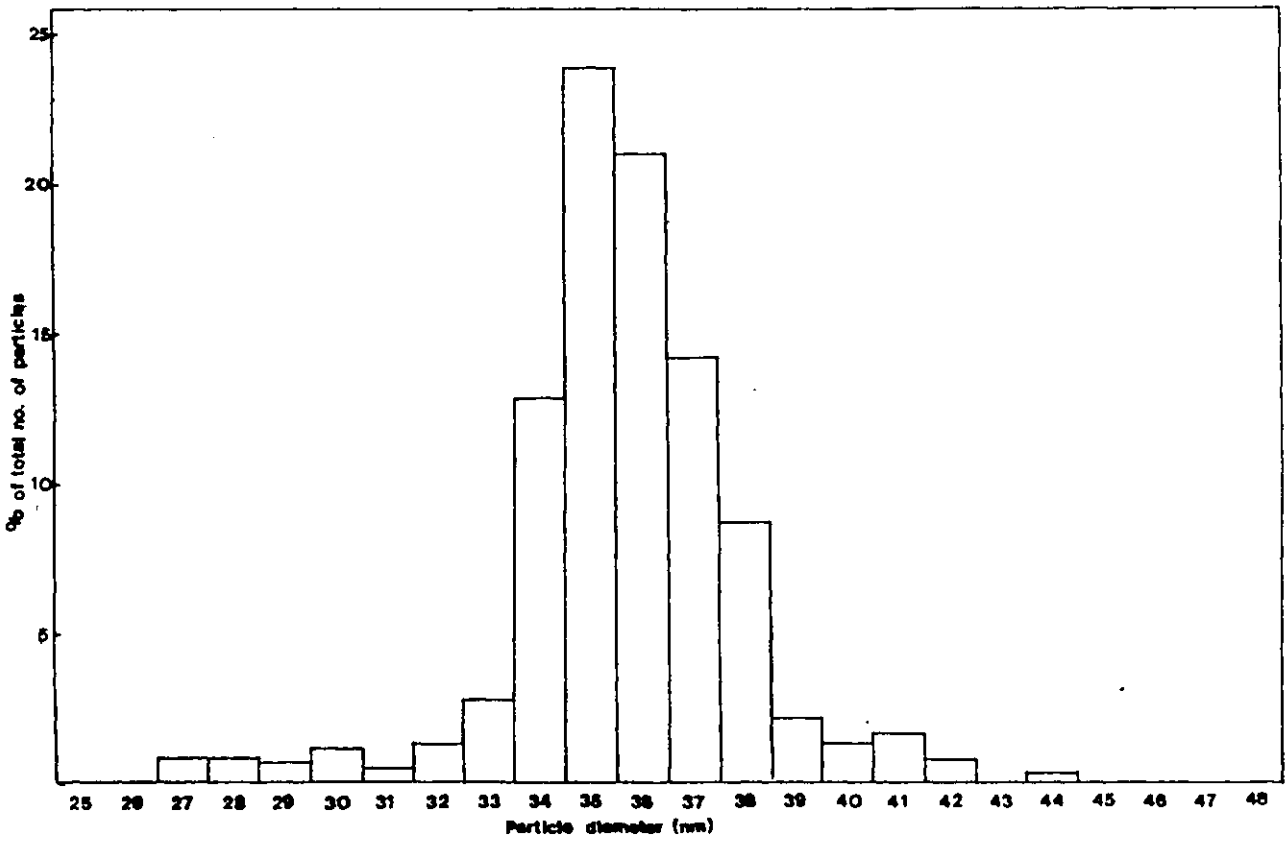


Fig. 40 Histograms of virus particle diameter of viruses from (a) 3b1a, sucrose gradient fraction 4, (b) virus extracted from 3b1a con E/A2.

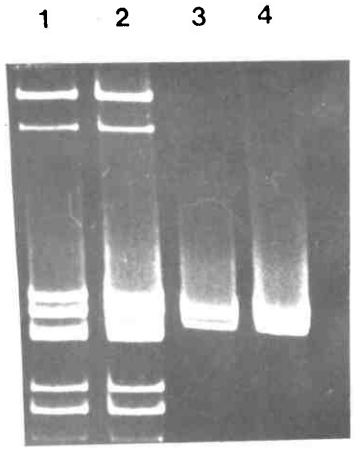
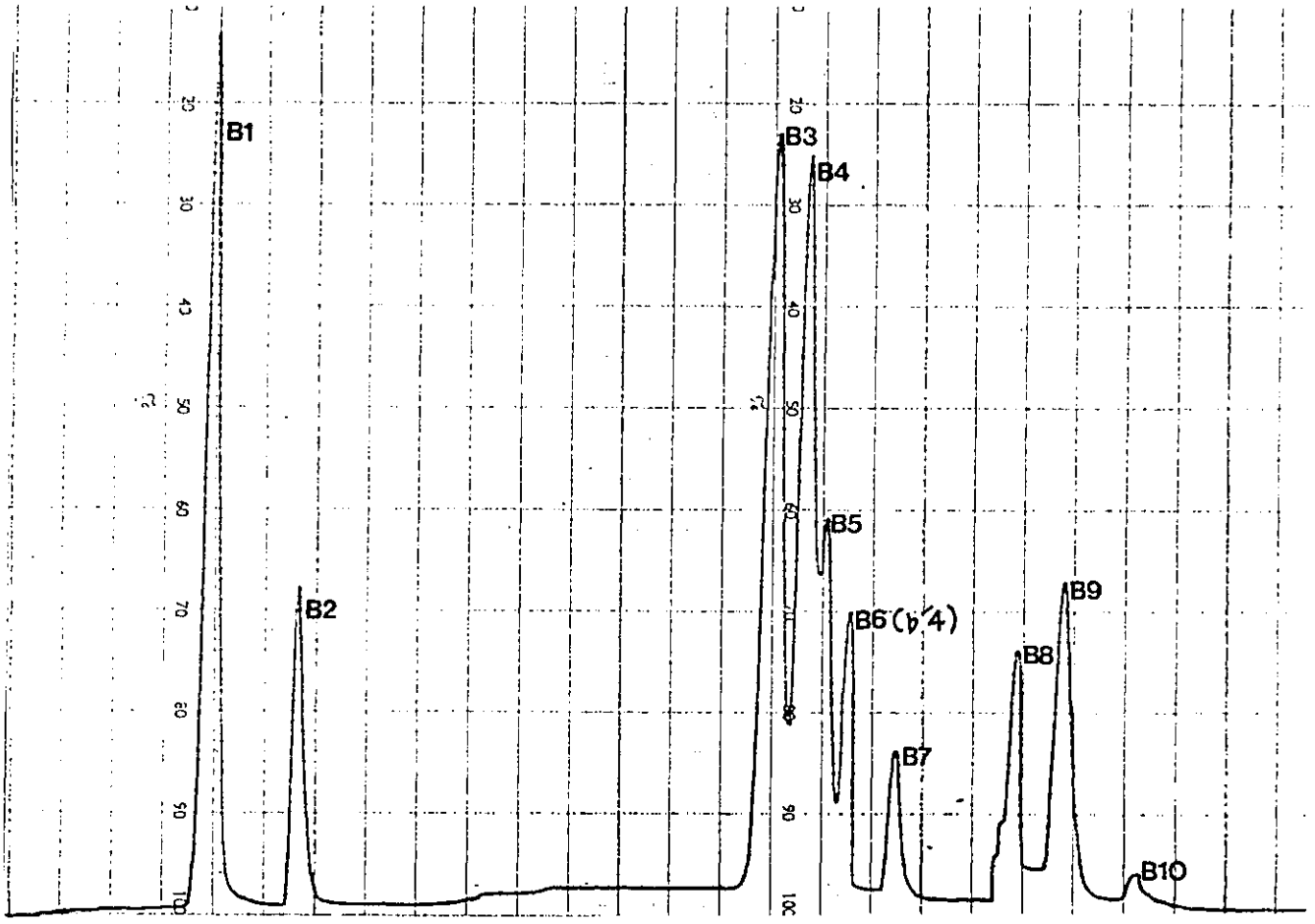


Fig. 41 PAGE of 3b1a and ascospore viral dsRNA (i) 3b1a viral dsRNA (sucrose gradient fraction 4), 30µl sample, (ii) 3b1a viral dsRNA (20µl) plus 5µl of ascospore virus (from 3b1a con E/A2), (iii) ascospore viral dsRNA 10µl sample from 3b1a con E/A2, (iv) dsRNA from ascospore isolate 3b1a con D/A1. The gel was electrophoresed at 60V for 24 hours and stained with ethidium.



a

Fig. 42 Gel scans of 3b1a and ascospore viral dsRNA. DsRNAs were subject to electrophoresis in 4% polyacrylamide cylinder gels at 6V/cm for 24h. The gels were then stained with toluidine blue and scanned at 550nm. The direction of electrophoresis was from left to right. (a) 3b1 dsRNA (sucrose gradient fraction 4) 20μl, (b) ascospore dsRNA (from 3b1a con E/A2 5μl, (c) (over page) 3b1a dsRNA (20 1) plus 1μl of ascospore dsRNA. The position of the ten dsRNA components present in 3b1a (labelled B1, B2 from 3b1a-A, B3-B7 from 3b1a-B1 and B2,) the components of virus 3b1a B1 are indicated B_13, B_14, B_16 and virus 3b1a B2 are indicated B_25 and B_27; B8-B10 from virus 3b1a-C. The position of the two dsRNA components of ascospore virus are labelled A1 and A2.

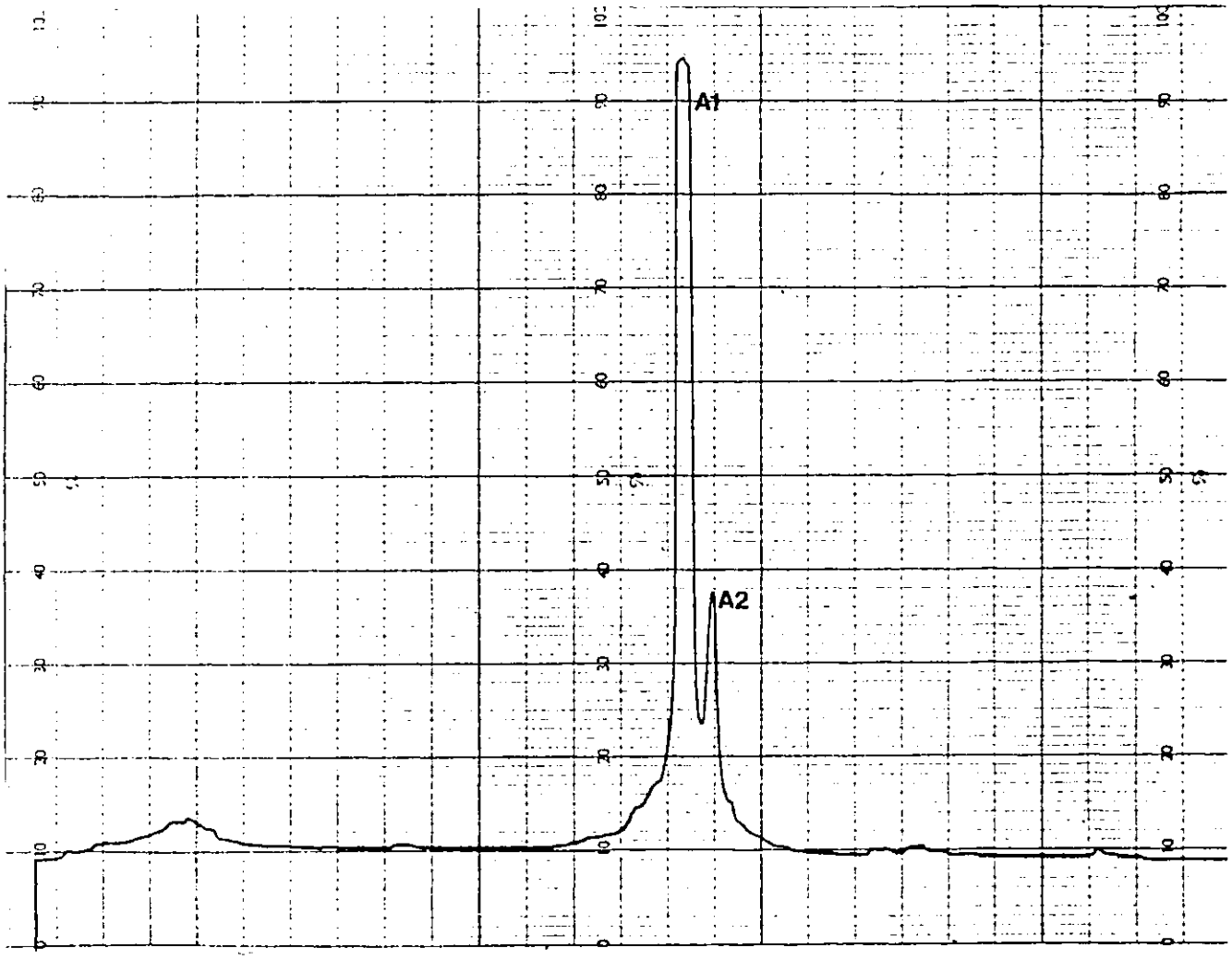


Fig. 42b

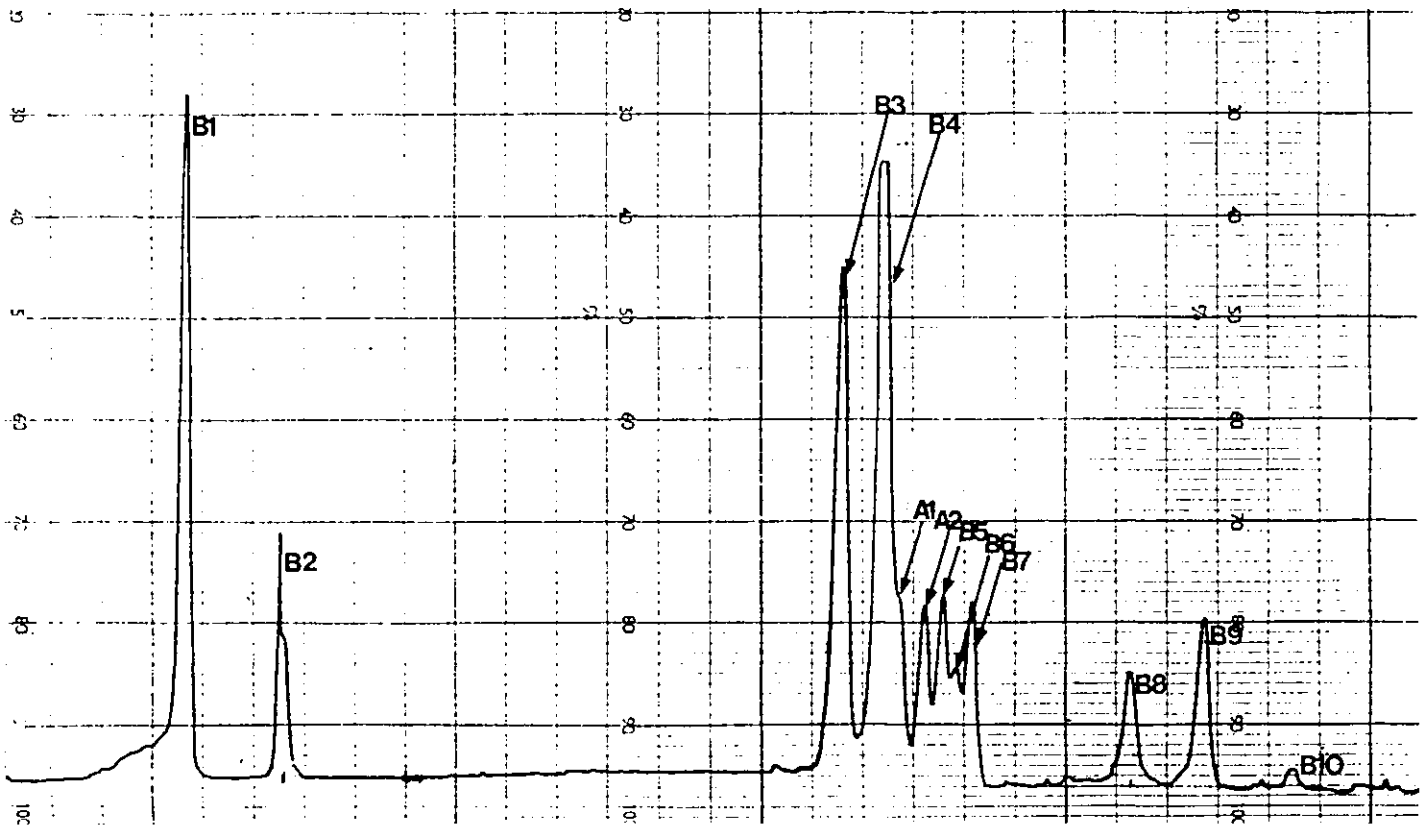


Fig. 42c

However, when ascospore and parental 3b1a dsRNA was electrophoresed on semi-micro tube gels (which give finer resolution), it was found (Fig. 42) that neither ascospore dsRNA bands co-electrophoresed with any parental 3b1a dsRNA bands. Spectrophotometric scans of these gels are shown in Fig. 42 and it was seen that 7 bands were clearly present in the co-electrophoresis (though the higher mol. wt. ascospore band was recorded on scanning as a shoulder on the 1.54×10^6 3b1a B1 band). The mol. wts. of the ascospore bands were determined as 1.52 and 1.48×10^6. Since A1 and A2 dsRNA components co-migrated (on all gel systems tried) these mol. wts. apply to both viruses.

3.3.8.3 Electrophoretic Mobility of Virions

The electrophoretic mobility of ascospore virus was examined by agarose gel electrophoresis, alongside 3b1a sucrose gradient fractions 2, 4 and 7 (Section 3.1.9). Three bands were seen to stain both with ethidium bromide (for nucleic acids) and coomassie blue (for proteins); one of these bands (middle) co-electrophoresed with the single band seen in both A1 and A2 tracks (Fig. 44). The bands co-electrophoresing in A1, A2 and 3b1a were excised, disrupted, SDS treated 60°C, and analysed by PAGE. The five dsRNA components of viruses 3b1a B1 and B2 were visible in the 3b1a band, the two dsRNA components of A1 and A2 were visible in the ascospore band. This demonstrates that ascospore virus migrates on electrophoresis as a single band and co-electrophoreses with viruses B1 and B2 from the parent 3b1a.

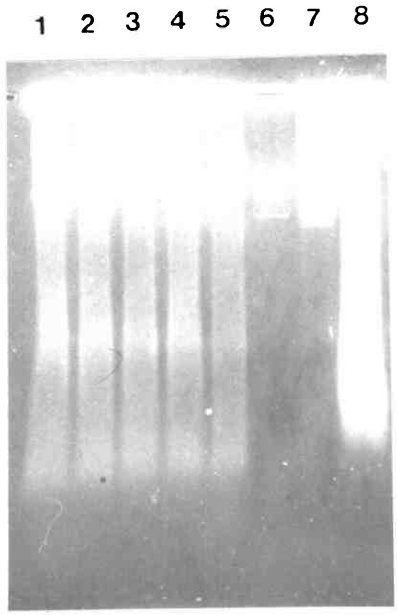


Fig. 43 Electrophoresis of intact virus in 0.8%-agarose gel. (i) - (v) ascospore virus (10μl of 3b1a con E/A2 virus), (vi) 3b1a viruses (sucrose density gradient fraction 2, see Fig. 13), 30μl, (vii) 3b1a viruses, sucrose gradient fraction 4, (viii) 3b1a viruses sucrose gradient fraction 7. The gel was electrophoresed at 100V for 4 hours and stained with ethidium.

3.3.8.4 Capsid Polypeptide of Ascospore Virus

The capsid polypeptide components of A2 virus were examined by SDS-PAGE. Many host proteins bands were visible in crude virus preparations and interpretation was impossible. Crude virus (1ml) was therefore further purified by rate zonal centrifugation in 20ml linear sucrose density gradients (10 to 45% in PBK buffer). Purified virus was analysed by SDS-PAGE in an 8% polyacrylamide gel.

The result (Fig. 45) shows ascospore virus to contain one major polypeptide that had approximately the same mobility as the 68000 mol. wt. polypeptide of virus 3b1a-B.

3.3.8.5 Serology

The serological relationships between parental 3b1a viruses and ascospore viruses was investigated:

(i) 3b1a mouse antiserum was titrated against ascospore virus. The result showed a single precipitin line that had an antiserum titre of 128 with both ascospore viruses (A1 and A2).

(ii) 3b1a mouse antiserum (1/64) was reacted against 3b1a viruses (sucrose gradient fraction 4), 3b1a bentonite supernatant viruses (as above) and ascospore A1 and A2 viruses in adjacent wells.

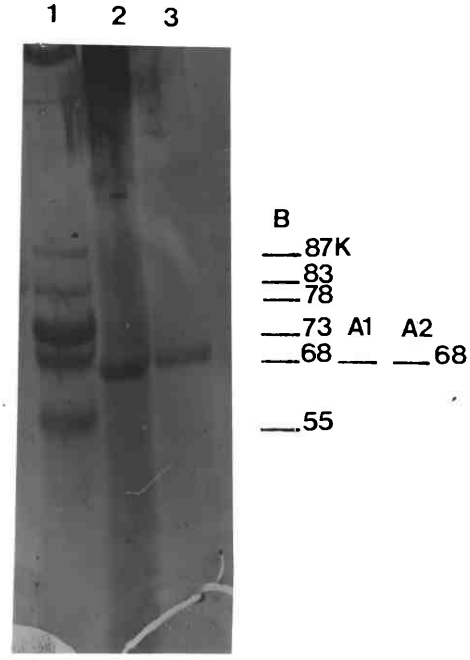
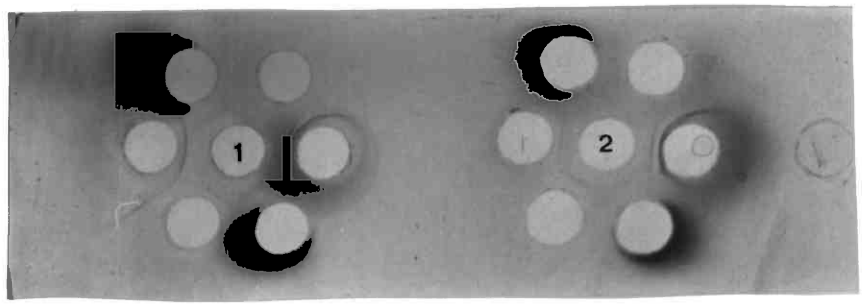


Fig. 44 SDS-PAGE of 3b1a and ascospore virus proteins electrophoresed in an 8% polyacrylamide gel. (i) 3b1a viruses, sucrose gradient fraction 4, (ii) ascospore virus from 3b1a con E/A2, (iii) ascospore virus from 3b1a con D/A1. The gel was electrophoresed at 80V for 18 hours and stained with coomassie blue.

The result (Fig. 45) showed the precipitin lines formed by A1 and A2 ascospore viruses to fuse, indicating that they are serologically identical (using this antiserum). This line also fused with the precipitin line of virus B1 in the bentonite supernatant fraction, indicating ascospore virus was serologically identical to virus 3b1a-B1 (using this antiserum). Ascospore virus (A2) however formed a spurred precipitin line with the line containing B1 + B2 in the 3b1a virus mixture. Like the reaction between the bentonite supernatant and 3b1a virus mixture the line formed between ascospore virus and 3b1a virus mixture spurred towards the ascospore virus well. This was interpreted similarly to the reaction between virus B1 and virus B2, i.e. that ascospore virus is serologically distinct to virus B2.

3.3.8.6 Discussion

The results indicate that ascospore viruses A1 and A2 are serologically identical, and identical to virus B1 of the parent 3b1a. The dsRNA components do not however have the same molecular weight as B1 dsRNA components and also the minor dsRNA component of virus B1 (b4) is not present in the ascospore virus. Ascospore virus contains one capsid polypeptide that has the same mobility on SDS-PAGE, as the 68000 mol. wt. capsid polypeptide of parent B1 and B2 viruses. It is not completely certain which virus (B1 and B2) contains which capsid polypeptide, or if indeed both polypeptides are contained in both viruses, or if the 68000 polypeptide is a proteolytic cleavage



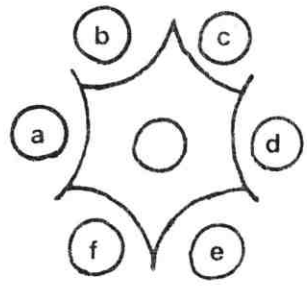


Fig. 45 Gel immunodiffusion tests showing serological relationships between 3b1a and ascospore virus. Mouse antisera at (i) 1:64 dilution of antiserum, and (ii) at 1:128 dilution of antiserum (in centre wells). (a) contains 3b1a viruses sucrose gradient fraction, (b) contains virus extracted from ascospore isolate 3b1a con E/A2, (c) contains fraction of 3b1a viruses (sucrose gradient fraction 2) not binding to bentonite (bentonite supernatant) and separated from bentonite bound material as described in Fig. 19, (d) contains 3b1a viruses sucrose gradient fraction 2, diluted 1:3, (e) contains virus extracted from ascospore isolate 3b1a con E/A2, (f) contains virus extracted from ascospore isolate 3b1a con D/A1. The gel was stained with coomassie blue. A diagrammatic representation of the result is shown beneath.

product of the 73000 polypeptide. The evidence suggests (Fig. 14) that virus B2 contains the 73000 polypeptide and B1 contains the 68000 polypeptide. The fact that the ascospore virus is serologically identical to virus B1 and contains a 68000 mol. wt. capsid polypeptide would support this conclusion. However, this would imply that the virus with the smaller dsRNA genome (virus B2) encodes the larger capsid polypeptide. This is theoretically feasible since the smallest dsRNA component of virus B2 (1.43×10^6) has enough coding capacity to encode a polypeptide of molecular weight 79000 (assuming 3 nucleotides encodes one amino acid of average mol. wt. 330 and 110 respectively).

3.3.9 Discussion

Virus particles and dsRNA were found in two ascospore isolates out of 16 tested. Virus particles and dsRNA were also found in hyphal tip isolates derived from isolate F6. dsRNA, in the absence of virus particles, was not detected in any ascospore isolates. The ascospore isolates in which virus was found were derived from different conidial isolates. Rawlinson et al, (1973) examined 56 ascospore isolates by direct electron microscopy, but did not detect virus particles. This contrasting result could be accounted for by any of the following explanations: (i) In the present study mycelial contamination of ascospores might have been the source of virus. This can be eliminated since in such a situation viruses isolated from the ascospore cultures would be identical with those obtained from the parent. In both cases this was plainly not the case. (ii) Virus particles might have been present in the 56 ascospore isolates investigated by Rawlinson, but at an undetected level. This is possible since the methods Rawlinson used for extraction and detection of virus were not as sensitive as the methods developed in the present work. However, the level of virus found in ascospore isolates and A2 here, was similar to that present in the parent 3b1a in which Rawlinson detected virus. So this possibility does not seem likely. (iii) Virus particles might be transmitted only very rarely to ascospores but, by chance, were transmitted to the isolates examined in this study. Since virus was found in two ascospore isolates this may not seem likely, however, in both cases, only a single virus

out of four present in the parent, was transmitted to the ascospore. Many Ggt isolates contain only one virus type, so it is possible that viruses capable of ascospore transmission are rare in Ggt, considerably reducing the chance of finding ascospore transmission. (iv) Virus transmission into ascospores produced by field isolates (used by Rawlinson et al, (1973) which could be mixtures of strains of heterokaryotic, might be much less frequent than into ascospores derived from the homokoryotic single conidial isolates in which virus transmission was found in the present study. This could explain why virus particles were not detected in the ascospore isolates supplied by D. Hornby (Rothamsted Experimental Station), which were derived from the field isolate 3b1a, although insufficient isolates were examined to be certain of detection of a one in four transmission of virus to ascospores. Mechanisms that could allow this would be purely conjecture, but may involve the preferential formation of perithecia, between hyphae containing low levels of virus in field isolates, where the level of virus within the hyphal may be more variable than in conidial isolates. This may be possible if the presence of virus inhibited production of perithecia, as seems to occur in Endothia parasitica (Day & Dodds, 1979). Relevant to this may be data (Blanch, 1979), (see Introduction p.29) that suggests that perithecial production may be affected by cytoplasmic factors.

These results are consistant with the observation by Lapierre (1973) that virus is not always eliminated during sexual reproduction. Moreover, Tivoli et al, (1979) reported that an

ascospore isolate 911-1 (F911-1 used in the present study is a sub-culture of this isolate), although initially virus-free, was shown to contain virus particles after storage in culture for several years. However, in the present study (Section 3.3) virus extraction was performed on large quantities of (fermenter grown) mycelia of F911-1 and examined for virus and no virus was detected; so the significance of Tivoli's finding is not clear. In both ascospore isolates in which virus was found in this study, it was present at levels approaching 'normal' levels for 3b1a; no evidence of virus existing for long periods in a 'suppressed' state was obtained in this study.

The question arises whether the results presented here account for the observations by Rawlinson et al, (1973, 1976) (see Introduction p. 71), on the appearance of virus in Ggt found in newly infected cereal crops. If virus were present in only a minority of infecting asco pores (possibly lower than found in this study since they would all be derived from field isolates) then it may spread to other isolates by heterokaryosis and subsequently be found in an increasing proportion of isolates, as was actually observed. This study does not shed any light on the appearance of predominantly 27nm particles in Ggt isolates from newly infected fields (Rawlinson et al, 1973, 1976) since these particles have not been purified or characterised. If they represent another group of Ggt viruses, then it is possible that they are transmitted to ascospores more frequently than other Ggt viruses. 27nm particles were not present in any of the isolates from which ascospores were derived in the present work.

In both ascospore isolates that virus was found in this study (3b1a con E/A2, 3b1a con D/A1), a single virus was present, and the virus was identical in both cases. The mechanism of exclusion of the viruses not found in the ascospores is unknown, but in yeast preferential inclusion of dsRNA in ascospores, relative to other cytoplasmic elements is found (Brewer and Frangman, 1960), suggesting possible nuclear association of dsRNA. A similar mechanism may operate here, i.e. preferential inclusion of ascospore virus relative to other viruses present in the parent. However, in yeast, transmission of virus to ascospores is the rule rather than the exception.

Perhaps the most surprising result is the demonstration that virus present in ascospores was not identical to any of the parent viruses. The ascospore virus was serologically identical to virus 3b1a-B1 in the parent, and contained a similar capsid polypeptide, but the dsRNA mol. wt. s were markedly different from those of the parent B1 dsRNA components. Additionally the minor dsRNA component of virus 3b1a-B1 (b 4) was not present in the ascospore virus. This loss of dsRNA component is similar to the loss of a single dsRNA component of virus F6-B in two conidial isolates of F6 (Section 2.1.3) and may be explained by a similar mechanism, i.e. loss of a non-essential dsRNA component. Since in this case, the dsRNA component that was lost was of lower mol. wt. than those retained, it may be either a satellite dsRNA component of virus 3b1a-B1 of unknown function, or a deletion product of one of the higher mol. wt. dsRNA components.

Romanos (1981) obtained evidence (as discussed in Introduction) of two defective interfering dsRNA components of another Ggt virus, 45/9, which were postulated to be deletion products of the functional dsRNA components of this virus.

The alteration in mol. wt. of the dsRNA components of the ascospore virus, relative to the parent virus 3b1a-B1 is more puzzling and without obvious precedent. If it is accepted that it is the minor dsRNA component (b'4) that is lost, then since the mol. wt. of B1 virus dsRNA, were 1.60×10^6 and 1.54×10^6 and the mol. wt. of ascospore dsRNAs were 1.5×10^6 and 1.48×10^6. If the highest and lowest molecular weight components are equated then the 1.60×10^6 component has lost 0.05 of its sequence length and the 1.54×10^6 component has lost 0.04 of its length, 121 and 91 base-pairs respectively. Alternatively, if the 1.52×10^6 component was derived from the 1.540×10^6 component only 30 base-pairs would have been lost; and if the 1.48×10^6 component was derived from the 1.60×10^6 component then 181 base-pairs would have been lost. This phenomenon does bear some similarities however to the changes observed in dsRNA profile by passage of virus in Endothia parasitica between strains by heterokaryosis and passage of virus between strains of Ustilago maydis by heterokaryon transfer and sexual crosses.

Anagnostakis and Day (1979) transferred dsRNA (via heterokaryosis) from hypovirulent strains of Endothia parisitica dsRNA containing isolates to virulent isolates (lacking dsRNA). dsRNA was transferred from one isolate (113) to a virulent isolate (155) with six replicates of the experiment. In each case the dsRNA pattern of the converted strain (155 - hypovirulent? was very similar to the dsRNA pattern of 113 although small differences in mobility may have occurred in some cases. However, when dsRNA from isolate 113 was transferred to a virulent isolate 78, again with six replicates, the dsRNA patterns of the resultant six converted strains were all different and none of them were identical to 113. In some cases bands seem to have been lost and bands of mobility not present in 113 have appeared. The dsRNA patterns of these strains were unstable on further subculturing; dsRNA bands disappeared and new bands appeared after five subcultures. The dsRNA pattern of the converted strain 155 was however stable on further subculturing. Transfer of dsRNA from 113 to other virulent isolates gave similar results, in some cases the converted strains had dsRNA profiles similar to 113, in other cases the band pattern was completely different.

When the dsRNA pattern for the derived strain was considerably altered from the pattern in 113, and was also unstable, the strain was usually derived from a strain of a particular vegetative compatibility group (v-c 17). The derived patterns were more complex than might be

expected from simple reassortment of dsRNA components if 113 carried a multi-component virus or several distinct viruses; the authors suggest that the configurational instability may result from the action of dsRNA nucleases present in v-c 17 strains, that are absent or ineffective in other strains.

Day (1981) transferred dsRNA by heterokaryosis from a compatible strain of Ustilago maydis carrying dsRNA to two strains A and C with no detectable dsRNA. Nine dsRNA containing strains were used containing nine different patterns of dsRNA. Extraction and characterisation of dsRNA from converted strains showed in general a high degree of fidelity for all transferred patterns except for three patterns. One of these (III) was changed in both backgrounds. The original isolate contained two major dsRNA bands, one being a doublet; in the converted strains the doublet became a single band and a new band, of lower molecular weight, appeared. For pattern IV, bands 2 and 4 (of 5 bands) were missing in A and band 4 was missing in C. For pattern VII, band 2 was missing in A and no dsRNA was recovered in C.

Day (1981) also examined the dsRNA pattern from the sexual spores, teliospores, produced from the same gall on a host; and also the sibling tetrad products from a single teliospore. It was found that not only was there variation in dsRNA pattern from different teliospores, but there was also variation between sibling products from single teliospores (roughly equivalent to individual ascospores from a single ascus), some bands being present in an individual product that were lacking in siblings.

The results with Endothia and Ustilago suggest that a dsRNA pattern that is stable within one host is unstable when transferred to another host. The loss of single or multiple dsRNA components during transfer by heterokaryosis could be attributed to incomplete transfer of virus or virus components. This would however suggest that transfer of virus involves only a very limited number of virus particles. Since fusion of single cells would probably allow hundreds of virus particles to move between hyphae, this seems unlikely. Moreover, this would not account for the alteration in molecular weight of dsRNA components after transfer. A more likely explanation is that the 'new' host somehow alters the virus genome. One method of doing this would be by nuclease digestion of genome components. either specifically in a manner analagou to the action of bacterial restriction endonucleases on bacteriophage genomes, or by some unspecific (possibly dsRNA specific) nuclease. Relevant to this may be the evidence of Koltin et al (1979) that killer protein in Ustilago has nuclease activity. An alternative explanation would be that the original virus is heterogeneous, con isting of a wide variety of dsRNA segments of differing sequence and that the dsRNA bands that are seen on a gel represent only the model size of these molecules, minor populations of molecules of different size may be undetected. When the virus is transferred to a new host, different conditions may select a different population of dsRNA molecules that are able to replicate most efficiently in the new environment.

Sequence heterogenicity has been detected in many RNA viruses from animals and plants. Donnis et al, (1981) examined Satellite Tobacco Necrosis virus (STNV) RNA by hybridizing viral ssRNA with short DNA oligonucleotides of specific sequence such that short lengths (4 nucleotides) of dsRNA are formed with homologous sequences. The RNA can then be digested with ribonuclease H (RNA-DNA hybrid specific) to produce specific fragments in a manner analagous to the use of DNA restriction endonucleases. The authors found that sequential passage of the virus through host produces several changes in digestion band pattern and these changes were gradual, indicating a heterogenous population.

Younger et al (1981) examined the production of temperature sensitive (TS) mutants by Vesicular stomatis virus (VSV) during sequential passage through host. The authors found a striking increase in the frequency of Ts mutants with repeated passage through cells and by examining cloned virus showed that a large degree of sequence heterogen ity was generated. However, different cell lines produced markedly differing results; some cell lines produced no increase in Ts mutants. This indicated that host cell functions may be involved in the generation of Ts mutants and sequence heterogen ity; perhaps some factor involved in the fidelity of RNA transcription.

Holland et al (1980) obtained similar results, when VSV produced by persistently infected cells maintained for 3 - 5 years was examined by RNA fingerprinting with T1 ribonuclease. The virus produced had low infectivity, contained many T1 map changes and a heterogenous population was generated.

The genome of influenza virus has been similarly analysed during sequential passage through host (Pons, 1980), Brand, 1980) and similar sequence changes and heterogencity were shown. Again, virus behaved differently in different cell lines indicating that host factors were involved.

Persistent intracellular infections probably provide a permissive environment where the selection on virus is least, allowing the evolution of a heterogenous population of sequences. If also the environment of the virus rapidly changes during intracellular growth then it is possible that the mechanism of production of heterogenecity may be selected for to allow rapid evolutionary change when the environment is altered by, for instance, transfer of virus to a new host (Pringle, 1979).

The situation may be analogous to polymorphism at the genomic and polypeptide level, found in E. coli (Milkman, 1973) Drosophila (Hubby and Lewontin, 1966) and man (Bostein et al, 1980). Virus infection in fungi bears many similarities to persistent infections; the exclusively intracellular life cycle probably provides the most permissive environment possible in allowing genome variation and heterogenecity. Virus isolated from persistent infections has low infectivity; one could imagine that a persistent intracellular intection maintained for perhaps hundreds of years might produce virus particles that had completely lost the ability

to infect extracellularly - a situation analogous to fungal virus infection.

Persistent intracellular infections also produce an increase in the number of defective interfering (DI) particles. These have been found in most RNA viruses studied and are generated by internal deletion of standard viral RNAs (Kennedy, 1976) Like the production of heterogencity and Ts mutants, the generation of DI particles is dependent on the host cell, different DI particles being produced by different cell lines (Stark and Kennedy, 1978) and often a whole series of DI particles being produced with continued infection. The production of DI particles undoubtedly reduces the infectivity of the virus population.

DI particles have been found in yeast. They are produced by internal deletion of M dsRNA and are known as S RNAs. The 3′ end of these S RNAs have been sequenced and are markedly heterogeneous (Bruenn and Brennan, 1980). They gradually displace M dsRNA in yeast cells producing a wide variety of defective dsRNAs (Bruenn and Kane, 1978). Yeast L dsRNA also shows sequence heterogencity (Brennen et al, 1981).

The results with Endothia and Ustilago can therefore be interpreted in two related ways. Firstly the multiple dsRNA

components of the parental viruses could be composed of several defective interfering RNAs, and these could rapidly evolve in the new environment to produce a different dsRNA pattern. The instability of dsRNA pattern on heterokaryon transfer between some strains of Endothia parasitica (Ananostakis and Day, 1979) certainly bears similarities to the divergence of DI particles in yeast virus. Alternatively the new environment may select, by, for instance, competition for a host factor involved in virus replication that is altered in the new host, a new population of RNA molecules derived from a previously heterogenous population. This need not involve the alteration of any dsRNA component, merely that a dsRNA component that was a minor constituent of the virus population in the donor host is able to replicate more efficiently in the new host and become the major component. The heterogencity in genome that this would imply may be selected for in order to allow rapid evolution in a new environment.

A similar situation may apply to the alteration in the genome of 3b1a B1 virus with passage through ascospores. Since virus is usually excluded from ascospores there must be some mechanism for exclusion. The most likely possibility is that cytoplasm is excluded in the ascospore since fungal virus particles seem to be located, from ultrastructure evidence, nearly exclusively in the cytoplasm (Weber, 1979). However, ascospores have to retain some cytoplasm and cytoplasmic genetic information, such as that contained

in mitochondria. If the population of virus in 3b1a is heterogenous then the ascospore virus may represent a minor population of particles that is able to either, replicate more efficiently in ascospores, or during the events leading to ascospore formation, or merely to be retained in the ascospore, possibly by being located in nuclei or mitochondria. In either case, the mechanism cannot be a random retention of a single virus component since the same virus was retained in ascospores from two separate conidial isolates. Alternatively, the mycelium derived from the ascospore may provide an altered environment from that in the conidial parent. Since the conidial parent and ascospore progeny have identical nuclear genomes this could result from the exclusion or alteration of a cytoplasmic factor involved in virus replication. Romanos (1981) found evidence of sequence heterogencity in some Ggt viruses by noting a large number of minor spots appearing in some T1 fingerprints.

An understanding of exactly what is occurring in virus passage through ascospores would require knowledge of the fate of virus in ascospores, and particularly the method of exclusion. Since genetic information has been lost in virus B1 (the shortening of dsRNA segments and loss of minor dsRNA component) on passage through ascospores this may imply that nuclease action attacked the dsRNA genome. The resultant genome may be resistant to nuclease action, (possibly if a sequence-specific nuclease is active, by lack of a site), or be able to replicate more efficiently in the presence of nuclease.

This latter situation would probably result in the surviving virus containing the minimum genetic information required for virus replication. This minimum would probably be the same in two different mycelia, explaining why the same ascospore virus was produced by two conidial parents. It may be that this same principle would apply to the selection of a single virus from a heterogenous population; that the virus containing the minimal genetic information is most likely to be inherited through ascospores. The determination of exactly what is occurring would require sequencing of individual dsRNA components.

The generation of virus with altered dsRNA genome in ascospores is probably at least partly responsible for the wide variation of dsRNA components in Ggt (Buck et al, 1981) and indeed in other fungal viruses.

3.4 Search for Provirus in Ascospore Cultures of Ggt

Total cell DNA was extracted, firstly from isolate 3b1a con E/A1, as described in Methods. The DNA was analysed to ensure purity and then applied to 'Southern' blots and probed with viral ssRNA prepared from 3b1a viruses, in order to detect provirus. DNA was also probed with ribosomal RNA prepared from Ggt in order to act as a positive control. dsRNA was also applied to the filters containing DNA and was used to measure the sensitivity of the hybridization technique.

3.4.1. Preparation of Ggt Total RNA

Preparations of total RNA from most organisms will typically consist of 90% ribosomal RNA (R. Cox personal communications). If this preparation of total RNA is radioactively labelled then since, in fungi, such as Neurospora crassa, ribosomal RNA sequences are present in about 185 copies per haploid genome and account for about 88% of the repetitive DNA content of N. crassa (Koumlanf & Marzluf, 1980), then it should be possible to use preparations of total RNA as probe to identify the ribosomal DNA genes in fungi and other organisms.

It was decided to use Ggt total RNA to identify the ribosomal RNA genes in Ggt in order to show that the techniques for detecting provirus in the Ggt genome were feasible. It should, however, be

remembered that the sensitivity for detecting proviral DNA should be at least 185 times the sensitivity needed to detect the repetitive ribosomal DNA genes.

Total RNA was prepared from 100g of Ggt mycelium as described in Methods. The RNA was analysed by urea-PAGE using wheatgerm RNA as marker. Ggt RNA was found to be quite degraded, presumably due to nuclease action during the extraction procedure. The size of the RNA was estimated to be between 100-500 base-pairs. This RNA was end-labelled directly (using polynucleotide kinase) for use in Southern blots of Ggt DNA.

3.4.2. Preparation and Analysis of Ggt DNA

Total cell DNA from Ggt isolate 3b1a con E/A1 was prepared as described in Methods and analysed as follows:

3.4.2.1 Spectrophotometry

The DNA was scanned between 320-220nm. The spectrum was typical of nucleic acid with an absorbance maximum at 256nm, a minimum at 230nm, an A_{260}/A_{280} ratio of 1.95 and no absorbance at 300nm. No shoulder at 290-270nm can be seen, which indicates absence of contamination with protein; the high A_{260}/A_{280} ratio confirms this.

The yield of DNA was estimated as 800μg from 100g (wet wt.) of mycelia. This is a low yield but sufficient for the purpose of these experiments.

3.4.2.2. Thermal Analysis

A thermal denaturation curve of total DNA is shown in Fig. 46. Initial attempts to denature the DNA in SSC buffer were unsuccessful as recording the complete absorbance profile necessitated maintaining the temperature above 98°C which was the highest temperature obtainable with the equipment available. The buffer used was therefore: 0.0025M-Na_2HPO_4, 0.005M-NaH_2PO_4, 0.001M-EDTA, pH, 6.8, which Marmur and Doty (1962) found gave melting temperatures 20°C lower than in SSC for eight different DNA samples. The initial absorbance at 260nm was 0.268 at 25°C, the final absorbance was 0.375 at 90°C (corrected for volume expansion) giving a hyperchromicity of 39.5% of the absorbance value at 25°C.

The Tm, defined as the temperature corresponding to half the final increase in absorbance (corrected for volume expansion) was 72.4°C. This would give a Tm in SSC of 92.4°C. The melting curve showed a steady fall in absorbance in the region before the DNA melts, indicating a lack of contamination with RNA or protein.

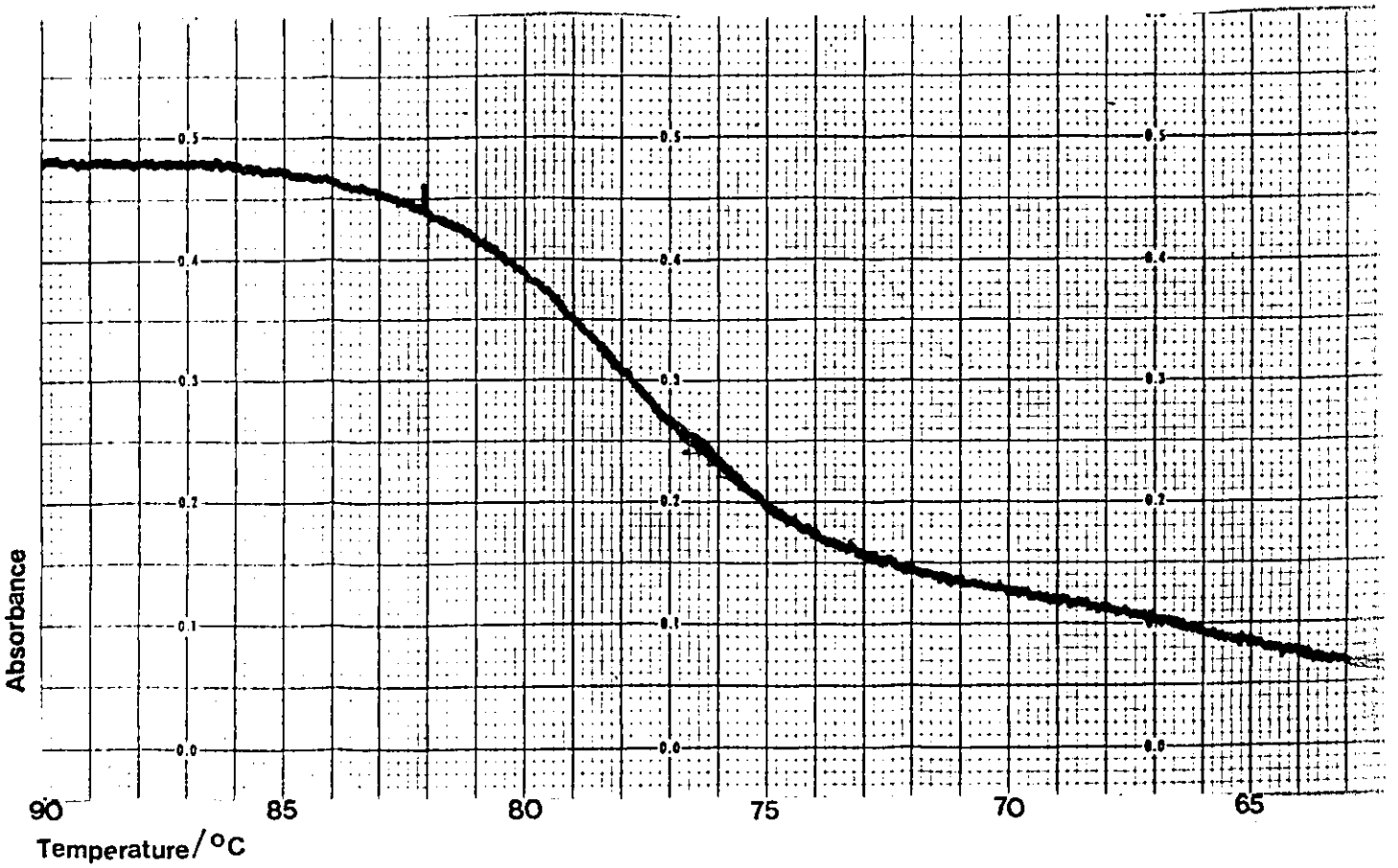


Fig. 46 Spectrophotometric recording of the thermal denaturation of Ggt DNA. 1.8ml of Ggt DNA (A_{260} = 0.8, prepared from isolate 3b1a con E/A1) in 2.5 M-Na_2HPO_4, 5mM-NaH_2PO_4, 1mM-EDTA, pH6.8 buffer as monitored in a Varian Cary uv/vis Spectrophotomater. The temperature was increased in 5°C increments and the absorbance change with temperature increase was recorded.

A pure sample of DNA should have a hyperchromicity of about 40% the value obtained shows the DNA to be pure without significant protein/ultraviolet absorbing contamination. The Tm has been used to calculate the guanine plus cytosine content of DNA by use of the equation:

$$(GC) = (Tm - 69.3)2.44 = (92.4 - 69.3)2.44 = 56.3\%$$

where (GC) is the mole percentage of quanine plus cytosine. (Marmur and Doty, 1962)

The melting curve however was not a simple sigmoidal curve but had two components; this would indicate that the DNA preparation did not contain a distribution of molecules (or lengths of DNA) whose GC content distributes about the mean value with a normal distribution, but contained two populations of DNA with different mean GC contents. This was clearer when the rise in absorbance per degree rise in temperature was plotted against temperature. There were two points of inflection (maxima) on the curve and these corresponded to two separate Tm values:

(in SSC) were $84.5^{o}C$ ($64.5^{o} + 20^{o}$) and $95.5^{o}C$ ($75.5^{o} + 20^{o}$)

which corresponded to GC contents of 37% and 64% respectively. This analysis assumed non-cooperative melting between the two populations.

3.4.2.3 Analysis of Ggt DNA by 'Southern Blotting'

Samples of Ggt DNA (from 3b1a con E/A1) were digested with various restriction enzymes, electrophoresed in agarose gels, transferred to nitrocellulose and hybridized with 1μg of Ggt total RNA (containing mostly ribosomal RNA - Section 3.4.1) in order to reveal the Ggt ribosomal DNA bands.

(i) Digestion with Eco R1

Samples of Ggt DNA (6.3 g) were digested with increasing amounts of the restriction endonuclease Eco R1. The digests were electrophoresed (Fig. 47) transferred and hybridized with Ggt RNA. A (^{32}p) labelled Hind III digest of phage λ DNA was also electrophoresed as size markers (sizes of 23.7, 9.8, 6.6, 4.5, 2.2 and 0.49 kilo-bases-kb). To ensure that Ggt was completely digested by Eco R1 enzyme, 1μg of λ DNA was added to the digest containing the largest amount of enzyme (60 units) prior to the digest. An autoradiograph of the hybridized filter is shown in Fig. 47. The ethidium stained gel (Fig. 48) showed that DNA was digested by Eco R1 and discrete bands of repetitive DNA were present. Additionally, the digest was shown to be complete since the DNA added to the digest was completely digested producing six fragments of mol. wt. 21.63, 7.49, 5.82, 5.62, 4.83 and 3.47 kilobase pairs (kb) (Philipsen et al, 1976). These can be compared with the fragments

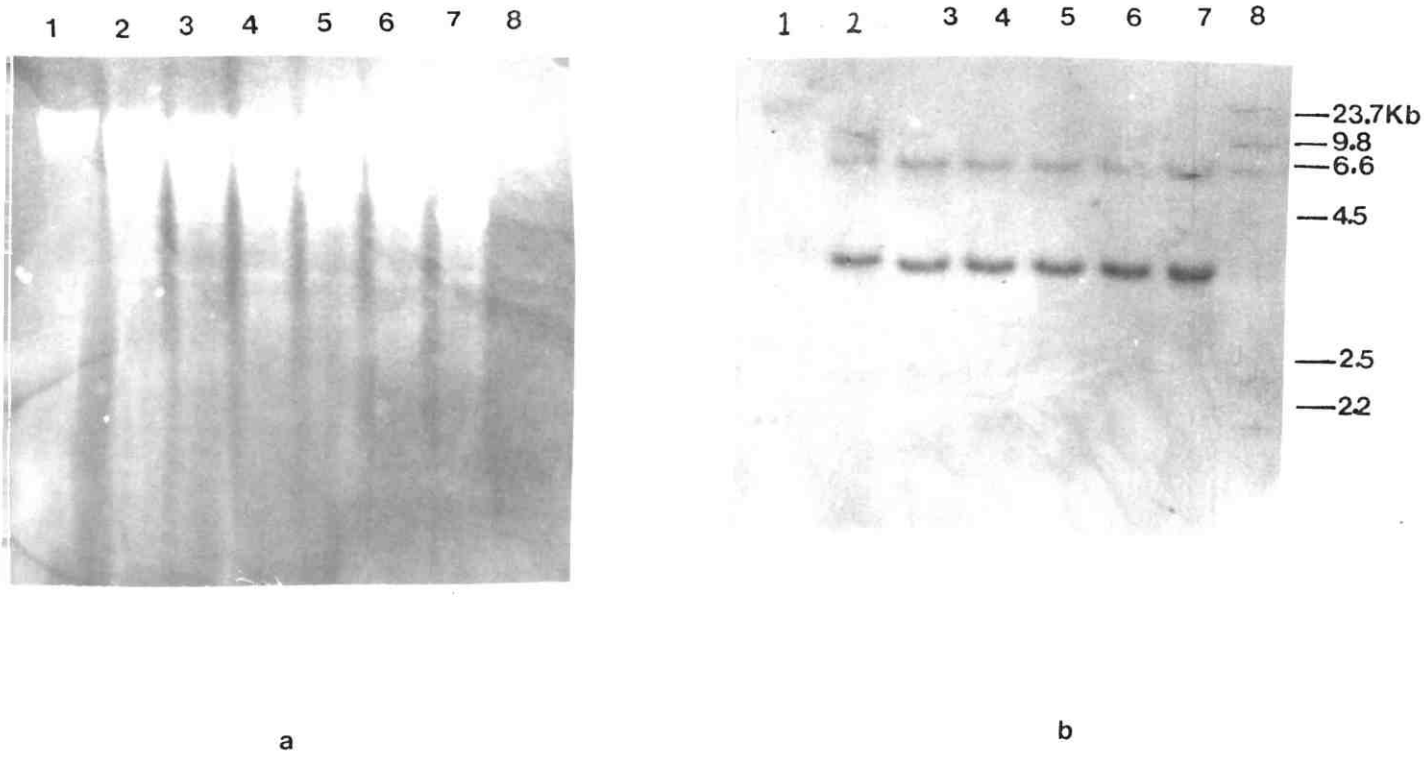


Fig. 48 EcoR1 digest of Ggt DNA. INcreasing amounts of enzyme were used to digest 6.3μg of Ggt DNA (prepared from 3b1a con E/A1). Undigested DNA was electrophoresed in track (i) (2μg), (ii) 2 units of EcoR1, (iii) 10 units of EcoR1, (iv) 20 units of EcoR1 (v) 30 units of EcoR1, (vi) 40 units of EcoR1, (vii) 50 units of EcoR1 containing 1μg of phage λ DNA to check extent of digestion, all in 30μl. A Hind III digest of phage DNA, that had been labelled with (^{32}p) using polynucleotide kinase was electrophoresed in track (viii) as mol. wt. marker. The mol. wt. s of these fragments (in kb) is indicated on the autoradiograph. The mol. wt. of the EcoR1 digest of phage λ DNA containing fragments of mol. wt. 21.63, 7.49, 5.82, 5.62, 4.83 and 3.47 Kb (Phillipsen et al, 1978). The gel was then transferred to nitrocellulose and hybridized with Ggt total RNA, kinase labelled with (^{32}p) to a specific activity of 0.9 x 10^6 ct/min/μg, and autoradiographed for 48 hours (b).

produced by the Hind III digest of DNA in the next track, of mol. wt. 23.7, 9.8, 6.6, 4.5, 2.2 and 0.49 kb. Two bands of repetitive DNA were clearly seen in the digests of Ggt DNA, the mol. wt.'s of these bands were determined as 6.8 kb and 2.4 kb. Undigested Ggt DNA had a mol. wt. of approximately 25 kb.

After hybridization with Ggt RNA, two bands were clearly seen in each Ggt track of the autoradiograph of the filter, (Fig. 47b) The mol. wt. of these bands were determined (using the (^{32}p) labelled Hind III digest of DNA as standards) as 6.8 kb (and therefore almost certainly identical with the repetitive DNA band of same mol. wt. seen in the ethidium stained gel) and 3.6 kb. In addition, an extra band was seen in the digest with only 2 units of enzyme, of mol. wt. 10.4 kb; this was probably a partial digest fragment of mol. wt. equal to the sum of the 6.8 and 3.6 kb fragments.

(ii) Digestions with Eco R1, Hind III and Bam H1

Samples of Ggt DNA were digested with Hind III, Eco R1 and Bam H1. Phage λ DNA was added to aliquots of digests and the completeness of digestion wa checked. The digests were electrophoresed alongside (^{32}p)-labelled (with polynucleotide kinase), phage DNA the gels ethidium stained, transferred and hybridized with Ggt RNA as before. The autoradiograph revealed identical bands, (Fig. 48)

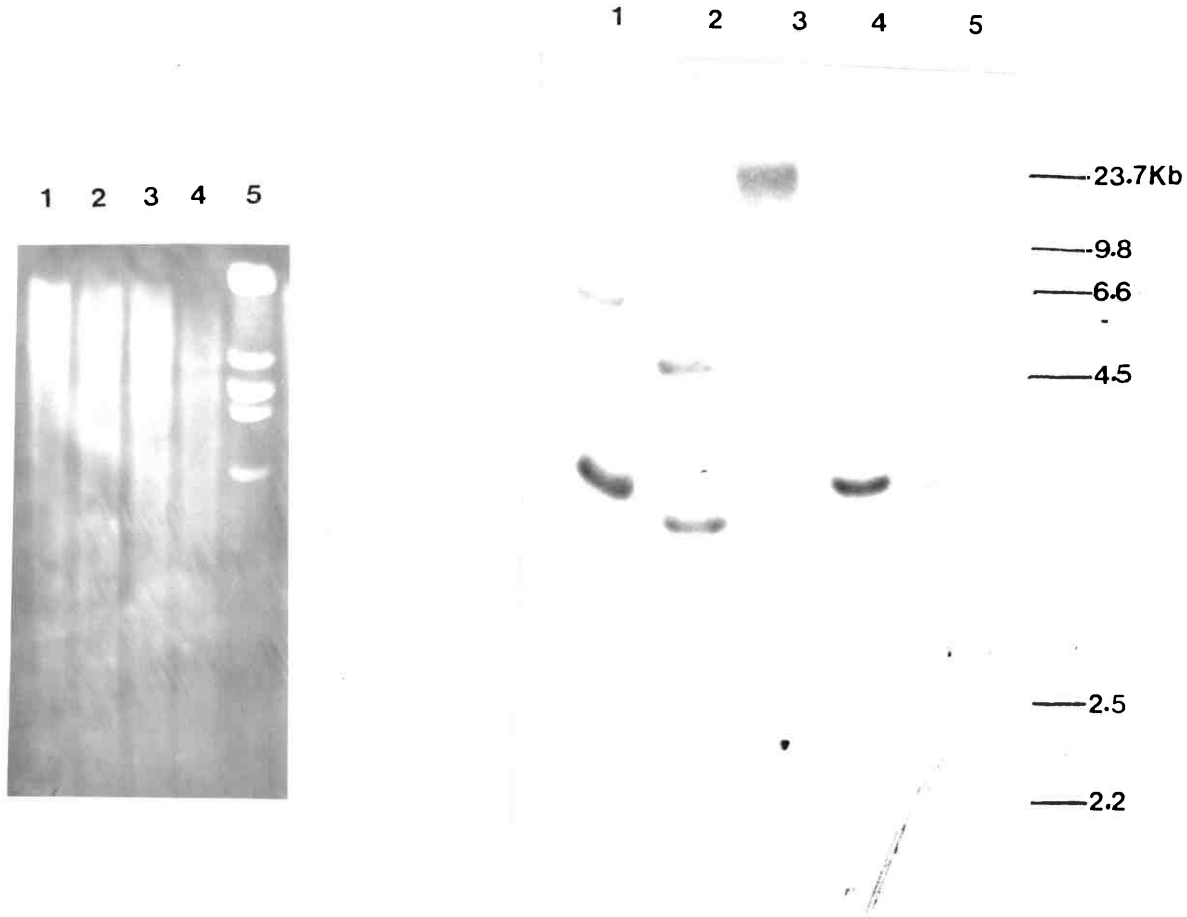


Fig. 48 EcoR1, Hind III and Bam H1 digest of Ggt DNA. Ggt DNA was digested with (i) 30 units of EcoR1, (ii) 30 units of Hind III, (iii) 30 units of Bam H1, (vi) 30 units of EcoR1 plus 30 units of Bam H1, (v) 30 units of EcoR also containing 1μg of phage λ DNA, electrophoresed, transferred and probed with Ggt RNA exactly as Fig. 48. The position of mol. wt. markers is shown. The ethidium stained gel is shown in (a) and the autoradiograph (48 hours) of the filter is shown in (b).

as above in the Eco R1 digest. Hind III digest of Ggt DNA gave three bands of mol. wt. 4.7, 3.5 and 2.7 kb. The Bam H1 digest did not produce discrete bands but hybrisization was seen to have taken place with material in the region of the gel of mol. wt. equal to that of undigested DNA (25kb), suggesting that the ribosomal DNA gene(s) did not contain a site for the enzyme Bam H1. This was confirmed by the result of the double digest of Ggt DNA with Bam H1 and Eco R1, when bands identical to those obtained using Eco R1 alone were seen.

These results show that the size of the ribosomal DNA repeat unit in Ggt DNA is approximately 10.6 kb (the size of the partial Eco R1 band, also the sum of the complete Eco R1 digest bands were 10.9 kb, the mean of these determinations was 10.6 kb), if the repeat unit consists of direct repeats; or 5.3 kb repeat units if they are inverted. In Neurospora crassa (Cox, 1980) the DNA coding for all three ribosomal RNA subunits (28S, 18S and 5S) is contained within a direct repeat unit of length 8.6kb. This suggests the repeat unit in Ggt DNA is 10.6 kb.

3.4.2.4 Isopycnic Caesium Chloride Density Gradient Centrifugation of Ggt DNA

Quantities of Ggt DNA from isolate 3b1a con E/A1 (0.1 - 1.5mg per centrifuge tube) were separated by isopycnic centrifugation in



Fig. 49 Isopynic caesium chloride gradient of Ggt DNA centrifuged to equilibrium. 50μg of Ggt DNA (from 3b1a con E/A1) was centrifuged to equilibrium in a isopynic caesium chloride gradient of initial density of 1.619g/ml containing 1 mg/ml of ethidium bromide, at 45000 revolutions min. for 24 hours in a Beckman vertical VTi rotor. The centrifuge tube was removed and photographed under u.v. (long wave) irradiation.

caesium chloride gradients, containing ethidium bromide. In each case, two DNA bands were seen although a band containing RNA was sometimes seen at the bottom of the tube (Fig. 49). The separated bands were removed and analysed as follows:

(i) Restriction Enzyme Analysis

Unfortunately the DNA obtained by this method was highly sheared and nicked (as shown by electrophoresis in alkaline agarose gels). Digests gave featureless smears on electrophoresis on agarose gels, and on probing transferred blots of digests with Ggt RNA a featureless smear of hybridization was seen with both samples.

(ii) Electron Microscopy

Samples of each DNA fraction were spread by the Kleinsmidt cytrochrome c technique, mounted, shadowed with platinum and examined by electron microscopy. DNA in both samples was linear with a wide variation in fragment length. There was no visible difference between the two DNA fractions.

(iii) Thermalanalysis

Thermal denaturation analysis of both DNA fractions were carried out spectrophotometrically. Each DNA gave a single melting

component. DNA from the upper caesium chloride gradient band designated DNA 1) had a Tm of 98.5°C; DNA from the lower band (designated DNA 2) had a Tm of 86.5°C (in SSC). These Tms corresponded to (G + C) contents of 41.9 and 71.2% respectively. Both DNAs showed the expected hyperchromicity of 40% DNA.

(iv) Yield

The yield of each DNA was determined spectrophotometrically. It was found that 1 mg of total DNA fractionated into approximately 250μg of DNA 1 and 750μg of DNA 2. The proportions of DNA however varied in different DNA preparations.

Lastly a preparation of DNA obtained from purified nuclei (see Section 2.40) was applied to a caesium chloride gradient. Only one band was seen, the quantity of DNA recovered did not allow a further examination of this band.

3.4.3. DNA-DNA Hybridization in Solution

Double-stranded DNA can be denatured by heating, when the strands separate to become single-stranded, this is commonly known as 'melting' the DNA. The reassociation of homologous single-stranded DNA is a spontaneous reaction and the rate-limiting step in the reaction is the collision of complementary DNA strands capable of forming a stable hybrid. The reaction follows

approximately second order kinetics with the reaction rate proportional to the product of the concentration of complementary DNA. Thus, for reactant A:

$$- d(A)/dt = K(A)^2$$

where (A) is the concentration of reactants and K is the rate constant t is time. If a is the initial concentration of A and x is the concentration of product then:

$$dx/dt = K\ (a - x)^2$$

and, on integration:

$$1 \ / \ (a - x) = Kt + c$$

At $t = o$, $x = o$ and $c = a$

therefore:

$$x \ / \ (a(a - x)) = Kt$$

Applying this to reassociation of complementary DNA then $a = Co$, the initial DNA concentration.

In moles nucleotide L^{-1}; (a - x) = C the concentration of complementary DNA in single-stranded regions. Then:

$(C_o - C)/Co\ C = Kt$, and

$c/Co = 1/(KCot + 1)$ ..... eqn. 1

C/Co = the fraction of DNA remaining single-stranded and is the parameter usually measured in renaturation experiments. It can be shown that simple DNA renaturation, as measured by separation of single and double-stranded DNA by hydroxyapatite chromatography follows this equation, (Britten et al, 1974). However, if the reaction is followed using single-strand specific S1 nuclease, then the reaction rate does not follow ideal second order kinetics but is described by the equation:

$C/Co = (1/\ 1+ KCot))^{0.45}$ .....eqn. 2

(J. Morrow, Ph.D. Thesis, Stanford University, California; referred to by Hastie et al, 1978).

From these equations the value of the rate constant K can be determined.

For sheared DNA without repitition, the concentration of DNA (mol nucleotide per litre) present in a set of like pieces that can reassociate with each other is:

$$Ci = P\ Co/G_i$$

where P is the average length of sheared pieces of DNA and G is the genome length (defined as the haploid content per cell). If there is present a nucleotide sequence (longer than P) repeated Ni times, the effective concentration for reassociation of this family would be:

$$Ci = Ni\ P\ Co/G$$

and if these repeated sequences reassociate independently of other sequences then:

$$Ci\ d\ Si/dt = -ki\ \ (Ci\ Si)^2$$

where Si is the fraction of this family remaining single-stranded. Therefore, on integration:

$$Si = 1/(1 + Ni\ Ki\ P\ Co\ t/G)$$

and since the piece size remains constant it can be incorporated into the rate constant

$$Si = 1/(1 + Ni\ K\ Co\ t/Gi) \ldots\ldots\ \text{eqn. 3}$$

It can be seen that eqn. 3 has the same form as eqn. 1 such that if the genome size and repitition frequency are incorporated into an apparent rate constant Ki, then:

$$Ki = Ni\ K\ /\ Gi \qquad \text{..... eqn. 4}$$

and $$Si = 1/(Ki\ Co\ t + 1) \qquad \text{..... eqn. 5}$$

or, if measured using S1 nuclease:

$$Si = 1/(Ki\ Co\ t + 1)^{0.45} \qquad \text{..... eqn. 6}$$

By measurement of reassociation rate the apparent rate constant can be calculated. If a reference DNA of known genome size and complexity is included in the experiment and its rate of reassociation measured separately, then these values can be inserted into eqn. 4 such that the value of the rate constant K for the reaction can be determined. If the reassociation of the unknown DNA sample is measured and the apparent rate constant Ki determined, then the value of Ni/Gi for the unknown DNA may be calculated. Knowledge of either of these parameters allows determination of the other (eg if it is known that the DNA sample is single copy then the genome size may be calculated).

Most reassociation experiments employ Escherichia coli DNA as the reference DNA. Since this DNA is known to be single-copy, and if the unknown DNA is single-copy also, then:

$$Gi/Gcoli = K_{coli} / Ki \qquad \text{..... eqn. 7}$$

The criterion of a nucleic acid hybridization reaction is defined as the difference between the temperature of incubation and the melting temperature (Tm) of the DNA under study, in the solvent in which the reassociation is taking place. The criterion is important as it will determine the 'stringency' of the hybridization conditions; that is, the level of sequence mismatching that will be tolerated under these conditions. The thermal stability of a double-stranded DNA decreases in proportion to the amount of mismatching; therefore at high temperatures only perfect or near perfect complementary sequences will be stable and will form in the reaction. If the hybridization reaction is performed at lower temperatures imperfect matching of DNA sequences can occur.

The reaction rate for DNA reassociation is directly proportional to temperature, up to about 20°C below the Tm of the DNA; above this the reaction rate falls. It is usual therefore to perform reassociation incubations at a temperature 25°C below the Tm of the DNA in the incubation solvent. This maximises the reaction rate and maintains 'stringent' incubation conditions.

The fragment length is also critical for DNA reassociation. Large fragments may contain DNA sequences of differing repitition frequency's if this occurs the rate equations are no longer valid.

It is customary to use fragments of about 450 nucleotides long. Because of possible ss breaks in dsDNA, for these purposes DNA is always sized in completely denaturing systems such as electrophoresis alkaline agarose gels.

3.4.3.1 Ggt DNA - DNA Hybridizations in Solution

Total Ggt DNA prepared as described in Section 2.40 from 3b1a con E/A1 was shown to be pure by all the criteria mentioned. It was decided to use DNA that had been fractionated by centrifugation in caesium chloride isoprynic gradients into the upper DNA band, designated DNA 1 and lower DNA band, designated DNA 2. As previously shown, these bands represent the two components of the melting curve of total DNA and are likely to represent two separate populations of DNA sequences. A combined hybridization curve might be complicated and difficult to analyse and so it was felt that analysing both fractions separately would give a clearer result.

E. coli DNA was included in the reassociation reaction in order to determine the rate constant. Commercial E. coli DNA (Sigma type VII) was dissolved, phenol extracted twice to ensure complete deproteinization, dialysed extensively and its purity was checked as described for Ggt DNA.

Aliquots of each DNA (0.5μg) were labelled by nick translation. DNA 1 was labelled to a specific activity of 1.06 x 10^6 ct/min/ g with (^{32}p); DNA 2 was labelled to a specific activity of 0.9 x 10^6 ct/min/μg with (^{32}p). E. coli DNA was labelled to a specific activity of 1.23 x 10^6 ct/min/μg with tritium. The labelled products were sized by electrophoresis alkaline agarose gels, and shown to be each about 1000 nucleotides long (single-strand length). They were therefore sonicated to a length of 500 nucleotides. Unlabelled DNA was sonicated to the same single-strand length, as measured on alkali agarose gels.

Tritiated E. coli DNA was used to determine the concentration of S1 nuclease, needed in the assay for determination of hybrid formed, to give maximum differentiation, between double and single-stranded DNA. Tritiated E. coli DNA plus unlabelled DNA, at the concentration of total DNA to be used in the reassociation mix, was digested with S1 nuclease before and after denaturation with varying amounts of enzyme between 0.015 units and 15 units. The amount of enzyme that gave maximal differentiation (native 94.5% resistant, denatured 97.6% digested) was 1.5 units so this amount of enzyme was used in all subsequent S1 assays. This experiment also showed that the nick-translation reaction did not produce snap-back sequences in the E. coli DNA. The Ggt DNA preparations were similarly assayed with 1.5 units of enzyme. Native DNA 1 was 93.4% resistant to S1 digestion, after denaturation it became 95.7% sensitive. Native DNA 2 was 94.1% resistant, denatured DNA 2 was 95.9% sensitive.

In order that renaturation of E. coli DNA would occur at about the same time as Ggt DNA in the reaction, (where most determinations would be required) it was necessary that E. coli DNA be at about one fifth of the concentration of Ggt DNA. This is because E. coli has a genome size of 4.5×10^6 base-pairs and if Ggt has a genome size similar to that of Aspergillus nidulans (Timberlake, 1978) it will be about five times the size of the E. coli genome and therefore will have to be at five times the concentration of E. coli DNA in order that unique sequences will be at the same concentration.

Two reassociation experiments were therefore performed in parallel. Reaction tube A contained DNA 1 with 0.01μg of nick translated DNA 1 (106,000 ct/min) at a total concentration of 110μg/ml, and E. coli DNA with 0.1μg of nick translated E. coli DNA (123,000 ct/min. at a total concentration of 24μg/ml. Reaction tube B contained DNA 2 with 0.01μg of nick tran lated DNA 2 (90,100 ct/min) at a total concentration of 110μg/ml and E. coli DNA at 24μg/ml (123,000 ct/min). Tube A was incubated for reassociation at 73°C (25°C below the Tm for DNA 1) tube B was incubated at 61°C (25° below the Tm for DNA 2).

The hybridization reactions were incubated for 2 weeks and samples were taken for S1 nuclease digestion. After S1 nuclease digestion the samples were frozen until the end of the experiment

when they were TCA-precipitated and filter assayed. In addition samples were taken for determination of total TCA precipitable radioactivity so that the amount of any thermal degradation during the incubation could be measured. This was found to be negligible.

The results are presented in Fig. 50 and 51 and Table 9.

From equation 6

$$Si = 1 / (K_i' \quad Co\ t + 1)^{0.45}$$

where Si is the fraction of DNA remaining single-stranded (as measured by digestion with S1 nuclease), K_i' is the apparent reassociation rate constant, Co is the DNA concentration (mols. nucleotide per litre) and t is time (seconds). The value of Co is determined from knowledge of the extinction coefficient of DNA (7200 moles l^{-1} cm^{-1} in 0.15M Na^+).

The concentration of E. coli DNA in the reaction mixtures was therefore 0.3333×10^{-3} moles nucleotide l^{-1}.

The concentration of Ggt (DNA 1 and 2) in the reaction mixtures was therefore 1.5277×10^{-3} moles nucleotide l^{-1}.

The value of Si is determined from measurement of the fraction of DNA that is sensitive to digestion with S1 nuclease, by measurement of acid-precipitable radioactivity before and after digestion with S1 nuclease. Since E. coli DNA is radiolabelled with tritium and Ggt DNA is labelled with (^{32}p), then the reassociation of both DNA species can be monitered.

The results are plotted in Figs. 50 and 51.

Equation 6 on rearrangement gives:

$$\left(\frac{1}{Si}\right)^{\frac{1}{0.45}} = K_i' \; Co\, t + 1 \quad \ldots\ldots \text{eqn. 8}$$

thus, if $(1/Si)^{2.222}$ is plotted against Co t then a straight line should be obtained with intercept of 1 and slope equal to Ki. The results, so transformed are plotted in Fig. 51. In each case it can be seen that a straight line was obtained. The slope of the lines was determined by insertion of the values in the least mean squares regression equation

$$b = \frac{N \quad \Sigma x \quad y - (\Sigma x)\,(\Sigma y)}{N\,\Sigma x^2 - (\Sigma x)^2}$$

where b is the slope of line described by the equation

$$Y = a + b\,X$$

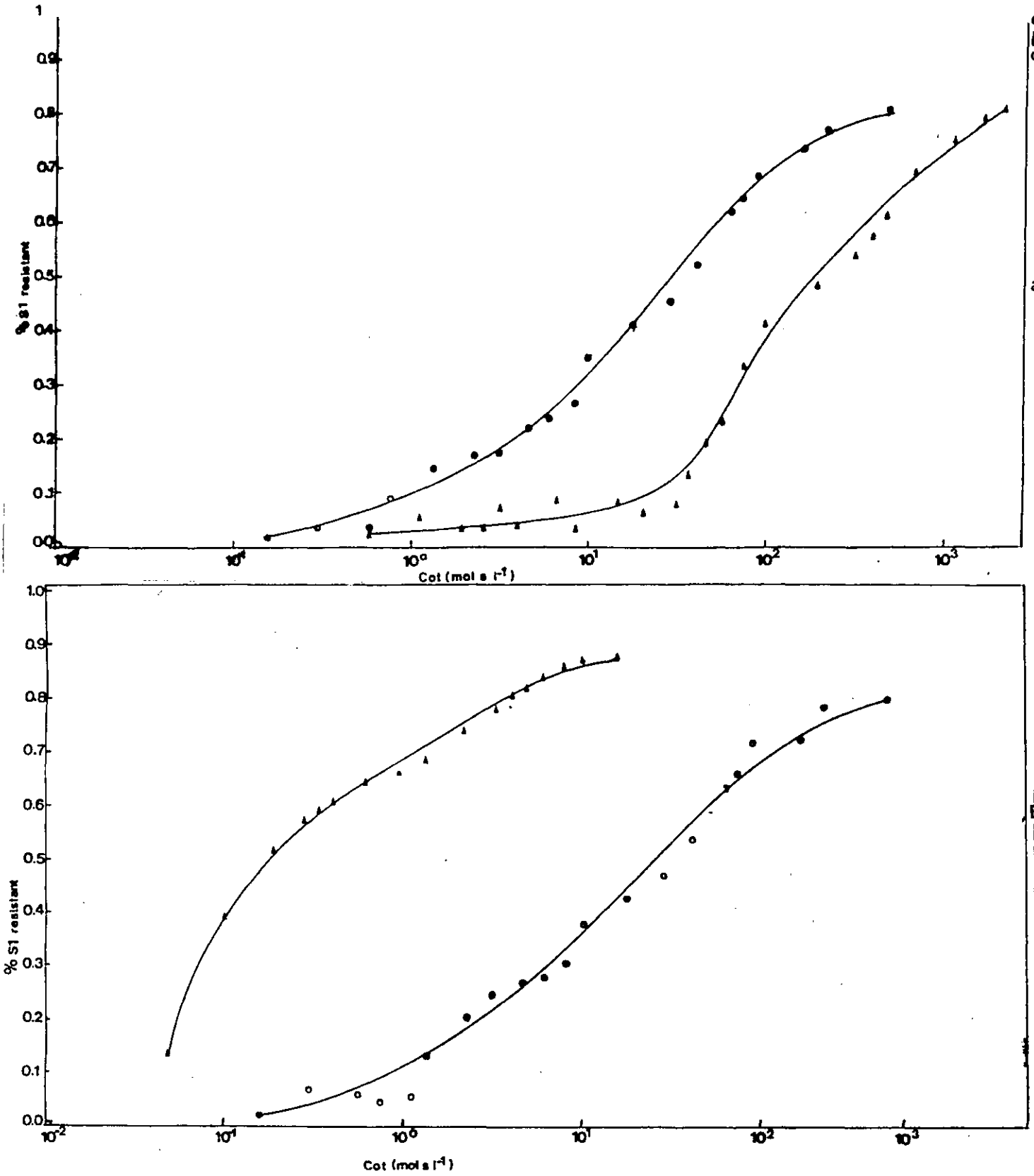
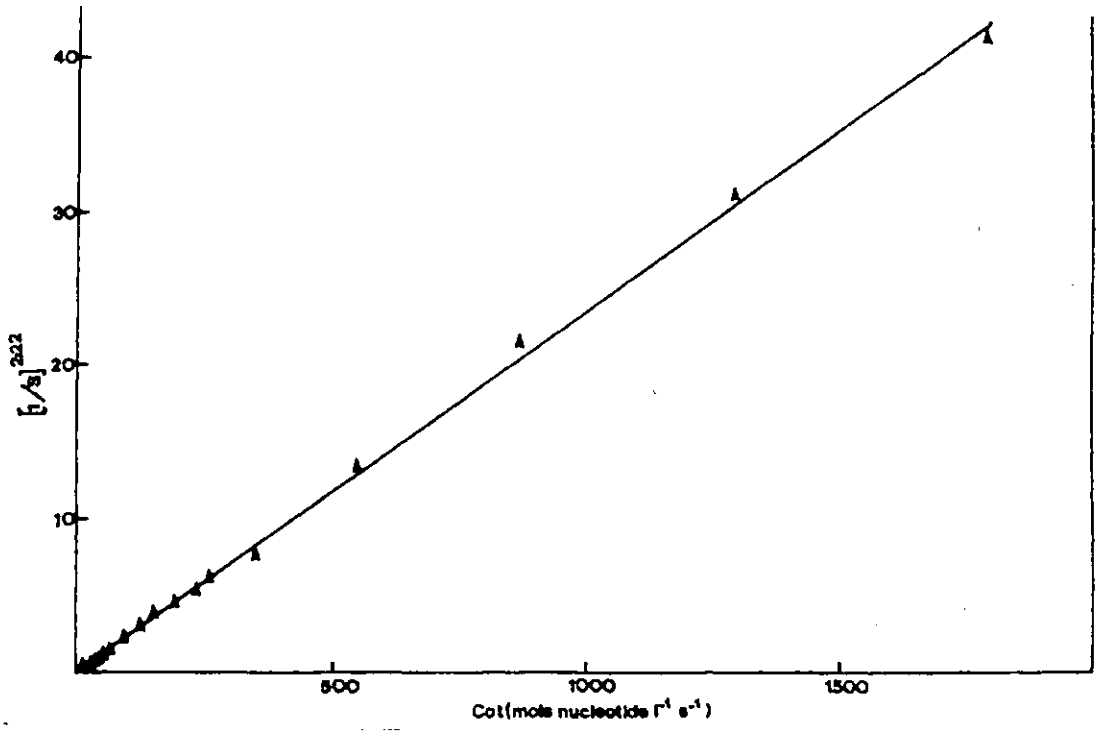


Fig. 50 Renaturation of (a) Ggt DNA1, (b) Ggt DNA 2 in the presence of E. coli DNA. Ggt DNA (▲) at 110μg/ml and specific activity of 192 ct/min/μg of (^{32}P) was denatured, and renatured at (a) 73°C and (b) 61°C in 0.15M-NaCl. 2mM-EDTA 10mM tris-HCl buffer in the presence of 24μg/ml of E. coli DNA (●) of specific activity 1025 ct/min/μg of (^{3}H) all in 5ml. The amount of each DNA in double-stranded form was determined by digestion of reaction samples with S1 nuclease



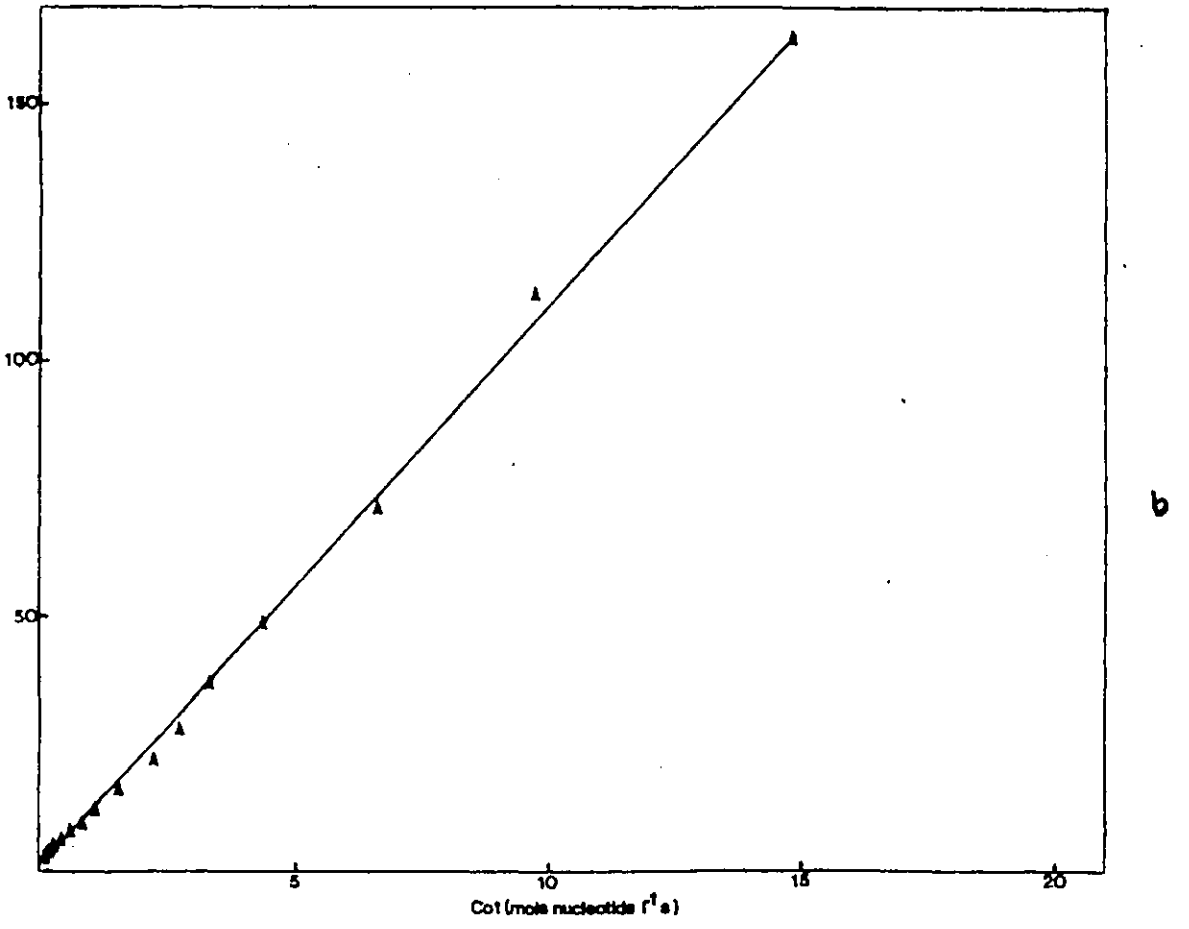


Fig. 51 Reciprical Cot plots of the renaturation of Ggt and E.Coli DNA. The reciprical of the values, obtained in the experiments described in Fig. 50, for the fraction of DNA remaining single stranded (transformed as shown) were plotted against the Cot values for (a) DNA1 (b) DNA2, (c) (over page) E. Coli DNA from both renaturation experiments: with DNA 1(O), with DNA 2 (▲). The lines were determined by regression analysis. For comparison of rates, in (c) is also shown the reciprical Cot lines for the renaturation of DNA 1 (— — —) and DNA 2 (—— ——— ———)

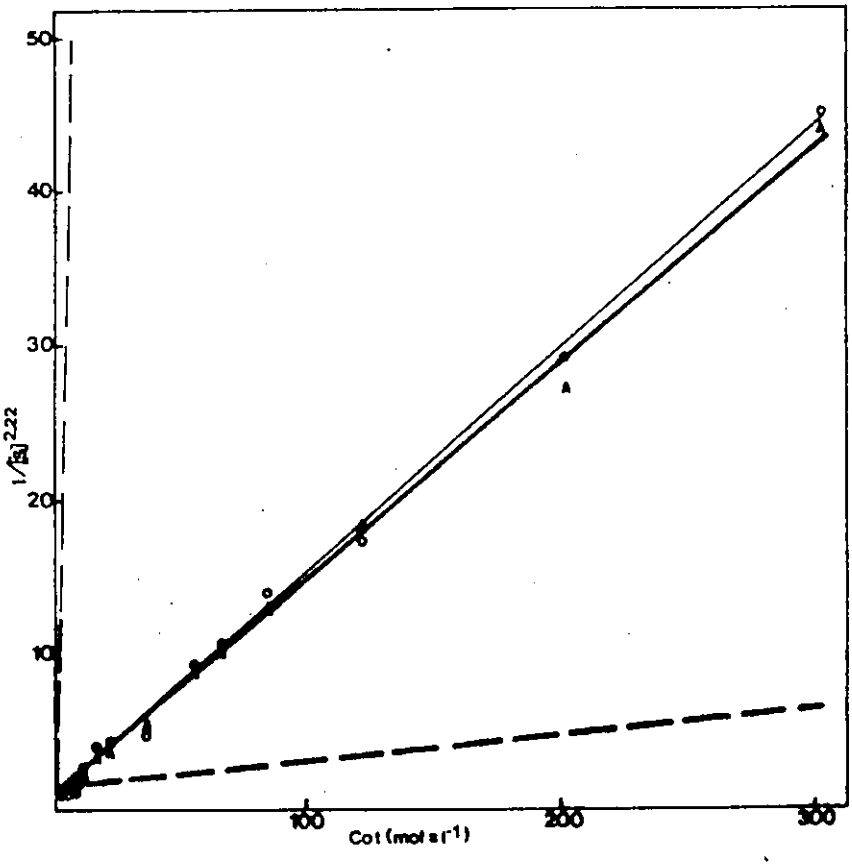


Fig. 51c

TABLE 9 Reassociation Rates of Ggt and E.coli DNA

Sample	Reassociation Rate Ki ($M^{-1}sec^{-1}$)	Root Mean Square Standard Error in Determination of Reassociation Rate	Genome Size[a] (base pairs)
E.coli DNA	0.139[b], 0.141[c]	0.002	4.6×10^6
Ggt DNA1[d]	0.0219	0.003	29.1×10^6
Ggt DNA2[e]	10.908	0.174	59.5×10^3

a Calculated assuming single copy DNA
b Value from determination with Ggt DNA1
c Value from determination with Ggt DNA2
d and e Ggt DNA fractions separated by centrifugation in isopynic caesium chloride gradients (Section 3.4.2.4)

The value of the intercept a, is of course 1.

The value of the slope of each plot gives the value of the renaturation rate constant for each DNA sample (Ki). These values are presented in Table 9, with the root mean square error for each Ki determination.

The determinations of the rate constants for E. coli DNA in both experiments was in good agreement (0.139 and 0.141), the difference may be due to the difference in temperature of incubation in the two renaturation reactions.

If it assumed that both Ggt samples contain only single-copy sequences then the genome length for both DNAs may be calculated from equation 7, taking the genome length for E. coli to be 4.6×10^6 base-pairs (Zimmerman & Goldberg, 1977). The genome lengths so calculated are shown in Table 10.

The genome length of Ggt DNA 1 (29.1×10^6 base-pairs is consistant with a total genome length of a fungus and is close to the genome length determined for Aspergillus nidulans, 26×10^6 base-pairs (Timberlake, 1978) another ascomycete. The value is also close to the genome size determined for Dictyostelium discoideum (32.2×10^6 base-pairs, Firtel et al, 1975) and Neurospora crassa (27×10^6 base-pairs Kaumlauf and Marzluf, 1980).

The genome length of DNA 2 was determined as 59.5×10^3 base-pairs, assuming it to be single copy DNA. This value is consistant with DNA 2 being mitochondrial DNA. (Yeast mitochondrial DNA = 78,000 base-pairs: Hollenburg et al, 1978); Neurospora crassa mitochondrial DNA = 98,000 base pairs: Wood & Tuck, 1969)

The hypothesis that DNA 2 is composed of mitochondrial DNA is supported by the finding (Section 3.4.2.4) that DNA purified from isolated nuclei of Ggt gave only one band when centrifuged on isopynic caesium chloride gradients. It is known that mitochondrial DNA from many organisms, including several fungi(D. discoideum, Firtel et al, 1975) migrates as a satellite band on buoyant density gradients.

There is no evidence of repetative DNA in Ggt from this hybridization data. This would suggest that repetitive DNA such as ribosomal DNA constitutes a small proportion (probably less than 10% of the total genome. This is consistent with what is known of the DNA of other fungi (Timberlake, 1979).

If it is accepted that DNA component 2 is mitochondrial DNA then the total genome size of Ggt can be calculated as (nuclear and mitochondrial) 29.2×10^6 base-pairs.

3.4.4. Use of dsRNA Transfer to Estimate Sensitivity of Technique for Detecting Provirus

As explained the intention of developing a technique for transfer and hybridization of dsRNA into nitrocellulose, was so that the technique could be used to estimate the number of copies of viral dsRNA detectable by hybridization and hence, the number of DNA copies of viral dsRNA detectable. This would be done by applying known amounts of dsRNA to an agarose gel, along with DNA, and hybridizing these with viral probe. By applying decreasing amounts of dsRNA it would be possible to determine the minimum amount detectable. A number of assumptions would then allow one to estimate the sensitivity for detection of a DNA copy of viral dsRNA. These are: (i) that the transfer of dsRNA from agarose to nitrocellulose is 100% efficient; (ii) that dsRNA is not eluted from the filter during the hybridization incubation; (iii) that all of the dsRNA transferred is available for hybridization; (iv) that the sensitivity for detection of DNA is the same as that for detection of dsRNA.

The first and second assumptions were tested by electrophoresing three (equal) samples of (^{32}p)-labelled dsRNA in an agarose (formaldehyde gel, and transferring two agarose gel tracks (containing these samples) to nitrocellulose. The nitrocellulose filter was then autoradiographed alongside the untransferred agarose gel track. The result (not shown) showed the autoradiograph of the agarose gel to be slightly less

intense than that of the bands transferred to nitrocellulose. This is presumably due to the greater thickness of agarose. The second assumption was tested by cutting out a strip of the nitrocellulose containing one of the transferred dsRNA bands, and incubating the other in a hybridization mixture (50% formamide, 42°C) for 24 hours. After autoradiography of both nitrocellulose strips it was clear that most dsRNA remained on the filter in the hybridization mix, but an estimated 10-20% was lost. For this reason, no more than 24 hour incubations were used for detection of dsRNA on filters.

The third assumption would be difficult to prove; however, if it were not true then the sensitivity of the experiment would have been underestimated, so its proof was not important. The fourth assumption would again be difficult to prove. However, when ssRNA was used for detection of provirus on normal 'Southern' blots, a 48 hour hybridization incubation was used in order that any annealing reaction taking place would go to completion. It is possible that in the 24 hour incubation to detect dsRNA on nitrocellulose filters, the reaction did not go to completion. In this case however, lower amounts of dsRNA than those applied to the gels would have been detected, so again the sensitivity of the technique would have been underestimated. It therefore concluded that such an experiment as that described here, would be valid.

The experiment consisted of adding discrete amounts of 3b1a virus mixture dsRNA to a series of samples of 6μg of Ggt DNA (from 3b1a con E/A1) that had been digested with the enzyme Eco R1. The mixtures were then denatured (formamide/formaldehyde) electrophoresed, transferred and hybridized with 1μg of the same (^{32}p)-labelled 3b1a ssRNA probe as was used for detection of DNA provirus (next section) of specific activity of 4.7×10^7 ct/min/ g, for 24 hours. The amount of dsRNA detectable was 5×10^{-5} μg dsRNA. It is probable that if only a single dsRNA component was present, then the sensitivity of the technique for detecting a single component would be one eighth of that for detection of the mixture of 3b1a dsRNA components.

3.4.5. Detection of Proviral DNA in the Genome of Ggt

This experiment, central to this work, has been outlined in the Introduction. Digests of Ggt DNA were electrophoresed, transferred to nitrocellulose and probed with 3b1a viral ssRNA probe.

Samples of Ggt DNA 6 g) extracted as described in Methods, from the following 3b1a isolates: 3b1a con E/A1, 3b1a con E/A2, 3b1a con D/A2 and the 45/10 ascospore isolate, 45/10 con B/A1, were digested with the following enzymes: Eco R1 and Hind III, and the fragments electrophoresed alongside undigested DNA, transferred and

hybridized with 1μg of 3b1a ssRNA end-labelled (with polynucleotide kinase) with (^{32}p) to a specific activity of 2.7 x 10^7 ct/min/μg. The 3b1a ssRNA was prepared as described in Section 3.2.2.3, from a polymerase reaction mix and was alkaline base-cleaved to achieve maximal labelling. The blot was incubated for 72 hours, washed and autoradiographed (7 day exposure).

Nothing was visible on the autoradiograph, indicating that there was no homology between 3b1a ssRNA and Ggt DNA, i.e. no provirus. In order to be certain that hybridization conditions were not too stringent for formation of hybrids, the experiment was repeated with the hybridization reaction taking place at 30°C (50% formamide). Again nothing was seen on the autoradiograph.

In order to be certain that Ggt DNA on the filter was available for hybridization, the filters were hybridized (24 hours) with Ggt total RNA probe (Sections 3.4.1 and 3.4.2.3). Ribosomal RNA bands identical to those described in Section 3.4.2.3 were seen in each Ggt DNA track on the filter, therefore the Ggt DNA was available for hybridization with homologous probe.

3.4.6. Sensitivity of Experiment to Detect Proviral DNA

The detection of proviral DNA in the genome of Ggt is essentially the detection of a single-copy gene in the total genome. The genome

size of Ggt has been determined as (Section 3.4.3) 29.2 x 10^6 base-pairs. If a single dsRNA component of 3b1a virus is represented as cDNA in the Ggt genome, then, to take the smallest dsRNA component (1.11 x 10^6 molecular weight), this would contain approximately 1.6 x 10^3 base-pairs and would represent approximately 0.006% of the Ggt genome. 6μg of Ggt DNA were applied to the gels and transferred to nitrocellulose for detection of provirus; this would therefore contain approximately 6 x 10^{-4} μg of viral cDNA.

This was then probed with viral message labelled to a specific activity of 2.7 x 10^7 ct/min/ g. If 90% hybridization with the cDNA was obtained (with 72 hour hybridization using ssRNA probe) (this would be a low estimate: P. Rigby, personal communications) then 1600 cpm of (^{32}p) would be bound to a single cDNA band, or 8000 ct/min if the cDNA was cut once by the restriction enzyme. Since 50 cpm of (^{32}p) in a single band is detectable by autoradiography (2 day exposure at -70^{o}C using intensifier screen) then 8000 ct/min would be easily detectable. If only 10% hybridization of cDNA with probe was achieved then the signal (800 ct/min) would still be easily detectable; if only one haploid cell in ten contained the viral cDNA then the signal, if only 10% hybridization, would still be detectable.

The experiment (Section 3.4.5) to directly measure the quantity of viral dsRNA detectable by transfer of dsRNA and DNA to nitrocellulose and hybridization with viral ssRNA probe determined that the lowest amount of viral dsRNA detectable was 5 x 10^{-5} μg.

This is less than one tenth of the quantity (of cDNA) that would be necessary to detect in order that a single-copy proviral cDNA is detected. It can be concluded, therefore, that these experiments demonstrate that a proviral form of 3b1a viruses B and C was not present in the 3b1a genome.

3.5.7. Discussion

Proviral forms of viruses 3b1a B and C were not present in the genome of Ggt isolates 3b1a con E/A1, 3b1a con E/a2, and 3b1a con D/A2. A purpose of this work was to demonstrate the absence of viral information in 'virus-free' ascospore isolates. Since 3b1a con E/A1 and 3b1a con D/A2 contained no virus particles or viral RNA (Section 3.3) and have here been demonstrated to contain no homologous viral DNA, it can be concluded that these isolates contain no virus B or C information. Isolate 3b1a con E/A2 contained viral information in the form of dsRNA but again no proviral DNA was present. Isolate 45/10 con B/A1 contain no detectable virus particles or dsRNA (Section 3.3) and it was shown in this section to contain no provirus homologous to viruses 3b1a - B or C. Some homology between 45/10 viruses and 3b1a viruses was demonstrated in Section 3.3.4.2.

The sensitivity of the experiments to detect proviral DNA was determined (previous section) and was found to be capable of

detecting less than 0.1 viral copies per Ggt genome. Therefore if provirus were present it must have been so in less than one haploid nucleus per ten cells. Since these isolates developed from a single (diploid) nucleus then if provirus were present it would have been contained in one of the conlugating nuclei and therefore present in at least 50% of derived mycelial cells. Provirus could not therefore have been present in the ascospores from which these isolates were derived.

It could be argued that proviral DNA was present in ascospores but was excised during mycelial growth. For this to have any significance to the virus or host then virus particles and/or dsRNA would have had to be produced prior or after excision of the provirus.

Virus was found in two ascospore isolates, one derived from con D and one derived from con E. As described in Methods, each of the con E ascospore isolates and each of the con D ascospore isolates were produced from a single peritheium. Blanch (1977) demonstrated that only two nuclei are involved in the production of all the ascospores in a single perithecium in several Ggt isolates, and therefore it is likely that each of the con D ascospore isolates developed from the same (diploid) nucleus, as did all the ascospore progeny of 3b1a con E. If virus resulted from provirus present in the parent nuclei then it would have been expected to be present in all of the daughter ascospore isolates (or 50% if present in only one of the haploid nuclei, but

since con E and con D mycelia contained only identical nuclei, this is unlikely), but was present in only one of four asco pore isolates in both cases.

As discussed in Section 3.1, Ggt viruses have been classified by Buck et al (1981) using the information on F6 viruses presented in this thesis, together with the properties of other Ggt viruses). Viruses 3b1a-B and C are typical members of groups II and I respectively and are related to other viruses in these groups. It is very unlikely that any of the viruses in these groups would be so radically different from 3b1a viruses as to be capable of producing provirus. The lack of an RNA-directed DNA polymerase activity detectable in 3b1a viruses (Section 3.2.2.7) is consistent with these conclusions.

The situation with virus 3b1a-A is, however, unclear. No RNA polymerase activity capable of producing ssRNA products, was detected in this virus, and since virus F6-A also had no RNA polymerase activity, this may be a general property of group III viruses. It is possible that their RNA polymerase activity is inactivated during purification. Since no ssRNA probe was available it was not possible to probe genomic DNA for sequences homologous to this viral RNA. As discussed in Section 3.3.4.3 dsRNA would not be suitable for detection of homologous DNA, because of removal of the probe into

dsRNA hybrids. However, an attempt was made to detect 3b1a virus-A provirus using kinase-labelled dsRNA, probing the filter described in Section 3.4.5; no hybridization was detected. It cannot, however, be concluded that no proviral form of virus 3b1a-A is present in the Ggt genome.

3.5 Possible Effect of Virus Infection on Inhibition Reactions of Ggt and Phialophora spp Isolates

Romanos et al (1980) discovered that certain isolates of Ggt produced diffusible inhibitors of the growth of other fungi when grown on PDA buffered at low pH. It was thought that this inhibitor may be similar to the 'killer' factors produced by yeasts and Ustilago maydis. The inhibition reaction, was first discovered in an isolate of Ggt, designated 45/8. This isolate (as discussed in Introduction) sectored into an inhibitor-producing sector, designated 45/8Q and a sector that did not produce inhibitor designated 45/8P. Virus particles were found in 45/8, 45/4P, 45/8Q and a single co idial isolate of 45/8 that did not produce inhibitor; however no dsRNA bands were detectable in nucleic acid extracts from these isolates. Subsequently, two ascospore isolates (with no detectable virus particles) of Ggt, designated F911-1 and 3b1a A52 were shown to produce inhibitor, therefore it seemed that the production of inhibitor was not dependent on the presence of virus particles. However, such a conclusion would be subject to the same criticisms as the conclusion that virus has no effect on pathogenicity as discussed in the Introduction. It was therefore decided to make a more definitive study of the effect of virus on the production of inhibitor, and also the sensitivity to inhibitor of the virus-containing, and virus-free isogenic strains produced in this study.

McGinty (1981) discovered that a number of isolates of Ggt, that had not produced inhibitor at low pH on buffered PDA, would produce diffusible inhibitor at low pH on buffered Lilley and Barnet media (Lilley and Barnet, 1963), and buffered Haskin's MB-50 media (Haskin, 1972) It was decided to use these media to test inhibition reactions of 3b1a isolates. It was thought useful to test previously untested Ggt and Phialophora spp isolates for ability to produce inhibitor on these media.

3.5.1. Inhibition by Ggt Isolates on Lilley and Barnet and Haskins MB-50 Media

The 3b1a and F6 isolates generated in this study and other Ggt isolates were tested for their ability to produce diffusible inhibitor at low pH on buffered Lilley and Barnet and Haskins MB-50 media. The isolates tested for production of inhibitor at pH 4.0 and pH 4.5 were Ggt isolates: 1-9, 01-1-4, 64/14, 5-9, 1-9, 5b3, 40/4, 64/4, 45/8, 45/8P, 45/8Q, F6, 3b1a; the isolates generated in this study and tested were: F6 con 1, F6 con 8, F6 con 8 hyphal tip cultures 1 and 2 (F6 HTI and F6 HT2), 3b1a con D, 3b1a con E, 3b1a con D/A1 3b1a con D/A2, 3b1a con E/A1 and 3b1a con E/A2. In addition the following isolates were also tested for production on inhibitor at pH 4.0 and pH 4.5: Phialophora graminicola isolate 1348-2, Phialophora sp. with lobed hyphopodia isolates 55-1 and 17-3. Phialophora sp. with

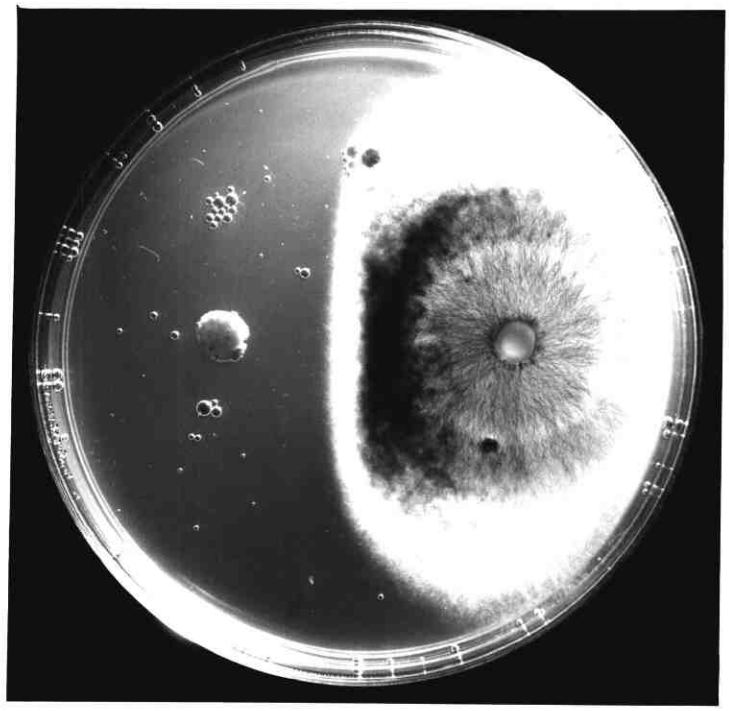


FIG. 52 Inhibition by Ggt Isolate 3b1a of the growth of Phialophora radicicola sp. Isolate 2/2 on Lilley & Barnet media at pH 4.5. Incubated for 7 days. The Ggt Isolate is on the left.

lobed hyphopodia isolate 2-2 was used as the tester sensitive strain. Standard inhibition tests were set up and incubated at 24°C for up to one month. A positive result was recorded when solates 2-2 was inhibited in its growth towards the isolate under test, and when, on examination by low power microscopy, there was no observable mingling of opposing hyphal before the inhibition. The results are summarised in Table 10 and an example of an inhibition reaction is shown in Fig. 52.

As can be seen from Table 10, only one Ggt isolate, 40/4 did not produce inhibitor on any of the media tested. None of the Phialophora isolates produced inhibitor on any of the media. Growth on both media of the inhibiting species, was slow, showing a very dense type of growth on Haskins MB-50 medium and a white aerial growth on Lilley and Barnet medium. The rate of growth of the inhibiting isolate was inversely proportional to the strength of inhibition. Haskins MB-50 medium was most successful in inducing isolates to produce inhibitor.

There was no observable difference in production of inhibitor between ascospore, conidial and parent cultures of a single strain. The hyphal tip culture gave identical results to the conidial and parent F6 strain.

TABLE 10 Inhibition by Ggt Isolates of Growth of Phialophora sp. Isolate 2.2, at low pH on Lilley and Barnet and Haskins' MB-50 Media

	Haskins MB-50		Lilley and Barnet	
Isolate	pH 4.0	pH 4.5	pH 4.0	pH 4.5
3b1a con D	+	+	+	+
3b1a con E	+	+	+	+
3b1a con D/A1	+	+	+	+
3b1a con D/A2	+	+	+	+
3b1a con E/A1	+	+	+	+
3b1a con E/A2	+	+	+	+
F6 con 1	+	+	+	-
F6 con 8	+	+	+	-
F6 con 8 HT1	+	+	+	-
F6 con 8 HT2	+	+	+	-
3b1a	+	+	+	+
F6	+	+	+	-
45/8Q	+	+	+	+
45/8P	+	+	+	+
45/8	+	+	+	+
64/4	+	-	+	-
40/4	-	-	-	-
5b3	+	+	+	+
1-9	+	-	+	+
5-9	+	+	+	+
64/14	+	+	+	+
01-1-4	+	+	+	+
Pg. 1348-2	-	-	-	-
P. sp. 55-1	-	-	-	-
P. sp. 17-3	-	-	-	-

Isolates were tested for ability to inhibit the growth of Phialophora sp. as described in Section 2.10. A positive result (isolate inhibited P. sp.) is indicated by +; - indicates no growth inhibition produced.

3.5.2. Discussion

All of the isolates tested, but one (40/4) produced inhibitor on one of the media tested. Since at least most of these isolates contained different dsRNA components. Then no single virus (or dsRNA component) could be responsible for production of inhibitor. Additionally, the studies on the isolates generated in this work, shows that in the case of 3b1a, virus is not responsible for the production of inhibitor, since isogenic strains with and without virus (or viral information in the form of DNA or dsRNA), all produce inhibitor. This distinguishes this inhibitor produced by Ggt from the diffusable 'killer' type inhibitor activity produced by some strains of yeast (Vodkin, 1979) and Ustilago maydis (Koltin, 1979) which are known to be associated with the presence of dsRNA molecules. This is in agreement with the results of Romanos et al (1980), who found that the properties of this inhibitor were quite unlike the properties of the 'killer' toxins of yeast and U. maydis.

4. GENERAL COMMENTS AND CONCLUSIONS OF THIS WORK

This work described the first purification and characterisation of three Ggt viruses from the same isolate, F6; including the first purification of a Ggt group III virus (Buck et al, 1981). Additionally isolate 3b1a was shown to contain four separable viruses including two related group II Ggt viruses.

The RNA polymerase activities of Ggt viruses of 3b1a and F6 were examined and this study constituted the first of its kind for Ggt group II viruses (Buck et al, 1981). Evidence was obtained to suggest a semi-conservative transcriptase activity in these viruses. The release of transcripts was examined by direct electron microscopy, the first published study of this kind for a mycovirus.

Conidial progeny of isolate F6 were shown to contain virus and dsRNA but two progeny had lost a dsRNA component of virus B, suggesting that this component may be a satellite RNA. Techniques were developed for highly sensitive detection of virus and dsRNA in ascospore isolates; this included the first report of transfer of viral dsRNA from gels to solid support and hybridization with a labelled probe. This technique was shown to be convenient, highly sensitive for detection of dsRNA, and useful for the detection of homology between various Ggt viral RNAs, confirming the results of Buck et al, (1981) on relatedness of Ggt viruses. Using these

techniques ascospore progeny were examined and in most cases shown to be free of dsRNA and virus particles; however in two isolates virus and dsRNA were found. Although Lapierre (1971) reported that virus was not always eliminated during sexual reproduction, this study was the first completely documented report of this occurring and was the first occasion that virus transmitted to Ggt ascospores was characterised. Three 3b1a viruses were shown to be eliminated in ascospores when transmission occurred and the transmitted virus was shown to have undergone genomic alteration whilst remaining serologically identical to the parent virus. This was the first description of this phenomenon occurring in a mycovirus.

Ascospore isolates were examined for the presence of provirus and were shown not to contain any proviral form of viruses 3b1aB and C. In the course of this work the genome of Ggt was analysed, the genome size and repetitive DNA content was determined; also, the organisation of the ribosomal RNA genes was investigated.

REFERENCES

Adler, J. (1979) in "Fungal Viruses" ed. H.P. Molintoris et al, Apringer-Verlag

Adler, J. (1980) in "Viruses and Plasmids in Fungi" ed. P.A. Lemke, M. Dekker, New York

Anagnostakis, S. and Day, P.R. (1976) Phytopath; 69 1226-9

Al-Aidroos, K. and Bussey, H. (1978) Can. J. Microbiol, 24 228-37

Alboug, J, and Lapierre, H. (1971) Ann. Univ. et A.R.E.R.S. Reims 9: 333-339

Almond, M.R. (1979) Ph.D. Thesis, Imperial College, London

Almond, M.R. Buck, K.W. and Rawlinson, C.J. (1977), Abstr., p.13 Int. Mycol. Congr. 2nd, Tampa

Anagnostakis, S. (1977) Exp. Mycol. 1 308-316

Anagnostakis, S. (1978) "The American Chestnut: new hope for a fallen giant" Bulletin 777, 9pp New Haven: The Connecticut Agricultural Experimental Station.

Avrameas, S. and Ternynck, T. (1969) Immunochem. 6 53-66

Baimbridge, B.W. (1971) J. Gen. Microbiol. 66 319-340

Baker, K.F. and Cook, R.J. (1974) "Biological Control of Plant Pathogens" W.H. Freeman & Co., San Francisco pp. 182-183

Ballis, C. (1970) Ann. Appl. Biol., 66 59-73

Baltimore, D. (1974) Cold Spring Harbour Symposia Quant. Biol. 39 1187-1200

Baltimore, D. (1976) Science 192 632-636

Baluda, M. and Nayak, D. (1970) Proc. Natl. Acad. Sci. US 329-336

Bancroft, J.B. McLean, G.D., Rees, M.W. & Short, M.N. (1971) Virology 45 707-715

Banks, G.T., Buck, K.W., Chain, E.B., Darbyshire, J.E. & Himmel-Weit, F (1969a) Nature, 222 89-90

Banks, G.T., Buck, K.W., Chain, E.B., Darbyshire, J.E. & Himmelweit, F. (1969b) Nature, 223, 155-8

Banks, G.T., Buck, K.W., Chain, E.B., Himmelweit, F., Marks, J.E., Tyler, J.M., Hollings, F., Last, F.T. and Stone, O.M. (1968) Nature, 218, 542-5

Bartler, N.M., Gillies, S.C., Ballivant, S., and Bellamy, A.R. (1974) J. Virol, 14 315-326

Barton, R.J. and Hollings, M. (1979) J. Gen. Virol., 42 231-240

Berke, J.D., and Sharpe. M. (1977) Cell 12, 721-32

Bertrand, H., Collins, R.A., Stohl, L.L., Goeuvert, R.R. (1980) Proc. Nat. Acad. Sci. USA 77 6032-36

Bevan, E.A. and Herring, A.J. (1976) pp. 153-162 in "Genetics, Biogenetics and Bioenergetics of Mitochondria" ed. W. Bandelow et al, Walter de Gruyter, Berlin.

Bevan, E.A., Herring, A.J. & Mitchell, D.J. (1973) Nature 345 81-6

Bevan, E.A. & Mitchell, D.J. (1979) pp. 161-199 in "Viruses and Plasmids of Fungi" ed. P.A. Lemke, M. Dekker, New York.

Bevan, E.A., and Somers, J.M. (1969) Genet. Res., 14, 71-7

Biraghi, A. (1951) 5 pp. Firenze, Inst. Patologica Forestale Agraria.

Blanch, P.A. (1977) D. Phil. Thesis. Oxford University.

Border, D.J., Buck, K.W., Chain, E.B., Kempson-Jones, G.F., Ohoas, P., Ratti, G. (1972) Biochem, J. 127 47-67

Bostien, D., White, R.L., Skolnick, M, and Davis, R.W. (1980) Am. J. Hu. Genet. 32 314

Botcham, M., Topp, W., and Sambrook, J. (1976) Cell 9 269-287.

Bozarth, R.F. (1972) Environmental Health Perspectives 2, 23-29

Bozarth, R.F. (1977) Virol., 80, 149-57

Bozarth, R.F. (1979) in "Viruses and Plasmids in Fungi" pp 44-93, ed. P.A. Lemki, M. Dekker, New York.

Brewer, B.J. and Fangman, W.L. (1980) Proc. Natl. Acad. Sci. 77 5380-84

Bruenn, J.A. and Brennan, V.E. (1980) Cell, 19 923-33

Britten, R.J. and Kohne, D.E. (1968) Science, 161 529-62

Brennan, V.E., Field, R., Cizdzid, P., Bruenn, J.A. (1981) Nuc. Ac. Res. 9 4007-4021

Britten, R.J., Graham, D.E., Neufield, B.R. (1974) in "Methods in Enzymology", (L. Grossman and K. Maldave eds.) Vo. 29, Part E. 363-418 Academic Press, New York.

Brown, E.G. and Prevec, L. (1979) Virol., 95 244

Bruenn, J. and Kane, W. (1978) J. Virol., 26 762-72

Buck, K.W. (1975) Nucl. Acids Res., 2, 1889-1907

Buck, K.W. (1977) in "Biologically Active Substances - Exploration and Exploitation" ed. D.A. Hems. Wiley.

Buck, K.W., (1978) Biochem. Biophys, Res. Comm., 84, 639-45

Buck, K.W. (1979a) in "Viruses and Plasmids of Fungi" pp 1-43 ed. P.A. Lemke, M. Dekker, New York.

Buck, K.W. (1979b) in "Viruses and Plasmids of Fungi" pp 93-160

Buck, K.W. (1979c) in "Fungal Viruses" ed. H.P. Molintoris et al., pp 62-77, Springer-Verlag.

Buck, K.W. (1980) in "Eukaryatic Microbial Cell" Soc. Gen. Microbiol, Symp. 30. eds. G.W. Gooday et al, Cambridge Univ. Press.

Buck, K.W. Almond, M.R., McFadden, J.J.P., Romanos, M.A. and Rawlinson, C.J. (1981) J. Gen. Virol., 57 157-168

Buck, K.W. and Girvan, R.F. (1977) J. Gen. Virol., 34, 145-54

Buck, K.W. Girvan, R.F. and Ratti, G. (1973) Biochem. Soc. Trans., 1, 1138-40

Buck, K.W. and Kempson-Jones, G.F. (1974) J. Gen. Virol., 22 441-45

Buck, K.W. and Ratti, G. (1975) J. Gen. Virol., 27, 211-24

Buck, K.W. and Ratti, G. (1977) J. Gen. Virol., 37, 215-19

Buck, K.W., Romanos, M.A., McFadden, J.J.P. and Rawlinson, G.J. (1981) J. Gen. Virol. 57, 157-168

Burnet, J.H. (1975) "Mycogenetics" pp. 259-287, Wiley, J.

Cairns, J. (1963) Cold Spring Harb. Symp. Quant Biol. 28, 43

Carpenter, J.M., and Rawlinson, C.J. (1974) p. 119 in Rothamstead Expt. Stn. Rep., 1973.

Castanho, B. and Butler, E.E. (1978a), Phytopathology, 68, 1505-10

Castanho, B. and Butler, E.E. (1978b), Phytopathology, 68, 1514-1515

Castanho, B., Butler, E.E. and Shepherd, R.J. (1978c), Phytopathology 68, 1515-18

Chambers, S.C. and Flentge, N.T. (1967) Aus. J. Biol. Sci. 20, 927-40

Christensen, B., Dam (1967) Tiddskr. PLAvl, 71, 64-69

Clark, M.F., and Adams, A.N. (1977) J. Gen. Virol. 34, 415-483

Collins, R.A., Stohl, L.L., Cole, M.D., Lambouritz, A.M. (1981) Cell 24, 443-452

Cox, R.A. and Padenk, K. (1978) Biochem-Soc. Trans. 6, 1230-1232

Cox, R.A. and Thompson, R.D. (1980) Biochem. J. 187, 75-90

Cross, R.K. and Fields, B.N. (1977) pp 291-340 in "Comprehensive Virology" ed. H. Fraenkel-Conrat and R.R. Wagner, vol. 9 Plenum Press, New York

Gunge, N. & Sakeguchi (1981) J. Bact. 147, 155-60

Cunningham, P.C. Spillane, P.A., Foreman, B.T. and Conriffe, D. (1968), Ir. J. Agric. Res., 7, 183-93

Cummings, J. and Brown T. (1979) Mol. Gen. Genet. 171, 239-250

Davis, R.J. (1925) J. Agric. Res., 31, 801-25

Day, P.R. (1981) Mycologia 73, 379-391

Day, P.R. and Anagnostakis, S.L. (1973) Phytopath, 63, 1017-18

Day, P.R. and Dodds, J.A. (1979) Chapt. 5 in "Viruses and Plasmids of Fungi" ed. P.A. Lamke, M. Deter, New York

Day, P.R. Dodds, J.A., Elliston, J.E. Jaynes, R.A. and Anagnostakis, A.L. (1977) Phytopath, 67, 1393-6

Deacon, J.W. (1973a) Pl. Pathol., 22, 88-94

Deacon, J.W. (1973b) Trans. Brit. Mycol. Soc. 61, 471-85

Deacon, J.W. (1974a) Trans. Brit. Mycol. Soc. 63, 307-27

Deacon, J.W. (1974b) Pl. Pathol. 23, 85-92

Deacon, J.W. (1976a) EPPO Bulletin 6, 349-363

Deacon, J.W. (1976b) Soil Biology and Biochemistry 8, 275-283

Deacon, J.W. and Henry, C.M. (1978) Ann. appl. Biol (1978), 89 401-409

de Deken, R.H. (1966) J. Gen. Microbiol. 44, 157-65

de Marini, D.M., Kurtzman, C.P. Fennell, D.I. Worden, K.A. and Detroy, R.W. (1977) J. Gen. Microbiol., 100, 59-64

Denhardt, J.D. (1966) Biochem. Biophys, Res. Comm. 23, 641-46

Dickson, H. (1936) Ann. Bot. 50, 719-33

Dieleman-van Zaayen, A. (1979) pp 239-324 in "Viruses and Plasmids of Fungi" ed. P.A. Lemki, M. Dekker, New York

Dieleman-van Zaayen, A. and Temmink, J.H.M. (1968) Neth, J. Pl. Pathol. 74, 48-51

Dobos, P., Hallett, R., Kells, D.T.C., Hill, B.J., Brecht, and Teninges, D. (1978) Abstr. 4th Int. Congr. Virol., p. 335. Wageningen: Centre for Agric. Publ. Document.

Dodds, J.A. (1979) in American Chestnut Symposium Proceedings, ed. W. MacDonald, W. Virginia Univ. Agric. Expt. Stn. and U.S. Dept. Agric.

Donnis, M., Keller, M., Browning K., Clark, J. (1981) virol 110 43-5[illegible]

Dusenberg, R. (1975) Biochem. Biophys. Acta. 378 363

Dutta, S.K. (1973) Biochem. Biophys. Acta. 324 482

Ellis, L.F. and Kleinschmidt, W.J. (1967) Nature, 215, 649

Etten, J.V. and Dean, J.W. (1976) J. Virol. 18 652-658

Falkou, D. "Infectious Multible Drug Resistance", London, Pion

Firtel, R.A., Cockburn, A., Frankel, G. and Hershfield, V. (1976) J. Mol. Biol. 102 831-852

Flor, H.H. (1971) A.r. Phytopathology, 9, 275-96

Franklin, T.A. (1966) P.N.A.S. (USA) 55 (1504-1511)

Frick, L.J. and Lister, R.M. (1978) Virol., 85, 504-17

Fried, H.M. and Fink, G.R. (1978) P.N.A.S. USA 75, 4224-28

Garret, S.D. (1970) "Pathogenic Root-Infecting Fungi", Cambridge University Press.

Gealt, M.A., Sheir-Ness, G. and Morris, J. (1976) J. Gen. Microbiol 94 204

Gerlagh, M. (1968) Neth. J. Pl. Pathol., 73 Suppl. 2, 97 pp

Ghabriel, S.A., Sanderlin, R.S. and Calvert, L.A. (1979) Phytopath., 69, 312-15

Gillespie, D., and Spielgelman, S. (1965), J. Mol. Biol. 12 829-42

Gomori, G. (1959) Meth. Enzymol., 1, 138-40

Grente, J. (1965) C.R. Hebd. Seances Acad. d'Agric., France, 51 1033-7

Grimaldi. G., Guardiola, J., Martini, G. (1978) T.I.B.S., November 1978 248-49

Guerineau, M. in "Viruses and Plasmids in Fungi" ed. Lemki, P.A. Dekker, New York

Gunger and Sakaguehi (1981) J. Bacteriol. 47 155-160

Hareford and Roskash (1977) Cell 10 453

Haskins, J.A. (1980) in "Biology and Growth of Smut Fungi" ed. Fisher and Holten.

Hastie, N.D. Brennan, V., and Bruenn, J.A. (1978) J. Virol, 28 1002-5

Herring, A.J. and Bevan, E.A. (1977) Nature, 268, 464-6

Hill, M., Hillova, J., Dantchev, D., Mariage, R., and Goubin, G. (1974). Cold Spring Harbor Symposia Quant. Biol. 39 1015-26

Holden, J. (1976) Soil. Biol. Biochem., 8, 109-19

Holland, J.J., Grabau, E.A., Jones, C.L. and Semlev, B.L. (1979) Cel. 16, 495-504

Hollings, M. (1962) Nature 196, 962-5

Hollings, M. (1979) Adv. Virus Res., 22 2-54

Hollings, M and Stone, O.M. (1971) Ann. Rev. Phytopath, 9, 93-118

Hooper, J.E., Bostian, K.A., Rowe, L.B. and Tipper, D.J. (1977) J. Biol. Chem. 252, 9010-17

Horby, D. and Brown, M.E. (1977) Plant and Soil, 48, 455-71

Jinks, J.L. (1958) Proc. Roy. Soc. B 148 314-21

Jinks, J.L. (1969) Heredity, 13, 525-28

Jinks, J.L. (1966) Heredity, 21, 227-39

Jinks, J.L. (1966) Nature, 210, 778-80

Jamet-Vierry, L., Begel, O., Belcour, L. (1980) Cell 21 189-94

Kane, W.P., Pietras, D.F., and Bruenn, J.A. (1979) J. Virol. 32 692-703

Kennel, D.E. (1971) Proc. Nat. Acad. Sci. 11 259-301

Koltin, Y. and Day, P.R. (1975) Appl. Microbiol. 30, 694-6

Koltin, Y. and Day, P.R. (1976a) Proc. Nat. Acad. Sci., USA, 73 594-8

Koltin, Y. and Day, P.R. (1966) Genet., 82, 629-37

Koltin, Y and Kandel, J.S. (1978) Genet, 88, 267-76

Koltin, Y., Mayer, I. and Steinlauf, R. (1978) Mol. Gen. Genet, 166 181-6

Koltin, Y., Levine, R., Kande, J. (1979) Nuc. Ac. Res. 6, 3717

Laemmli, U.K. (1970) Nature, 277, 680-5

Lapierre, H. (1973) Colloque Sci. Int. Stn. Fed. Recherches Agron. de Lausanne, Switzerland, pp 62-4

Lapierre, H., Lemaire, J.M., Jouan, B. and Molin, G. (1970) C.R. hebd. des Seances d l'Acad. des Sciences D. Paris 271, 1833-6

Lapierre, H., Molin, G., Kusiak, C. and Faivre-Amiot, A. (1974) Ann. Phytopathol., 5, 322-3

Last, F.T., Hollings, M. and Stone, O.M. (1967) Amn. Appl. Biol. 59 451-462

Lazarus, C.M., Earl, A.J. & Turner, G. (1981) Eur. J. Biochem. 106 633-41

Lemke, P.A., Nash, C.H. and Pieper, S.W. (1973) J. Gen. Microbiol. 76, 265-75

Lemki, P.A. and Nash, C.H. (1974) Bacteriol. Rev. Mar. 1974, 29-56

Lesemann, D.E. and Koenig, R. (1977) Phytophytopathologische Zeitschrift, 89, 161-9

Levine, R., Koltin, Y. and Kandel, J. (1979) Nucl. Acids Res., 6 3717-33

Uhoas, P. (1971) J. Gen. Virol., 13, 365-7

Lilley and Barnet (1963), Phytopathol. 53 354

Manella, D.A. (1979) J. Bact. 137, 1449-51

Marmur, J. (1961), J. Mol. Biol., 3, 208

Mandel, M. and Marmur, J. (1967) "Methods in Enzymology" 12 B 195-108

McConaughy, B.L., Laird, C.D., and McCarthy, B.J. (1969) Biochemistry 8, 3289

McDonnel, J. (1977) J. Mol. Biol. 110 119-149)

Mitchell, D.J., Herring, A.J., Bevan, E.A., (1976) pp 91-105 in "Microbiol and Plant Protoplasts" ed. J.F. Peberdy et al, Academic Press

Mittempergher, L. (1979) in American Chestnut Symp. Proc., ed. W. MacDonald, W. Virginia Univ. Agric. Expt. Stn. and US Dept. Agric.

Newton, M., Johnson, T. and Brown, A.M. (1930) Sci. Agr. 10 721-31

Nilsson, H.E. (1969) Ann. Agric. Coll. Sweden, 35, 275-807

Novotry, J. (1979) Zbl. Bakt. II Abt. 134, 419-22

Nygard, A.P. and Hale, B.D. (1965) J. Mol. Biol. 9, 125-42

Padwick, G.W. (1936) Ann. Appl. Biol., 23, 45-56

Pallett, I.H. (1976) pp 107-124 in "Microbiol and Plant Protoplasts" ed. J.F. Peberdy et al, Academic Press.

Partridge, J.E., van Etten, J.L., Burbank, D.E. and Vidaver, A.K. (1979) J. Gen. Virol., 43, 299-307

Person, C., Samborski, D.J. and Rohringer, R. (1962) Nature, 194, 561-2

Philliskirk, G. and Young, T.W. (1975) Ant. van Leeuwenhoek, 41, 147-51

Pons, M. (1980) Virol 100 43-52

Pope, A.M.S. and Jackson, R.M. (1973) Phytopath, 63, 882-90

Pulhalla, J.E. (1968) Genet., 60, 461-74

Rasmussen, C.R., Mitchell, R.E. and Slack, C.I. (1972) Mushroom, Sci., 8, 839

Ratti, G. and Buck, K.W. (1972) J. Gen. Virol., 14, 165-75

Ratti, G. and Buck, K.W. (1968) Nucl. Acids Res., 5, 3843-54

Rawlinson, C.J. and Buck, K.W. (1981) in "Biology and Control of Take-all" ed. M.J.C. Asher and P.J. Shipton, Academic Press.

Rawlinson, C.J., Hornby, D., Pearson, V. and Carpenter, J.M. (1973) Ann. Appl. Biol, 74, 197-209

Rawlinson, C.J. and Muthyalu, G. (1974) Rothamstead Expt. Stn. Rep., 1973, p. 119

Rawlinson, C.J. and Muthyalu, G. (1975) pp. 228-29 in Rothamstead Exp. Stn. Rep. 1974.

Rawlinson, C.J. and Muthyalu, G. (1976), pp. 255-6 in Rothamstead Exp. Stn. Rep. 1975.

Rawlinson, C.J. and Muthyalu, G. (1977) pp. 215-6 in Rothamstead Exp. Stn. Rep. 1976

Rawlinson, C.J., Muthyalu, G. and Deacon, J.W. (1977) 2nd Int. Mycol. Congr. Tampa, 1977, Abstr. p. 558

Rawlinson, C.J. and Pearson, V. (1972) pp. 133-4 in Rothamstead Expt. Stn. Rep. 1971.

Rigby, P.R., Diekman, M., Rhodes, C., Berg, P.J. Mol. Biol. 113 237

Riley, R. and Macer, R.C.F. (1966) Canad. J. Genet. Cytol. 8, 640-53

Rimon, A. and Haselkorn, R. (1978) Virol., 89, 218-28

Robertson, H.D., Matthews, M.B. (1968) Froc. Natl. Acad. Sci. USA 70, 225-9

Robinson, R.A. (1973) Rev. Pl. Pathol. 52, 483-501

Rogers, D. and Bevan, E.A. (1978) J. Gen. Microbiol., 105, 199-202

Romanos, M.A., Rawlinson, C.J., Almond, M.R. and Buck, K.W. (1980) Trans. Br. Mycol. Soc. 74 79-88

Roos, U.P., Khandjian, E.W. and Turian, G. (1976) J. Gen. Microbiol 95 87

Rovira, A.D. and Wildermuth, G.B. (1981) in "Biology and Control of Take-all" ed. M.J.C. Agher and P.J. Shipton, Academic Press.

Russell, R.C. (1939) Scient. Agric. 19, 662-669

Sanderlin, R.S. and Ghabrial, S.A. (1978) Virol, 87, 142-51

Scholtissek, C., Harms, E., Rohde, W., Orlich, M. and Rott, R. (1976) Virol., 74, 332-44

Scott, P.R. (1970) Trans. Brit. Mycol. Soc., 55, 163-7

Seghel, T.R. and Turner, A.S., (1977) Virol. 77, 1-11

Shipton, P.J. (1975) pp 137-44 in "Biology and Control of Soil-borne Plant Pathogens" ed. G.W. Bruchl. The Am. Phytopath. Soc., St. Paul, Minn. USA

Shalitin, C. and Fischer, I. (1975) Biochem. Biophys. Acta 414, 263-72

Silverstein, S.C., Christman, J.K. and Acs. 6 (1976) Ann. Rev. Biochem 45, 375-408

Simpson, R.W. and Hirst, G.K. (1961) Virol. 15 436-45

Sivasithamparam, K. and Parker, C.A. (1976) Soil Biol. Biochem. 8 71-73

Skou, J.P. (1981) in "Biology and Control of Take-all", ed. M.J.C. Asher and P.J. Shipton. Academic Press

Smiley, R.W. and Cook, R.J. (1973) Phytopath, 63, 882-90

Smith, K.M. (1977) p. 30 "Plant Viruses" 6th ed. Chapman and Hall

Smotkin, D., Yoshimuru, F.K. and Weinberg, R.A. (1976) J. Virol., 20, 621-26

Southern, E.M. (1975) J. Mol. Biol., 98, 503-19

Speakman, J.B. and Lewis, B.C. (1978) New Phytol. 80, 373-80

Stahl, U., Lemke, P.A., Tudzynski, P., Kuck, V. and Esser, K. (1978) Mol. Gen. Genet. 162, 341-3

Stanworth, D.R. (1960) Nature 188, 146-59

Stehelin, D., Guntaka, R.V., Varmus, H.E. and Bishop, J.M. (1976a) J. Mol. Biol. 101, 349-365

Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt P.K. (1976b) Nature 260 170-173

Tokanami, Y. (1981) Virol 109 120-26

Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA, ##, 5201-5205

Timberlake, W.E. (1978) Science 202, 973-975

Tivoli, B., Lemaire, J.M. and Jouan, B. (1974) Ann. Phytopath, 6 395-406

Tivoli, B., Ferault, A.C., Lemaire, J.M. and Spire, D. (1979) Ann. Phytopath, 11, 259-61

Ushigami, R. and Nakai, Y. (1975) Sing. Abst. 2nd Int. Mycol. Cong. 1975, 696

van Alfen, N.K., Bowan, J.T. and Simmons, J.R. (1979) in American Chestnut Symp. Proc. ed. W. MacDonald, Virginia Univ. Agric. Expt. Stn. and U.S. Dept. Agric.

van Alfen, N.K. Jaynes, R.A. Anagostakis, S.L. and Day. P.R. (1975) Science, 189, 890-1

Vandewalle, M.J. and Siegel, A. (1976) Virol, 73, 413-18

Vinograd, J.R. Bruner, R. and Wigle, J. (1963), Proc. Natl. Acad. Sci. USA 49, 902-910

Vodkin, M. (1977) J. Virol., 21, 516-21

Vodkin, M. (1979) in "Fungal Viruses" ed. H.P. Molintoris et al Springer-Verlag.

Vodkin, M., Katterman, F., and Fink, G.R. (1974) J. Bact., 117 681-6

Walker, J. (1975) Rev. Pl. Pathol., 54, 113-44

Walker, J. (1981) in "Biology and Control of Take-all". ed. M.J.C. Asher and P.J. Shipton. Academic Press.

Walker, P.J. Mansbridge, J.N. and Gorman, B.M. (1980) J. Virol, 34 583-91

Wallace, D.H. Wilkenson, R.E. (1975) in "Biology and Control of Soil-Borne Plant Pathogens", pp. 177-184, Am. Phytopath. Soc. Minnesota.

Weber, K., Pringle, C.R. and Osborn, N. (1972), Methods in Enzymology 16 3-27

Welsh, J.D., Lubowitz, M.J. Wickner, R.B. (1980) Nuc. Ac. Res. 8 2349-75

Wetmur, J. and Davidson (1968) J. Mol. Biol. 113, 237

Wickner, R.B. (1974) Genet, 76, 423-32

Wickner, R.B. (1979) Plasmid, 2, 303-22

Wickner, R.B. and Leibowitz, M.J. (1977) Genet., 87, 453-69

Wong, P.T.W. (1975) Soil Biol. Biochem., 7, 189-94

Wood, H.A., and Bozarth, R.F. (1972) Virol., 47, 604-9

Wood, H.A., and Bozarth, R.F. (1973) Phytopath, 63, 1019-21

Wood, H.A., Bozarth, R.F., Adler, J. and Mackenzie, D.W. (1974) J. Virol., 13, 523-4

Woods, D.R. and Bevan, E.A. (1968) J. Gen. Microbiol., 51, 115-26

Woods, D.R., Ross, I.W. and Hendry, D.A. (1974) J. Gen. Microbiol., 81, 285-9

Wood, D. and Cuck, D. (1969) J. Mol. Biol. 41, 211-24

Young, T.W. and Yagiu, M. (1978) Ant. van Leeuwenhoek, 44, 59-77

Younger, J.S., Kelly, E.V. and Frielle, D.B. (1981) Virol. 81, 87-97

Zimmerman, J.A. and Goldberg, E.K. (1977) Chromosoma 59, 277