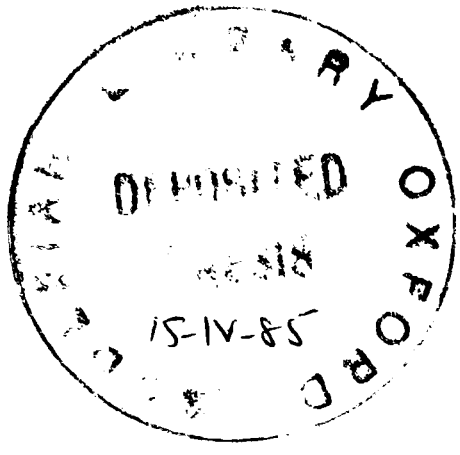
THE ISOLATION AND CHARACTERIZATION OF ORBIVIRUSES FROM TICKS (IXODES URIAE)



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ABSTRACT

The Isolation and Characterization of Orbiviruses from Ticks (Ixodes uriae)

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Ticks [Ixodes (Ceratixodes) uriae] were collected from two seabird colonies on the Isle of May in Scotland. Viruses were isolated from three tick pools, one from ticks collected during 1979 and two from ticks collected during 1981. The viruses replicated in suckling mouse brain, chick embryo fibroblasts, Vero and BHK-21 cells, but not in Xenopus cells. By virtue of their morphology in infected cells, physicochemical properties and reactions in complement fixation tests, they were identified as Kemerovo serogroup viruses belonging to the Great Island Complex (Orbivirus:Reoviridae). The three isolates were distinguished from each other by plaque reduction neutralization tests. After three cycles of plaque purification, the replication of one isolate, Mill Door/79 virus, was examined in Vero and BHK-21 cells. The virus grew to maximum titres 8 to 9 hours post infection (p.i.); over 99% of the infectivity was cell-associated. Twelve virus-specified polypeptides, p141, p93, p69, p65, p53/51, p44, p37, p36, p30, p27, p21 and p20 were identified in infected Vero cell lysates by polyacrylamide gel electrophoresis (PAGE). Similar polypeptide profiles were observed in infected BHK-21 cell lysates. Attempts to purify the virus, by polyethylene glycol-6000 precipitation, resulted in the detection of p93, p69, p53/51, p37, p21 and p20 after PAGE, whereas only four, p93, p69, p53/51 and p37 were detected after attempts at purification using ether extractions. Results using protease inhibitors and partial proteolysis indicated that three virus-specified polypeptides (p36, p30 and p27) may be cleavage products. All virus-specified polypeptides, with the exception of p30 and p20, were labelled in infected cell cultures with both [^{14}C] mannose and [^{14}C] glucosamine. Incorporation of these sugars was unaffected by tunicamycin, but the virus-specified polypeptides were not labelled with [^{14}C] glucosamine in the presence of 2-deoxy-D-glucose. Double-stranded (ds) RNA expressed in infected Vero cells by Mill Door/79 virus comprised 10 segments, with a total molecular weight of 11.64 x 10^6. Ten of the products of *in vitro* translation, in a rabbit reticulocyte lysate system, co-migrated in polyacrylamide gels with virus-specified polypeptides. Polypeptides co-migrating with p30 and p27 were not detected *in vitro*, whereas an additional polypeptide, p75, not detected in infected cell lysates, was synthesized *in vitro*. dsRNA coding assignments of Mill Door/79 virus were derived by translating RNA segments individually *in vitro*. Mill Door/79 virus was compared with North Clett/81 and Mill Door/81 viruses. dsRNA profiles of the three isolates were distinct, whereas few differences were detected by comparing the polypeptide profiles, or by partial proteolysis. Immune precipitation failed to distinguish between the three isolates. The properties of the Isle of May isolates were compared with those of other members of the family Reoviridae.

ABSTRACT

Ticks [Ixodes (Ceratixodes) uriae] were collected from two seabird colonies on the Isle of May in Scotland. Viruses were isolated from three tick pools, one from ticks collected during 1979 and two from ticks collected during 1981. The viruses replicated in suckling mouse brain, chick embryo fibroblasts, Vero and BHK-21 cells, but not in Xenopus cells. By virtue of their morphology in infected cells, physicochemical properties and reactions in complement fixation tests, they were identified as Kemerovo serogroup viruses belonging to the Great Island Complex (Orbivirus:Reoviridae). The three isolates were distinguished from each other by plaque reduction neutralization tests. After three cycles of plaque purification, the replication of one isolate, Mill Door/79 virus, was examined in Vero and BHK-21 cells. The virus grew to maximum titres 3 to 9 hours post infection (p.i.); over 99% of the infectivity was cell-associated. Twelve virus-specified polypeptides, p141, p93, p69, p65, p53/51, p44, p37, p36, p30, p27, p21 and p20 were identified in infected Vero cell lysates by polyacrylamide gel electrophoresis (PAGE). Similar polypeptide profiles were observed in infected BHK-21 cell lysates. Attempts to purify the virus, by polyethylene glycol-6000 precipitation resulted in the detection of p93, p69, p53/51, p37, p21 and p20 after PAGE, whereas only four, p93, p69, p53/51 and p37 were detected after attempts at purification using ether extractions. Results using protease inhibitors and partial proteolysis indicated that three virus-specified polypeptides (p36, p30 and p27) may be cleavage products. All virus-specified polypeptides, with the exception of p30 and p20, were labelled in infected cell cultures with both

[^{14}C] mannose and [^{14}C] glucosamine. Incorporation of these sugars was unaffected by tunicamycin, but the virus-specified polypeptides were not labelled with [^{14}C] glucosamine in the presence of 2-deoxy-D-glucose. Double-stranded (ds) RNA expressed in infected Vero cells by Mill Door/79 virus comprised 10 segments, with a total molecular weight of 11.64×10^6. Ten of the products of *in vitro* translation, in a rabbit reticulocyte lysate system, co-migrated in polyacrylamide gels with virus-specified polypeptides. Polypeptides co-migrating with p30 and p27 were not detected *in vitro*, whereas an additional polypeptide, p75, not detected in infected cell lysates, was synthesized *in vitro*. dsRNA coding assignments of Mill Door/79 virus were derived by translating RNA segments individually *in vitro*. Mill Door/79 virus was compared with North Clett/81 and Mill Door/81 viruses. dsRNA profiles of the three isolates were distinct, whereas few differences were detected by comparing the polypeptide profiles, or by partial proteolysis. Immune precipitation failed to distinguish between the three isolates. The properties of the Isle of May isolates were compared with those of other members of the family Reoviridae.

DEDICATIONS

In memory of my father

For Joy and my mother

for their support and understanding

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ABBREVIATIONS

AMD	actinomycin D
BHK-21	baby hamster kidney cells
bis	N-N'methylenebisacrylamide
BPA	bovine plasma albumin
BTV D	bluetongue virus "dense" particle
BTV L	bluetongue virus "light" particle
CEF	chick embryo fibroblasts
CTF	Colorado tick fever virus
CMC	carboxymethyl cellulose
cpe	cytopathic effect
CPV	cytoplasmic polyhedrosis virus
2DG	2-deoxy-D-glucose
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase 1	deoxyribonuclease type 1
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
EHDV	epizootic haemorrhagic disease virus
ELISA	enzyme-linked immunosorbent assay
EMEM	Eagle's modification of minimum essential medium
endo H	endo-p-N-acetyl glucosaminidase H
FCS	fetal calf serum
FDV	Fiji disease virus
GMEM	Glasgow's modification of minimum essential medium
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
i.c.	intracerebrally
IOA	iodoacetamide

L-15	Leibovitz (L-15) medium
MRDV	maize rough dwarf virus
mRNA	messenger ribonucleic acid
M.W.	molecular weight
PAG	polyacrylamide gel
PAGE	SDS-polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBSA	PBS, PBA, penicillin, streptomycin and kanamycin
PBSa	PBS and BPA
pfu	plaque forming unit
p.i.	post infection
PMSF	phenylmethyl sulphonyl fluoride
RDV	rice dwarf virus
RGDV	rice gall dwarf virus
RNA	ribonucleic acid
SDC	sodium deoxycholate
SDS	sodium dodecyl sulphate
ssRNA	single-stranded RNA
S.aureus	Staphylococcus aureus
TCA	tricholoroacetic acid
TEMED	N-N-N'-N' tetramethylethylenediamine
TLCK	N-α-p tosyl-L-lysine chloromethyl ketone
Tm/TM	tunicamycin
TPCK	L-1-tosylamide-2-phenyl ethyl chloromethyl ketone
Tris	Tris (hydroxymethyl)-aminomethane
Vero	African green monkey cells
WTV	wound tumour virus
ZnAc	zinc acetate

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I. INTRODUCTION TO THE FAMILY REOVIRIDAE

In 1962 Gomatos et al., using acridine orange, demonstrated that the nucleic acid of reovirus type 3 consisted of double-stranded (ds) RNA. This was the first report of a virus containing dsRNA. In the years that followed other viruses were shown to have similar genomes. These included wound tumour virus (Gomatos & Tamm, 1963), cytoplasmic polyhedrosis virus (Miura et al., 1968), bluetongue virus (Verwoerd, 1969) and neonatal calf diarrhoea virus (Welch, 1971). The dsRNA genome, genome organization, and replication strategies, led these viruses to be classified together within the family Reoviridae. Table 1.1 shows the six recognized genera of the Reoviridae together with their type species and host ranges. Other viruses which appear to be members of the Reoviridae but do not fit into any recognized genus include Drosophila virus F (Brun & Plus, 1980), Ceratitis 1 virus (Plus et al., 1981), Housefly virus (Moussa, 1978), Chum salmon virus (Winton et al., 1981) and $13p_2$ virus of oysters (Meyers, 1979).

The genomes of members of the Reoviridae are divided into 10-12 segments, depending on the genus. The segments are thought to represent single genes, which are not related as they do not hybridize with each other (Bellamy & Joklik, 1967). Other properties common to members of the Reoviridae include the possession of enzymes required to transcribe dsRNA into mRNA. This has been demonstrated for reoviruses (Borsa & Graham, 1968; Shatkin & Sipe, 1968b), orbiviruses (Martin & Zweerink, 1972; Verwoerd & Huismans, 1972; Verwoerd et al., 1972), rotaviruses (Cohen, 1977), Cytoplasmic Polyhedrosis Viruses (Lewandsowski et al., 1969) and plant reoviruses (Black & Knight, 1970). Unlike

other icosahedral viruses, the genome of members of the Reoviridae is not completely uncoated following infection: only the outer shell is removed leaving the genome associated with the subviral particles (Cohen et al., 1979; Borsa et al., 1981). Uncoating probably does not occur with cytoplasmic polyhedrosis viruses (see Section IV).

In the 22 years since Gomatos et al. (1962) first described the possible nature of the reovirus genome much has been discovered about the Reoviridae. The viruses isolated and characterized during the course of my thesis research were members of the Reoviridae, belonging to the Orbivirus genus. To contrast these viruses with other members of the Reoviridae the following literature review describes the morphology, nucleic acid and proteins of the six Reoviridae genera but with greater emphasis given to the Orbiviruses.

II. THE ORTHOREOVIRUSES

A. Introduction

Reoviruses (reo: respiratory enteric orphan; Sabin, 1959) were first isolated as a result of vaccination programmes for poliomyelitis (Ramos-Alvares & Sabin, 1954) but these viruses were too large (diameter 70nm) to be classified with the Picornaviridae. Three serotypes could be recognized: serotype 1 (prototype Lange strain), serotype 2 (prototype D5 Jones strain), and serotype 3 (prototype Dearing strain), although all the viruses were morphologically identical (Sabin, 1959). Reoviruses have been isolated from primates, cattle, monkeys, mice, dogs and cats (Rosen, 1962; Lou & Wenner, 1963; Scott et al., 1970) and antibodies to reoviruses have been identified in camels, guinea

pigs, hares, horses, marsupials, pigs, rabbits, rats and sheep (Stanley, 1967). All the reoviruses so far isolated from mammals belong to these serotypes. The reoviruses isolated from other vertebrates are not so easily classified serologically. Avian reoviruses have been classified, on the basis of neutralization tests, into five serotypes (Kawamura et al., 1965); they cross react serologically with mammalian reoviruses (Deshmukh et al., 1968). The only reoviruses which have been investigated in depth at a molecular level are the mammalian isolates and of these the Dearing strain of serotype 3 is the most studied.

B. Morphology

The reovirus capsid was shown to be comprised of an outer shell, having a diameter of 76nm and an inner shell or core of 52nm (Luftig et al., 1972). These results, obtained from negatively stained preparations, differed from those derived from diffusion coefficients which showed that when the outer capsid was hydrated, its outer shell diameter was 98nm (Harvey et al., 1974). The outer shell and core can be separated by various physical and chemical treatments (Mayor & Jordan, 1968; Amano et al., 1971) and by digestion with chymotrypsin (Shatkin & Sipe, 1968a). In CsCl gradients the intact virion has a density of 1.36g/ml and the cores, 1.43g/ml (Smith et al., 1969).

The detail of the outer capsid has proved difficult to elucidate. Using negative contrast electron microscopy Palmer and Martin (1977) found the capsid to be composed of 32 morphological units. They also noted the sharing of subunits, a unique feature of the Reoviridae. Palmer and Martin (1977) demonstrated 12 indentations in the reovirus outer capsid. The cores were distinct in possessing 12 projections, located on the

five-fold vertices of the virus icosahedron (Luftig et al., 1972). In the intact virion these projections are thought to be in alignment with the indentations in the capsid shell (Joklik, 1983). Other data supports this view (see below).

C. The Genome

Several properties have been used to demonstrate the double-strandedness of the reovirus genome: the failure of formaldehyde to produce hyperchromicity (Gomatos & Tamm, 1963), X-ray diffraction patterns consistent with double-stranded nucleic acid (Arnott et al., 1966), sharp melting point profiles, dependence of RNA melting point on ionic strength (Bellamy et al., 1967), resistance to ribonuclease, which is dependent on both cation and enzyme concentration (Bellamy et al., 1967), lower density of dsRNA compared to ssRNA in Cs_2SO_4 gradients (Iglewski & Franklin, 1967), and the base composition, which shows equality of A + U as well as G + C (Bellamy et al., 1967).

The segmented nature of the genome was first indicated by electron microscope studies which revealed a trimodal length distribution (Vasquez & Kleinschmidt, 1968). When the RNA was analysed on sucrose density gradients, three size classes were distinguished (Bellamy et al., 1967). Subsequently, polyacrylamide gel electrophoresis separated the RNA into 10 discrete molecular species having a combined weight of 15×10^6 daltons (Shatkin et al., 1968) (Fig. 1.1). Hrdy et al. (1979) showed that reoviruses isolated from humans, cattle and mice varied extensively in the genome profiles of their dsRNA. This variation was found in all three serotypes, and involved all ten genome segments. Variation within the genome of the Reoviridae is discussed in more detail in Section VI.

The terminal sequences at both the 3' and 5' ends of the plus strands has been determined for each genome segment of the Dearing strain of serotype 3 (Antezak et al., 1982). At the 3' end a pentanucleotide, UCAUC-3', is shared by all 10 segments. At the 5' end a tetranucleotide, GCUA-, is also shared by all segments. It is not yet clear what the significance of these conserved sequences may be.

Reoviruses contain up to 25% of their RNA in the form of small single-stranded oligonucleotides, and one third of these are composed solely of adenine (Shatkin & Sipe, 1968a). They are probably the products of abortive gene transcription (Bellamy et al., 1972).

D. Proteins

Table 1.2 summarises the properties of the proteins associated with reovirus particles, and those found in infected cells. Ten proteins are primary gene products (McCrae & Joklik, 1978). This would be expected from ten monocistronic genome segments. The other four proteins are derived from cleavage of primary gene products (Zweerink et al., 1971; Lee et al., 1981a). The outer capsid proteins, μ1c, σ1 and σ3, have been studied by Lee et al. (1981a) using monoclonal antibodies. These workers demonstrated that σ1, a minor constituent of the shell, was type specific: little cross reaction was found between the σ1 of different reovirus serotypes, and σ1 was the target for neutralizing antibodies. Monoclonal antibodies raised against σ1 also inhibited haemagglutination. Lee et al. (1981b) demonstrated that σ1 functioned as the cell attachment protein, as antibodies raised against it prevented reovirus adsorption and free σ1 present in lysates of infected cells was capable of

adsorbing to cells and competing with reovirus particles for cell surface receptors. Proteins μ1c and σ3 (Table 1.2) were not involved in neutralization reactions, and were sometimes found complexed together (Huismans & Joklik, 1976). The smaller protein, σ3 was shown to bind RNA strongly, and it may be important in viral morphogenesis. Rubin and Fields (1980) showed that protein σ1c [a cleavage product of μ1 (Zweerink et al., 1971)] is responsible for the susceptibility of the outer capsid to chymotrypsin degradation.

The major components of the reovirus core, λ1, λ2 and σ2 have not yet been assigned any precise functions, although λ2 appears to be the sole protein comprising the spikes described by Luftig and co-workers (1972) (White & Zweerink, 1976). Iodination studies demonstrated that λ1 was the more accessible of the other two major core proteins and was probably situated on the outer surface of the core, σ2 being situated on the inner surface. The exact arrangement of the minor core proteins, λ3, μ1 and μ2 is not known, although they may be associated with transcription or capping (Drayna & Fields, 1982).

The non-structural proteins, μNS and σNS, were found in infected mouse L-fibroblasts incubated at 31°C (Zweerink et al., 1971). These workers also detected, in infected cells incubated at 37°C, three other non-structural proteins derived from μ1 or possibly μ1c. They suggested that the latter may be formed as a result of cytopathic damage of infected cells incubated at the higher temperature. Although functions for the non-structural proteins have not yet been assigned, they may be concerned with transcription, as a poly (C)-dependent RNA polymerase activity in infected cells has been associated with σNS (Gomatos et al., 1980).

Apart from cleavage, other forms of protein modification have been reported for reoviruses. Krystal et al. (1976) reported the presence of an oligosaccharide trimer or tetramer, linked to a serine or threonine residue of the μlc protein, which contained N-acetyl neuraminic acid, N-acetyl galactosamine and galactose. Only about 3% of the polypeptides were glycosylated in this manner, hence the significance of these findings was not clear. Lee (1983) using labelled sugars, showed that all viral proteins, apart from σ2, became labelled with [^{3}H] glucosamine and this incorporation was inhibited by 2-deoxy-D-glucose (2DG) but not by tunicamycin (Tm). Lee (1983) demonstrated that 2DG inhibited the uptake of glucosamine into infected cells and suggested that this was partly or totally responsible for the reduction in glucosamine incorporation. Since 2DG is a mannose and glucose analogue this would be expected. The failure of Tm to inhibit sugar incorporation suggests that N-linked glycosylation does not occur, and the inhibition of sugar uptake into cells by 2DG, probably due to competition for membrane sites, makes uncertain its action through the inhibition of O-glycosidic bond formation. More work is required to determine the function of glycosylation and the type of bonds involved. Krystal et al. (1975) reported that, when grown in the presence of [^{32}P] orthophosphate, the protein μlc was labelled. They also demonstrated the presence of phosphoserine residues in μlc. The phosphorus may be associated with the protein in forms other than the simple orthophosphate and this possibility was investigated by Carter et al. (1980) and Mora & Carter (1983). They showed that protein μlc and "component viii" were polyadenylated and oligo (ADP) ribosylated. Since these proteins are both cleavage products of μl it may be that this protein is the target for phosphorylation.

Mora and Carter (1983) suggested that phospho- proteins could play a rôle in reovirus morphogenesis.

E. Gene coding assignments

Both translation systems, capable of synthesizing proteins in vitro, and genetic approaches have been used to determine the gene coding assignments of the reovirus specified proteins. McCrae and Joklik (1978) translated the separated dsRNA segments in a wheat germ translation system, after first denaturing them using dimethyl sulphoxide. Fig. 1.2A summarises their coding assignments. Mustoe et al. (1978) used recombinants derived from crosses between reovirus serotypes 1, 2 and 3 to identify the dsRNA segments encoding polypeptides of the μ and σ size classes. Their assignments were in agreement with those of McCrae and Joklik (1978). It is clear from Fig. 1.2 that the largest dsRNA genome segments do not always code for the largest proteins.

III. THE ROTAVIRUSES

A. Introduction

Apart from the Colorado tick fever viruses and some Kemerovo group viruses of the orbivirus genus, rotaviruses are the only members of the Reoviridae family that cause clinical disease in humans (Bishop et al., 1973; Bishop et al., 1974). Rotaviruses can also infect, and cause disease in many other species of mammal and bird (McNulty, 1978). Typically they cause acute diarrhoea in the young of the species they infect (Flewett et al., 1974). At present the type species is human rotavirus (Table 1.1) although simian rotavirus, SA 11 (Malherb & Strickland-Cholmley, 1967), is better characterized and may become

the new type species.

A common group antigen shared between rotaviruses infecting different species was demonstrated by Flewett et al. (1974) using both immunofluorescence and immunoelectron microscopy. Bridger (1978) located this antigen on the inner capsid shell and produced evidence that the antigen involved in neutralization reactions was present on the outer shell. When Thouless et al. (1977) used convalescent sera in cross-neutralization reactions between isolates from a range of mammalian species, significant levels of cross-reaction occurred. However, hyperimmune ascitic fluid, prepared in either previously uninfected guinea-pigs or rabbits, showed much less cross-reactivity (Wyatt et al., 1980).

It has become clear that the serological classification of rotaviruses is complex, and a new classification system has recently been introduced. Other methods of comparing rotaviruses are available and are discussed below (Section IIIC).

B. Morphology

The morphology of rotaviruses is similar to reoviruses, except that the periphery of the complete virion is smooth in negatively stained preparations (Flewett et al., 1974). Cores are commonly found in virus preparations. These closely resemble the inner capsid of orbiviruses (see Section VI) and in fact rotaviruses were described as "orbivirus-like" in early reports (Bishop et al., 1973; Middleton et al., 1975; Tam et al., 1976). The complete virion has a diameter of 65-75nm, and the core a diameter of 55-65nm (Woode et al., 1976). The core possesses icosahedral symmetry and probably comprises 260 trimeric subunits (Roseto et al., 1979; Holmes, 1982). Roseto et al. (1979) suggested that the outer capsid symmetry mirrored that of the

core, although they did not determine its structure. Tam et al. (1976) showed that the complete virion had a density of 1.36g/cm^3, whereas the cores, which were produced on heating the virus at 37°C, were 1.38g/cm^3.

C. The Genome

Welch (1971) demonstrated that the rotavirus genome contained dsRNA, and Newman et al. (1975) and Rodger et al. (1975) showed it to be divided into 11 segments. The dsRNA profile of rotaviruses was easily distinguished from the profiles of other members of the Reoviridae (Schnagl & Holmes, 1976). The total molecular weight of the genome, calculated for a bovine rotavirus using reovirus as a standard, was 10.75 x 10^6 daltons (Barnett et al., 1978). Recent calculations, using either glyoxal-denatured RNA or cDNA copies with DNA standards, suggest that the molecular weight should be 10% higher (Holmes, 1983).

Large quantities of rotavirus can be purified from the faeces of infected animals, including children. For this reason, and because serological analysis of rotaviruses had proved difficult, gel electrophoresis of the genome RNA (extracted from faecal samples) became recognised as a useful method for distinguishing between isolates. Several studies have been carried out using electropherotypes to examine rotavirus variation during epidemics (Espejo et al., 1980a; 1980b; Schnagl et al., 1981). Rotavirus genomes can have either "short" or "long" profiles; these terms refer to the migration of genome segments 10 and 11. In a "long" profile, segment 10 migrates with segment 11 of a "short" profile. Kalica et al. (1981) showed that rotavirus isolates with "short" genome profiles belonged to subgroup 1 (as defined by immune adherence

haemagglutination assay) whereas those having "long" genome profiles belonged to subgroup 2. This apparent correlation between genome profile and serological subgroup does not always hold true (especially since the subgroup antigen is coded by genome segment 6) and may be coincidental (Kapikian et al., 1981; McCrae and McCorquodale, 1982).

Other methods, such as RNA-RNA hybridization have been used to compare rotavirus genomes (Matsuno & Nakajima, 1982). These workers demonstrated an 88%-100% homology between two human rotavirus isolates, but no homology between the human isolates and bovine and simian strains. However, the bovine and simian strains showed a 30% homology. Street et al. (1982) and Schroeder et al. (1982) used northern blot hybridization to compare sequence diversity amongst human, and human and animal isolates, respectively. Considerable sequence diversity was detected amongst the human isolates and Street et al. (1982) suggested that this variation might be caused by both shift, due to the interchange of genome segments, and drift due to the accumulation of small changes in the sequence of the rotavirus genome. Schroeder et al. (1982) showed that human and animal rotavirus isolates possessed distinct nucleic acid sequences, thereby exhibiting a low order of relatedness.

The sequences of rotavirus RNA at both the 3' and 5' ends have been determined by McCrae and McCorquodale (1983). They found long terminal sequence conservation (at least eight bases) for each segment. This conservation was present in both bovine and human isolates. The degree of conservation was probably due to some functional role, such as: RNA transcriptase binding, RNA replicase binding, ribosome binding, or genome species selection during virus assembly. Until the interactions between the viral

proteins and the terminal sequences are elucidated, their exact function will remain unknown. Clark and McCrae (1983) were able to demonstrate that, adjacent to the absolute terminal conserved sequences, there was a region of approximately 30 nucleotides of moderately conserved sequence for each RNA segment, which was species specific. The function of these species-specific conserved regions is not known.

D. Proteins

The structural proteins of human, simian and bovine rotavirus isolates have been analysed by several workers (Rodger *et al.*, 1975; 1977; Matsuno & Mukoyama, 1979; Espejo *et al.*, 1981; Estes *et al.*, 1981; Dyall-Smith & Holmes, 1981b; McCrae & Faulkner-Valle, 1981; Arias *et al.*, 1982). These workers do not agree on the total number of polypeptides that are structural (see Table 1.3). Rodger *et al.* (1977) and Matsuno and Mukoyama (1979) identified eight structural polypeptides, three comprising the outer shell and five the core. However, Espejo *et al.* (1981) reported that the use of trypsin by previous workers to increase virus infectivity resulted in cleavage of the structural polypeptides VP2 (p96) and VP3 (p84). They concluded that the virus particle comprised only five proteins, two in the outer shell and three in the core. Further work by Estes *et al.* (1981) demonstrated tryptic cleavage of VP2 to VP4, and VP3 to VP5 and VP8. Dyall-Smith and Holmes (1981b) reported that VP4 (p84), the supposed cleavage product of VP2 (p96), was a primary gene product. It is clear that much confusion still exists as to the presence and arrangement of proteins in the rotavirus capsid.

It is generally agreed that two rotavirus proteins are glycosylated. These are the outer shell protein gp34 (VP7),

which is the outer shell's major component, and a possible additional outer shell protein, gp25 (VP10c, NCVP5). The protein gp25 is unusual in that its apoprotein precursor (pNCVP5) is present in infected cells treated with Tm (Arias et al., 1982). Arias et al. (1982) also tentatively suggested the presence of a non-structrual glycoprotein NCVP6, since its non-glycosylated precursor, pNCVP6 (molecular weight 16 x 10^3) was also detectable in Tm treated cells. The oligosaccharides of gp34 and gp25 were cleaved by treatment with endo-p-N-acetyl glucosaminidase H (endo H), suggesting that they were residues of the "high mannose" type (Arias et al., 1982) [endo H cleaves mannose rich precursor oligosaccharides, and will not attack the Golgi processed oligosaccharides (Robbins et al., 1977)]. Graham et al. (1983) using Tm, demonstrated that gp34 was N-linked, and that the oligosaccharide residue was cleaved with endo H, confirming the results of Arias et al. (1982). Using a mutant of simian rotavirus (SA 11), lacking the sugar moiety on gp34, Petrie (1983) demonstrated that glycosylation was not required for either haemagglutination, infectivity, replication, or stability against proteolytic digestion. Since gp34 is the protein that elicits neutralizing antibody (McCrae & McCorquodale, 1982) the exact function of the sugar moiety is still obscure. Petrie (1983) demonstrated the involvement of the glycoprotein, gp25 in SA 11 maturation.

E. Gene coding assignments

The gene coding assignments of SA 11 virus are given in Fig. 1.2B. Smith et al. (1980), using a wheat germ translation system, determined the products of the genome segments 1-6. Segments 1-4 coded for proteins p130,p93, p88 and p82, segment 5

coded for p55, and segment 6 coded for p42. The molecular weights of the protein products derived above differed slightly from those calculated by Dyall-Smith and Holmes (1981b) (see Table 1.3). Dyall-Smith and Holmes (1981a) determined the coding assignments of segments 10 and 11 of SA 11 virus and two human rotavirus isolates displaying "short" and "long" dsRNA profiles. For SA 11 virus, segment 10 coded for p21 and segment 11 coded for the protein moiety of gp25. The "long" profile human rotavirus coded for similar size proteins but the "short" profile isolate had the opposite assignments. It was not understood why the gene coding for gp25 shifted so dramatically in relative mobility yet encoded a protein of almost equal molecular weight. Arias et al. (1982) translated nine of the 11 segments of the SA 11 virus genome separately in a rabbit reticulocyte lysate system and found that segments 1 to 3 coded for p113, p96 and p84 respectively, segment 4 coded for p91, segment 5 for p57, segment 6 for p42, and segments 7, 8 and 9 for p33, p31 and the protein moiety of gp34 respectively. They found that segment 10 coded for the protein moiety of gp25, and although they could not translate segment 11 they suggested it coded for p16 (a possible precursor of the glycoprotein NCVP6). Their assignment of segment 4 differed from Smith et al. (1980), but it is possible that this could be due to either virus strain variation or repeated virus passage. McCrae and McCorquodale (1982) determined the gene coding assigments of a bovine rotavirus isolate and their data was in close agreement with the assignments of Smith et al. (1980) and Dyall-Smith and Holmes (1981). They assigned a protein of molecular weight between 20 x 10^3 and 25 x 10^3 to segment 11, which was cleavaged to produce a slightly smaller protein.

IV. THE CYPOVIRUSES

A. Introduction

Cytoplasmic polyhedrosis viruses (CPVs), although possessing many biochemical characteristics in common with other members of the Reoviridae, are easily distinguished in having virus particles lacking a double capsid and by their inclusion within proteinaceous inclusion bodies or polyhedra (Matthews, 1982). Unlike the other members of the Reoviridae (excluding phytoreoviruses), the host range of CPV is restricted almost entirely to insects. This host restriction may be associated with the inhibition of virus replication at temperatures above 35°C, which is probably due to the inhibition of viral RNA synthesis (Tanada & Chang, 1968; Kobayashi & Kawase, 1980).

Research on CPVs has been carried out because of the economic importance of their insect hosts. The CPV of the silkworm, Bombyx mori, causes extensive losses in the Japanese silk industry (Payne & Mertens, 1983) whereas CPVs of insect pest species could be used as control agents (Payne, 1982). In the latter case the biochemical similarities CPVs share with reoviruses, orbiviruses and rotaviruses have delayed their widespread use, although recently it has been shown that CPVs do not cause serious pathological changes when injected into or fed to test vertebrates, so their use as control agents could be extended (Katagiri, 1981).

In common with the rotaviruses, CPVs are compared by their dsRNA profiles and a classification scheme has been devised using this approach (Payne & Rivers, 1976). As yet no system for serological classification exists, partly because of the limited number of cell culture systems in which the viruses will

replicate, precluding the use of infectivity neutralization tests. Cunningham and Longworth (1968) demonstrated extensive cross-reactions, using complement fixation tests, between closely related CPVs. The CPV of B.mori (type 1) did not cross react with the CPVs of Aglais urticae, Nymphalis io, Vanessa cardui, Arctia caja, Porthetria dispar, Phalera bucephala and Euproctis chrysorrhoea, although, with the exception of A.urticae and P.bucephala these seven isolates were indistinguishable. Payne (1976) showed that the CPVs isolated from Nymphalis io and Spodoptera exempta were antigenically distinct; a mixture of the viruses could be resolved into two fractions using antisera against one isolate. Payne et al. (1978) were able to distinguish CPV isolates from Dendrolimus spectabilis, and Lymantria dispar from an isolate from B.mori, by gel immunodiffusion tests, but were unable to distinguish D.spectabilis CPV from L.dispar CPV. When these serological reactions were analysed in relation to the classification using electropherotypes (Payne & Rivers, 1976) it appeared that the virus antigens were largely type specific. Intertype cross-reactions did not occur or were rare. This conclusion was supported by Payne et al. (1983) who used ELISA; no cross-reactions were observed between CPV types 1, 2 and 5, although antigenic relationships were demonstrated between the two type 1 isolates and a type 12 isolate. The two type 5 isolates also cross-reacted. These reactions were a feature of the virus surface antigens, since antibodies prepared against artificial dsRNA poly rI:rC, which react with CPV-RNA, produced no positive reactions when used in ELISA in place of specific antiviral antibody (Payne & Churchill, 1977; Payne et al., 1983). Polyhedral protein reacted in serological tests in much the same manner as the intact virion (Payne et al., 1983).

B. Morphology

In the later stages of infection with CPVs, large proteinaceous inclusion bodies within cells can be seen clearly through the light microscope. They vary considerably in size and shape. For example, the polyhedra of CPVs infecting blackflies are between 0.3 and 1μm in diameter and have an irregular shape, whereas those from lepidopera can be as large as 5μm in diameter and possess dodecahedral symmetry (Ignoffo & Adams, 1966; Arnott et al., 1968; Bailey et al., 1975). Virus particles are occluded randomly within the crystalline protein lattice of the polyhedron and each polyhedron can hold up to 10,000 virus particles (Arnott et al., 1968).

CPV virus particles have a density of 1.44g/cm^3 although a range of 1.43-1.48g/cm^3 has been reported (Lewandowski & Traynor, 1972; Payne & Kalmakoff, 1974; Rubinstein, 1979). Hosaka and Aizawa (1964) used negative staining to study the CPV of B.mori. They reported that the virus particles were icosahedral, having a diameter of 69nm. Cunningham and Longworth (1968) calculated a mean diameter of 51nm for several CPV isolates, while Andreadis (1981) reported the diameter of the CPV of Aedes cantator to be 70nm. This variation may be attributable to either the methods of sample preparation or size standards used, rather than to structural differences amongst the isolates. Andreadis (1981) described CPV virus particles in midgut and gastric caecal cells of A.cantator as icosahedral in shape, consisting of electron-dense cores measuring 35nm in diameter surrounded by capsids having six projections. Lewandowski and Traynor (1972) had previously recognised particles having a dense core area surrounded by an outer shell membrane possessing spikes. No outer capsid analogous to those of reoviruses or rotaviruses was

described. In many respects the CPV particle is similar to a reovirus core, and it may be that the polyhedral protein of CPVs as suggested by Payne and Mertens (1983), serves as the outer capsid or shell.

The arrangement of capsomers on the surface of CPV particles has not been defined with certainty. Lewandowski and Traynor (1972) described a six-sided outline, with 20 capsomers visible along the circumference and distinct pyramidal spikes at the corners, in negatively stained CPV preparations. They contrasted the pyramidal spikes with those seen on reovirus cores which were more chimney or tube-like (Luftig et al., 1972). Although they could only readily see six spikes in their preparations, Lewandowski and Traynor (1972) predicted that 10, or more (probably 12) were present in complete virions. More elaborate studies by Yazaki and Miura (1980), using a combination of staining and shadowing methods, prompted them to describe the CPV particle as "just like a 12-starred pyrotecnic mine". On mild disruption of the particles with EDTA, genome dsRNA was observed being released through one of the spikes. This led Yazaki and Miura (1980) to suggest that genome dsRNA was transcribed by passing through the base of the spike, completed messenger RNA being released through the spike. Recent evidence suggests that the spike may consist of two sections, a "B spike" attached to the virus capsid (7-9nm high and 13-15nm wide) and a narrower "A spike" (8.9nm high and 9-11nm wide) (Payne & Mertens, 1983).

C. The Genome

During the course of studies on the nucleic acid metabolism of the silkworm, Hayashi and Kawase (1964) identified an RNA component in CPV infected larvae which had distinctive physical

properties. They analysed its base composition and demonstrated a G/C and A/U ratio of close to 1. This led them to suggest that the CPV RNA was double-stranded. Miura et al. (1968) carried out a detailed analysis of the RNA extracted from the CPV of B.mori. They confirmed the basic pairing results of Hayashi and Kawase (1964) and went on to demonstrate the double-stranded nature of the viral RNA by optical rotatory dispersion and circular dichroism, thermal denaturaion profiles, non-reactibility with formaldehyde, resistance to RNase and X-ray diffraction patterns. The possibility that the CPV RNA was single-stranded within the virion and only annealed on extraction was repudiated when Yamakawa et al. (1981) demonstrated the double-stranded nature in situ. They reported that CPV RNA displayed a much reduced yield of methylated adenine compared to single-stranded RNA on methylation with [methyl-^{3}H] dimethyl sulphate. This was because the hydrogen bonding of adenine-uracil base pairs, in double-stranded RNA, shielded the adenine methylation sites.

Miura et al. (1968) divided the CPV dsRNA into two components, having different sedimentation coefficients, and calculated that the genome was 4.7×10^{6} daltons. They also examined the dsRNA under the electron microscope and found a bimodal length distribution which corresponded to molecular weights of 1×10^{6} and 3×10^{6}. Kalmakoff et al. (1969) were able to further separate the dsRNA into 9 components on polyacrylamide gels. Using reovirus type 3 dsRNA as a standard, they calculated the CPV genome to be 12.7×10^{6} daltons. Further analysis of the genome on polyacrylamide gels by Fuji-Kawata et al. (1970) resolved 10 components, present in equimolar proportions and having a total molecular weight of 14.6×10^{6} daltons.

Furuichi and Miura (1972) determined the 3' terminal bases of the dsRNA segments of B.mori CPV. The plus strand terminated in cytosine while the minus strand terminated in uracil. Later Furuichi and Miura (1975) identified a "capping" structure at the 5' terminus of the plus strand, having a sequence of $m^7G^5'ppp^5AmpGpPyp$, the 5' end of the minus strand terminating in guanine. The minus strand, which does not bear this structure acts as the template for the polymerase-directed synthesis of mRNA (Miura, 1981).

When comparing the genome profiles of CPV isolates in polyacrylamide gels it became apparent that each one was characteristic of the isolate (Payne & Tinsley, 1974; Payne & Rivers, 1976; Harley & Rubinstein, 1978; Payne et al., 1978). This led Payne and Rivers (1976) to propose a classification system based on major differences between RNA profiles. The system used before this had classified CPVs by reference to the insect host from which the virus was isolated. The failings in this method were highlighted by the observations that distinct CPV types could replicate in the same insect species (Payne & Rivers, 1976; Harley & Rubinstein, 1978). Payne and Rivers (1976) defined separate CPV types as those having dsRNA profiles that differed from one another in the size of at least three genome segments, when resolved on 3% polyacrylamide gels using the Tris-acetate system described by Payne and Tinsley (1974). The original study of Payne and Rivers (1976) described eleven types. An additional type was described later by Payne et al. (1977). From these studies it can be shown that the genome size of CPVs range from 13.6×10^6 (type 9) to 15.6×10^6 (type 10) (Payne & Rivers, 1976). The discontinuous gel system of Laemmli (1970) was shown to provide greater resolution of the genome

segments than the Tris-acetate system used by Payne and Tinsley (1974) (Payne et al., 1983). Using both systems, Payne et al. (1983) demonstrated that, whereas Tris-acetate electrophoresis and serology distinguished virus types, the discontinuous system distinguished intra-type variations. In this way CPVs were shown to contain a high degree of genome heterogeneity. However, viruses that share the same dsRNA profile are not necessarily identical, since the base sequence of dsRNAs of the same molecular weight may differ (Walker et al., 1980). Sequence variations can only be resolved by RNA homology studies. The studies on RNA homology carried out to date appear to support the classification of Payne and Rivers (1976) (Payne and Kalmakoff, 1973; Payne et al., 1978).

D. Proteins

Lewandowski and Traynor (1972) studied the proteins of type 1 CPV virions on polyacrylamide gels after iodination. Five polypeptides were identified both when the particles had been iodinated before dissociation and when they were undissociated, but the relative levels of iodination in each case were not the same. As the position of the respective tyrosine residues on each protein was unknown it was not possible to confidently allocate positions to the polypeptides within the capsid (Table 1.4). Payne and Kalmakoff (1974) analysed the proteins of type 1 CPV on polyacrylamide gels after in vivo labelling with [^{14}C] amino acids. They resolved five polypeptides, but their molecular weights differed from the previous workers (Table 1.4). Payne and Rivers (1976) compared the capsid proteins of a series of CPV isolates and their size determinations agreed more closely with those of Lewandowski and Traynor (1972). The

calculations of Payne and Kalmakoff (1974) may have been erroneous as they did not use standard proteins of comparable size for their calibration.

The polyhedral protein was analysed by Lewandowski and Traynor (1972) and Payne and Kalmakoff (1974). The former workers suggested that the polyhedron was composed of two major polypeptides of 29.5×10^3 and 19.5×10^3 daltons respectively. They also recognised some minor polypeptides which they proposed could be virus genome products held with the virions within the polyhedra. Payne and Kalmakoff only detected two proteins unique to the polyhedra. One of these, P5, had a similar molecular weight to the larger polyhedral polypeptides described by Lewandowski and Traynor (1972). They also demonstrated that it covalently bound glucosamine. The other polyhedral polypeptide, P4, was possibly a dimer of P5. Table 1.4 summarises the polypeptides associated with B.mori CPV.

E. Gene coding assignments

Lewandowski and Traynor (1972) used the molecular weights of the virus dsRNA genome segments and the protein products to deduce the coding assignments of B.mori CPV. They proposed that polypeptides V1-V5 were coded for by genome segments I, II, III, VI and VIII, the other polypeptides of 122, 100 and 48×10^3 daltons were coded for by segments IV, V and VII, while the polyhedral protein, P5, and the small, 19.5×10^3 dalton protein were coded for by segments IX and X respectively. The same approach was used by Payne and Kalmakoff (1974) who calculated the theoretical protein size expected from each of the dsRNA species of B.mori CPV. The capsid proteins V1-V5 they assigned to segments I, II, III, V and VIII, and the polyhedral proteins

to segments VI and IX. Segment X, they calculated, should code for a protein of 19 x 10^3 daltons, similar in size to the small protein identified by Lewandowski and Traynor (1972).

These calcuations, although sometimes informative, do not take into account such factors as incomplete translation of segments, or the production of multiple products from single segments. A more precise approach developed relatively recently was the individual translation, in vitro, of the separated genome segments. After demonstrating that the in vitro translation products of viral messenger RNA and denatured dsRNA were identical, McCrae and Mertens (1983) separated the dsRNA genome segments and translated them individually, in vitro. The products were analysed on polyacrylamide gels and the coding assignments derived (Fig. 1.2C).

Discrepancies were evident between the gene products derived from in vivo studies (Lewandowski & Traynor, 1972; Payne & Kalmakoff, 1974; Payne & Rivers, 1976) and the in vitro products (McCrae & Mertens, 1983). In vivo polyhedrin is produced in large quantities [95% of viral products (Hukuhara & Hashimoto, 1966)], but in vitro it is only a minor product. McCrae and Mertens (1983) proposed that an in vivo mechanism existed for specifically increasing either the amount of the messenger RNA or the fecundity of translation.

V. PHYTOREOVIRUSES AND FIJIVIRUSES

A. Introduction

The phytoreovirus genus comprises three viruses, wound tumour virus (WTV), rice dwarf virus (RDV) and rice gall dwarf virus (RGDV) (Matthews, 1982). The genomes of these viruses

differ from those of other members of the Reoviridae (except Colorado tick fever virus) in consisting of 12 segments of dsRNA (Reddy & Black, 1973b) (Fig. 1.1). No serological relationship has been demonstrated between WTV and RDV and the recent isolate, RGDV, is distinct from both WTV and RDV (Iida et al., 1972; Francki & Boccardo, 1983). Antigenic relationships between WTV and reovirus, detected by complement fixation tests (Streissle & Maramorosch, 1963), were not confirmed (Gomatos & Tamm, 1963) and it is likely that the rabbits used for immunization in the original investigation carried reovirus antibodies. WTV causes neoplastic growth in infected plants (Black, 1970). RDV differs from the other plant reoviruses in that infection leads to stunted rather than neoplastic growth (Francki & Boccardo, 1983).

The Fijivirus genus comprises Fiji disease virus (FDV), maize rough dwarf virus (MRDV), oat sterile dwarf virus, cereal tillering disease virus, rice black-streaked dwarf virus, pangola stunt virus, Arrhenatherum blue dwarf virus and Lolium enation virus (LEV). All Fiji-viruses are transmitted by planthoppers (Delphacidae, Fulguroidae) (Francki & Boccardo, 1983). Their genome is composed of 10 segments of dsRNA (Reddy et al., 1975), and they can be separated into three serological groups which do not show any cross-reactions (Francki & Boccardo, 1983).

B. Morphology

Table 1.5 summarizes the sizes of some phytoreoviruses and Fijiviruses. The outer shell of WTV, present in the intact virus particle, conceals the internal or subviral particle capsomere structure in a manner analogous to orbiviruses (Section VI.D). WTV and RDV have a similar morphology and probably contain 32 morphological subunits (Kimura & Shikata, 1968).

Milne et al. (1973) described the Fijivirus MRDV particles as spherical with a double capsid. Each had 12 projections or spikes (designated A spikes) 11nm long located at the 5-fold symmetry axis. Chemical removal of the outer capsid and A spikes revealed the B spikes which were 8nm long. These workers proposed that the particles contained 92 capsomeres. Hatta and Francki (1977) studied the morphology of FDV and found it was very similar to that of MRDV described by Milne et al. (1973). They used negative staining, freeze-drying and shadowing techniques and demonstrated that FDV was a double-shelled icosahedral structure, consisting of a stable core surrounded by an unstable outer shell. The A spikes, visible on the intact particle were 8nm long and 14nm wide and were positioned at the 12 vertices of the icosahedron. The A spikes were attached to the B spikes located on the core, which were 9nm long and 19nm wide. Van der Lubbe et al. (1979) demonstrated that, after removal of the outer shell, dsRNA was released from virus particles in a similar manner to that described for CPVs (Section IV.B).

C. The Genome

Both WTV and RDV contain a 12 segmented dsRNA genome (Reddy & Black, 1973b). Reddy et al. (1974) resolved the genomes of WTV and RDV on polyacrylamide gels and showed that, although they were similar, they could be easily distinguished. The use of two buffer systems to resolve all the segments produced variations in the migration patterns such that the molecular weights calculated for the entire genomes in each system differed. WTV was either 16.04×10^6 or 16.67×10^6 daltons and RDV was 16.54×10^6 or 17.26×10^6 daltons, although the authors emphasized the

approximate nature of these molecular weight determinations, which also probably accounted for some of the variation.

Isolates of WTV that were maintained for long periods of time by vegetative propagation in sweet clover (from 2 to 24 years) lost their transmissibility by the vector and their infectivity for vector cell monolayers, either partially or completely. On close study it was shown that the genomes of these viruses lacked certain segments, or parts of segments (Reddy & Black, 1974). It is tempting to associate the reduced infectivity with the genetic changes (deletions), although a relationship has not been proven.

Reddy et al. (1975) resolved the dsRNAs of FDV and MRDV into ten species in polyacrylamide gels. The FDV genome size, depending on the buffer system used, was 19.26×10^6 or 19.85×10^6 daltons and the MRDV genome was 18.91×10^6 or 19.61×10^6 daltons. The genomes of the Fijiviruses are therefore significantly larger than those of the phytoreoviruses.

D. Proteins

Lewandowski and Traynor (1972) identified four polypeptides in WTV purified through sucrose and caesium chloride gradients (Table 1.6). They were not present in equal quantities and although the pattern on polyacrylamide gels remained the same, purification of the virus through caesium chloride gradients led to a loss of the outer capsid structure. They suggested that the sample preparation for electron microscopy may have accounted for this. Reddy and McLeod (1976) using a polyethylene glycol purification procedure for WTV, demonstrated seven polypeptides, comprising three major and four minor components, in the intact virion (Table 1.6). Since the two smallest polypeptides were

found in varying amounts in different preparations, the authors suggested that one may be a cleavage product of the other. When treated with chymotrypsin the intact virion lost two polypeptides, V2 and V4, although the remaining particles were infectious for insect monolayer cultures. When examined under the electron microscope these particles were shown to have lost their outer capsid structure. Reddy and McLeod (1976) concluded that the outer capsid contained two proteins, V2 and V4, the remaining five proteins being associated with the core. This structure is very similar to that of orbiviruses (Section VIF). Nuss and Peterson (1980) identified twelve virus-specific polypeptides in vector cell cultures infected with WTV. They also translated WTV mRNA *in vitro*, which resulted in the synthesis of products which comigrated with all, except one, of the WTV polypeptides expressed *in vitro*.

Boccardo and Milne (1975) studied the protein components of MRDV, and resolved six polypeptides in polyacrylamide gels (Table 1.6). When treated with either chloroform or heat the MRDV particles lost three polypeptides, V4, V5 and V6, and spiked cores were produced. Butanol treatments removed the spikes, leaving smooth cores with the loss of V3. Van der Lubbe *et al* (1979) analysed FDV core preparations recovered from sucrose density gradients and resolved three polypeptides. Two were similar in size to those associated with smooth cores (Boccardo & Milne, 1975) whereas the third was much smaller than the smallest polypeptide of MRDV and was possibly a degradation product. Boccardo and Milne (1975) found close agreement between calculated and theoretical protein sizes for MRDV.

VI. THE ORBIVIRUSES

In the preceding sections an outline of the recognised genera of the family Reoviridae has been presented. The following section, dealing with the orbiviruses, is more comprehensive and discusses, in detail, the serology, genome, genome products and replication of this large group of viruses.

A. Introduction

Orbiviruses, some of which are important pathogens of livestock, were classified historically as arboviruses, that is viruses which multiply in both vertebrates and their arthropod vectors (Casals, 1959). Most arboviruses possess a lipid envelope and are, therefore, susceptible to lipid solvents and detergents (Theiler, 1957). Orbiviruses were considered resistant and hence were regarded as an unclassified group of arboviruses. Subsequent work on the structure of orbiviruses permitted proper classification into genus and family (see below and Table 1.1).

The most studied orbiviruses are those of the bluetongue virus group or complex which presently comprises 21 serotypes; other groups contain viruses of both veterinary and medical importance and these will also be discussed to emphasize the diverse nature of the orbivirus genus. Studdert et al. (1966) showed a strain of bluetongue virus, originally isolated from an outbreak of bluetongue on the Isle of Cyprus, to be structurally similar to the reoviruses (Section II). Verwoerd (1969) demonstrated a dsRNA genome in bluetongue virus type 10 which could be separated into at least three components in sucrose gradients. He suggested bluetongue virus be classified with

reoviruses and other dsRNA viruses in a new group, the diplornaviruses (Verwoerd, 1970b). The results of morphological studies by Murphy et al. (1971) and physicochemical and serological studies by Borden et al. (1971) led to the formation of a new virus group, with bluetongue virus as the type species. Based on the appearance of the virus, as seen by electron microscopy, Borden et al. (1971) proposed the name orbivirus, derived from the latin ORBIS, meaning ring or circle. Orbiviruses are now classified as a genus within the family Reoviridae (Matthews, 1982). There are several reviews of the orbivirus genus (Gorman, 1979; Verwoerd et al., 1979; Gorman et al., 1983; Spence et al., 1984), some of which are included in more general reviews on dsRNA viruses (Wood, 1973; Joklik, 1974).

Orbiviruses can be divided into thirteen serological groups using complement fixation tests (see Table 1.7), the serotypes within each group being distinguished by neutralization tests (Gorman, 1983). This serological classification is somewhat confused by the use of names to describe the members of most serogroups and numbers to describe the members of the bluetongue and African horsesickness groups. Gorman (1979) suggested a system whereby established orbivirus serogroups are clustered under letter headings and the individual serotypes within each group are given numbers (Table 1.8). Tests relying on a single, or small number, of antigenic determinants will not identify similarities between other determinants not involved in these tests. Hence the degree of genetic similarity between isolates may be underestimated when neutralization and complement fixation tests are used as the sole guides to classification. It has been shown recently that a more precise form of classification may be achieved through the use of monoclonal antibodies and immune

precipitation (Appleton & Letchworth, 1983).

B. Orbivirus serogroups

Table 1.8 lists the serogroups and the serotypes within each group. The classification system devised by Gorman (1979) is placed alongside each recognised group, and shows the serological relationship between serogroups.

Bluetongue serogroup. Members of the bluetongue, epizootic haemorrhagic disease virus and Eubenangee serogroups (Matthews, 1982) are considered a cluster of related viruses due to their serological relationships (Gorman, 1979). There is a reluctance to place viruses, such as those of the Eubenangee serogroup, into the bluetongue virus group because they are not known to cause disease in animals. For the purpose of this review these groups will be discussed separately.

Bluetongue viruses have a wide geographical distribution. The disease is endemic in Africa and periodic epizootics have occurred in Israel, the Middle East, Cyprus, Spain and Portugal (Gorman et al., 1983). Epizootics caused by serotypes 10, 11, 13 and 17 have also been reported in the United States (Barber, 1979). Although there is no evidence of the disease in Australia, three bluetongue virus serotypes have been isolated (Gorman et al., 1983).

Using regression analysis of plaque neutralization tests, Thomas et al. (1979) showed that the degree of cross-reactivity of American bluetongue virus isolates formed a spectrum from easily demonstrable antigenic differences to almost total relatedness. Della-Porta et al. (1981) demonstrated that bluetongue virus type 20 could be used to protect animals against challenge with virulent type 4 or type 17 viruses. In these

circumstances the practical value of designating discrete serotypes is questionable (Gorman, 1979; Gorman et al., 1983).

Eubenangee serogroup. Eubenangee virus was first isolated from mosquitoes collected in Northern Queensland (Doherty et al., 1968). In complement fixation tests a relationship was shown with Pata virus, a virus isolated in the Central African Republic (Borden et al., 1971). The third serotype belonging to this group, Tilligerry, was isolated in Nelson Bay, New South Wales (Gard et al., 1973). By complement fixation tests it is more closely related to Eubenangeee than to Pata virus (Marshal et al., 1980). Pata virus cross reacts in complement fixation tests with epizootic haemorrhagic disease (New Jersey) virus, and Eubenangee virus cross reacts with bluetongue virus (Borden et al., 1971). This serogroup, together with bluetongue and epizootic haemorrhagic disease viruses, is clustered together in group B (Gorman, 1979).

Corriparta serogroup. Members of the Corriparta serogroup have been isolated in Australia (Whitehead et al., 1968; Liehne et al., 1976), Ethiopia (Berge, 1975) and Brazil (Knudson, 1980). Doherty et al. (1970) demonstrated antibodies against Corriparta virus in man, cattle, horses, marsupials and birds.

Changuinola serogroup. Viruses of this serogroup have been isolated from Phlebotomines, mosquitoes and sloths in Central and South America (Berge, 1975) and are abundant in forested areas of tropical America (Travassos da Rosa et al., 1984). Changuinola virus has also been isolated from the blood of a human patient suffering from a mild febrile illness (Gorman et al., 1983). It has been suggested that sloths and possibly other edentates are involved in the natural cycle of these viruses (Travassos da Rosa et al., 1984).

Colorado tick fever subgroup. Colorado tick fever virus resembles orbiviruses in structure and in mode and site of maturation (Murphy et al., 1968). However, Knudson (1981) recently demonstrated that Colorado tick fever virus was distinct from other orbiviruses since it had a 12 segmented dsRNA genome. In the light of this evidence, Knudson (1981) suggested a re-evaluation of its taxonomic status.

Kemerovo subgroup. Kemerovo viruses are transmitted exclusively by ticks (Matthews, 1982); birds are the predominant vertebrate host. The serogroup is divided into four antigenic complexes (Libokova & Casals, 1971; Main et al., 1976). The Kemerovo complex contains two serotypes, Kemerovo, transmitted by Ixodes persulcatus and Tribec, transmitted by I.ricinus. Both these viruses have been associated with human disease (Libokova & Casals, 1971; Málková et al., 1980).

The Great Island Complex is maintained in seabird populations throughout the world; the main vector is I.uriae. These viruses cross-react in complement fixation tests to varying degrees, and are distantly related to viruses of the Kemerovo complex (Main et al., 1976).

The two other antigenic complexes are Chenuda, members of which are transmitted by soft ticks of the genera Argas and Ornithodoros, and Wad Medani virus, which is transmitted by hard ticks of the genera Boophilus, Hyalomma, Amblyomma and Rhipicephalus. These viruses are distantly related, serologically, to the Kemerovo serogroup viruses in other antigenic complexes (Main et al., 1980).

Palyam serogroup. Viruses belonging to this group have been isolated from species of Culicoides, Aedes and cattle in India, Australia and Africa; six serotypes have been described (Lee et

al., 1974; Berge, 1975; Swanepoel & Blackburn, 1976). Possible relationships with bluetongue viruses of serotypes 1 and 20 and Ibaraki virus from the epizootic disease of deer serogroup have been demonstrated (Della-Porta et al., 1979).

Epizootic disease of deer serogroup. These viruses were originally isolated from Virginia white tailed deer in south-eastern United States (Shope et al., 1960) and subsequently from various species of deer and cattle in several states of the US and Alberta, Canada (Foster et al., 1980; Hoff & Trainer, 1978). Viruses from this serogroup have also been isolated in Nigeria, Japan and Australia (Lee et al., 1974; Inaba, 1975; Lee, 1979; Gorman et al., 1983). Huismans and Erasmus (1981) located shared antigenic determinants in the nucleocapsids of bluetongue type 10 and the New Jersey strain of epizootic haemorrhagic disease virus.

Warrego serogroup. Viruses of this serogroup have been isolated only in Australia (Doherty et al., 1970). Two serotypes, Warrego and Mitchell River, are readily distinguished by complement fixation tests, although detailed comparisons of Warrego related viruses have not yet been made (Borden et al., 1971; Doherty et al., 1973). To date 27 strains of Warrego virus have been isolated and await characterization (Gorman et al., 1983).

Wallal serogroup. Viruses of the Wallal serogroup have been isolated only in Australia from Culicoides species (Doherty et al., 1973). A distinct serotype, Mudjinbarry virus, was subsequently isolated by Doherty et al. (1978). Results of sero-surveys suggest that the most likely vertebrate hosts of these viruses are kangaroos and wallabies (Doherty et al., 1973).

African horsesickness serogroup. African horsesickness has been recognised as a clinical entity probably since the occupation of

the Cape of Good Hope by the Dutch East India Company. It was shown to be a filterable agent by M'Fadyean (1900). Through the work of Theiler and others the complexity of the African horse-sickness group was determined (Theiler, 1921). These viruses do not appear to be closely related to other viruses in the genus, but the available evidence does not preclude the possibility of genetic relationships between viruses of the horsesickness and bluetongue serological groups (Verwoerd & Huismans, 1969). African horsesickness virus is transmitted mainly by nocturnal biting insects; Mellor et al. (1975) suggested Culicoides pallidipennis as a likely vector.

Equine encephalosis serogroup. Viruses of this group are found in South Africa. They have been isolated from the blood of febrile horses and from fatal cases, and were shown to be indistinguishable by serum neutralization tests (Erasmus et al., 1970). The prototype strain, Cascara, was shown to be an orbivirus on the basis of morphology and physicochemical properties (Lecatsas et al., 1973).

Umatilla serogroup. This recently described serogroup contains two serotypes (Gorman, 1983). The prototype, Umatilla virus, was isolated from a pool of Culex pipiens in Oregon in 1969 (Berge, 1975).

C. Physical and Physicochemical Properties

Physical properties. Determination of the physical properties of orbiviruses is hampered by their instability in solutions of high ionic strength. Neutral caesium chloride gradients remove one or more of the outer capsid polypeptides (Martin & Zweerink, 1972; Verwoerd et al., 1972). This instability is probably the reason why conflicting values have been reported for the buoyant density

of bluetongue virus. Verwoerd (1969) reported a density of 1.38g/cm^3 for infective virus and a similar value was reported by Verwoerd et al. (1972). However, Martin and Zweerink (1972) gave a value of 1.36g/cm^3 for infectious virus. All these workers reported a band of higher density in caesium chloride gradients with a lower specific infectivity.

Analysis of purified bluetongue virus indicated that the virion was composed of approximately 80% protein and 20% RNA (Verwoerd, 1969).

Stability in detergent and solvents. Members of the reoviridae are generally considered to be resistant to lipid solvents, reflecting the absence of a lipid envelope (Theiler, 1957; Berge, 1975; Theiler & Downs, 1973). However, orbiviruses can show a slight, but reproducible loss of infectivity, although responses vary (Borden et al., 1971). Gorman (1978), in a study of the Australian orbivirus groups, showed that Wallal virus was ether sensitive. The action of ether and chloroform on orbiviruses is not understood. Verwoerd et al. (1979) suggested that they either act as denaturing agents or remove external stabilizers such as cell membrane with which the virus may associate. Lipids are not considered to be essential for viral infectivity since, apart from their relative resistance to lipid solvents, compared with enveloped viruses, orbiviruses are not inactivated by phospholipase (Gorman, 1978).

pH sensitivity. Orbiviruses characteristically lose their infectivity at low pH and this contrasts with the stability of reoviruses and rotaviruses. Verwoerd (1969) concluded that bluetongue virus was more stable at pH 9. The mechanism of inactivation at low pH is not understood. As mentioned earlier, high salt concentrations lead to a reduction of infectivity

(Verwoerd, 1969). The reported lability of the virus in caesium chloride at neutral and low pH (Verwoerd et al., 1972) suggests a possible dual mechanism involving low pH and high cation concentration.

D. Morphology

Table 1.9 summarizes the basic morphology of orbiviruses. Studdert et al. (1966) examined the Cyprus strain of bluetongue virus by electron microscopy; using potassium phosphotungstate negative staining they calculated an average particle diameter of 53nm. They suggested that the capsid was icosahedral, with four capsomers per edge giving a total of 92 capsomers. The morphology resembled the reoviruses. In a study of bluetongue virus in mosquito salivary glands, Bowne & Jones (1966) observed filamentous networks associated with virus particles, and some enveloped particles. Els and Verwoerd (1969) described bluetongue virus type 10 as icosahedral but suggested it contained only 32 or possibly 42 capsomers. "Pseudo-envelopes" were also described but their importance was doubted as they could be removed without any loss of infectivity; they probably originated from the cell membrane (Els & Verwoerd, 1969). Murphy et al. (1971) studied several orbiviruses by electron microscopy and concluded that the most likely capsid structure contained 32 capsomers, rather than the 42 capsomer structure suggested by Els and Verwoerd (1969). Bowne and Ritchie (1970) studied negative stained preparations of bluetongue virus and reported diameters for the virus between 60-70nm, although both larger and smaller particles were seen. They described a progressive obscuring of reovirus-like symmetry with increase in particle diameter. They also demonstrated that the membranes of enveloped particles were

derived from the cell, and were probably unmodified.

The conflicting observations on virion structure and size were clarified by Verwoerd et al. (1972). They observed that virus purification through caesium chloride gradients removed the outer capsid, resulting in sub-viral particles; this did not occur in sucrose gradients. The sub-viral particles, which have a clearly defined structure, were probably the particles described by Studdert et al. (1966) and Els and Verwoerd (1969). The results of Verwoerd et al. (1972) indicated that the bluetongue virus particle contained a nucleocapsid 54nm in diameter and a diffuse, outer capsid 68-70nm in diameter.

Els and Verwoerd (1969) described the capsomers on the surface of the nucleocapsid. They suggested that each capsomer was a hollow cylinder 10-12nm long and 8nm in diameter with an axial hole of 4nm diameter. Each capsomer consisted of smaller structural units arranged in pentagonal or hexagonal array. In a study of an equine encephalosis virus Lecatsas and Erasmus (1973) proposed a core substructure of 12 spherical subunits, each with a diameter of 14.3nm. They suggested that these subunits may contain viral nucleic acid.

E. The Genome

Composition. Several criteria have been used to establish the double-strandedness of the orbivirus genome. Green (1970) showed that inclusion bodies formed in Vero cells infected with Colorado tick fever virus stained yellow-green with acridine orange, indicating double-stranded RNA. Double-stranded RNA has a distinct, sharp melting point which is dependent on ionic strength (Bellamy et al., 1967). This was demonstrated for bluetongue virus type 10 (Verwoerd, 1969), Warrego and Mitchell

River viruses (Gorman et al., 1977), and Ibaraki virus (Suzuki, 1977). The RNAs of Colorado tick fever virus (Green, 1970), bluetongue virus type 10 (Verwoerd, 1969), Ibaraki virus (Suzuki et al., 1977) and Kemerovo virus (Rosenbergová & Slavik, 1975) were resistant to degradation by ribonuclease. In addition, a base composition reflecting the pairing of adenine and uracil as well as guanine and cytosine was demonstrated for bluetongue virus type 10 (Verwoerd, 1969; Suzuki et al., 1977).

Replication of the orbivirus genome has been studied using inhibitors and analogues. Verwoerd (1969) assessed the effects of different metabolic inhibitors on the growth of bluetongue virus type 10 in BHK-21 cells. p-Fluorodeoxyuridine, a pyrimidine analogue which specifically inhibits DNA synthesis by blocking the thymidylate synthetase reaction, failed to inhibit virus growth. Guanidine had no effect, but the virus was sensitive to actinomycin D. The latter is known to interact only with double-helical DNA, intercalating between two G-C base pairs, therefore inhibiting transcription by blocking chain elongation. The mechanism by which actinomycin D reduces orbivirus yield is not understood. Other inhibitors, such as mitomycin C, do not affect orbivirus replication (Carley & Standfast, 1969; Ozawa, 1967).

Verwoerd (1969), in a study of the structure of the orbivirus genome, extracted double-stranded RNA from purified bluetongue virus type 10 and subjected it to sucrose density gradient centrifugation. Analysis of these gradients showed four peaks with sedimentation coefficients of 4S, 10S, 12S and 14-16S; improved resolution in the gradients resolved five peaks with sedimentation coeffients of 8.5S, 10S, 12S, 14S and 15.5S (Verwoerd et al., 1970). When the RNA was analysed on poly-

acrylamide gels ten components were resolved (Verwoerd et al., 1970). Using the reovirus double-stranded RNA genome as a standard, the total molecular weight of the bluetongue virus type 10 genome was calculated as 14.9×10^6 (Verwoerd et al., 1970). Verwoerd et al. (1972) repeated these determinations, co-running the reovirus genome with the bluetongue virus genome, and showed the genome molecular weight to be 11.8×10^6. The bluetongue virus genome is considerably smaller than the reovirus genome which was determined to be 15×10^6 (Shatkin et al., 1968).

All orbivirus genomes contain 10 segments of double-stranded RNA (for example see Fig. 1.1 and Fig. 1.3) except Colorado tick fever virus which contains twelve double-stranded RNA segments, with a total molecular weight of 18×10^6 (Knudson, 1981). This is significantly larger than the genome of any other orbivirus.

The 3' terminal sequences of all ten segments of bluetongue virus type 10 and 11 have been determined (Rao et al., 1983). The complementary strands from denatured double-stranded RNA were sequenced at the 3' end. Each segment was separated by polyacrylamide gel electrophoresis into 'fast' and 'slow' migrating bands. The fast migrating strands were found to have identical 3'-terminal sequences of six nucleotides while the slower migrating strands shared eight common nucleotides at the 3'-end. It was suggested that these conserved sequences served as recognition signals for the initiation of transcription by the viral transcriptase, RNA replication, ribosome binding to the plus-sense mRNA strands and genome assembly during viral morphogenesis (Kiuchi et al., 1983; Rao et al., 1983).

Heterogeneity of orbivirus genomes. Gorman et al. (1977) compared the genome profiles of Warrego and Mitchell River viruses, two viruses that exhibited low level cross reactions in

complement fixation, but not in cross-neutralization tests (Borden et al., 1971; Doherty et al., 1973). Their profiles were quite distinct: co-electrophoresis of the genomes produced eighteen bands. The total molecular weights and melting points of the genome RNAs also differed. Gorman and Taylor (1978) compared the genome of Tilligerry virus with that of Eubenangee virus, and found most segments had different molecular weights. The genomes of Wallal and Mudjinbarry viruses differed in six segments (Gorman et al., 1978). Bluetongue virus serotypes 1, 4, 10, 15, 17 and 20 were compared: the electrophoretic separation of the genome segments was distinct for each serotype (Gorman et al., 1981). The American bluetongue virus serotypes were also shown to differ in their genome profiles (Squire et al., 1983).

The genome profiles of orbivirus isolates vary between serogroups and serotypes. The possibility of grouping by genome profile should be considered as it has been for other double-stranded RNA viruses, namely cytoplasmic polyhedrosis viruses (Payne & Rivers, 1976) and rotaviruses (Rodger & Holmes, 1979). However, such grouping assumes that co-migrating genome segments have identical, or at least, very similar sequences. Walker et al. (1980) demonstrated that corresponding genome segments of Wallal and Mudjinbarry viruses produced significantly different fingerprint patterns when digested with ribonuclease T_1. Similar results were obtained for bluetongue serotypes 10 and 11, although some genome segments had identical, or very similar fingerprint patterns (Sugiyama et al., 1981).

The levels at which variations in the genome occur must be distinguished. Point mutations can lead to dissimilar oligonucleotide fingerprint patterns with ribonuclease T_1, although the overall similarities between the genomes might be high.

Hybridization experiments allow direct comparisons to be made between isolates and useful information has been derived from such studies. Verwoerd and Huismans (1969) hybridized the messenger RNA from cells infected with bluetongue virus strains, African horsesickness virus type 3, and reovirus type 1, with denatured double-stranded genomic RNA. By comparing homologous and heterologous hybridization they demonstrated that bluetongue virus type 10 and African horsesickness virus showed 4% homology while bluetongue virus type 10 and type 4 showed 64% homology. Sugiyama et al. (1981; 1982) reported that both genetic drift, involving point mutations in the viral genome, and genetic shift resulting from RNA segment reassortment, occur in nature. Accumulations of point mutations can eventually lead to new serotypes; this has been shown to occur with bluetongue type 11 which has generated a new serotype, type 17 (Sugiyama et al., 1982).

Huismans and Howell (1973) carried out cross-hybridizations on different isolates of bluetongue virus serotype 4 (4/Theiler, 4/1314 and 4/Cyprus) and demonstrated incomplete homology. Huismans and Bremer (1981) compared bluetongue virus types 4, 10 and 20 by cross immune-precipitation and hybridization. They found that while type 4 and type 20 were immunologically closely related, their genomes were only 20-30% homologous. Serotypes 4 and 10 were less closely related by immune-precipitation, but their genomes were 71-77% homologous. These observations are consistent with evolution of viruses in isolation (Bluetongue type 4 is of African and Asian origin while type 20 isolates originate in Australia) resulting in significant differences in the gene pools (Gorman, 1983; Gorman et al., 1983).

Gene reassortment. Orbiviruses contain segmented genomes, and

new virus serotypes can be formed by reassortment of the genome segments. This has been demonstrated in the laboratory using temperature sensitive mutants (Shipman & De La Rey, 1976; Gorman et al., 1978) and by comparison of field isolates (Osburn et al., 1981). Sugiyama et al. (1981), using oligonucleotide fingerprinting, demonstrated the existence of natural reassortments between bluetongue viruses in the United States. They compared the fingerprints of the prototypes of bluetongue virus types 10 and 11 and a recent isolate of bluetongue virus type 11 (Idaho). One RNA segment (segment 3) of the Idaho strain was more closely related to that of bluetongue virus type 10 prototype than the type 11 prototype. Moreover, segments 8 of the two prototype strains were almost identical while the pattern of the Idaho strain segment 8 was readily distinguished from both these.

The ability of orbiviruses to reassort in nature, and the incidence of point mutations in the genome leading to genetic drift (Sugiyama et al., 1982), may play an important role in generating diversity within serogroups. In order to understand the significance of reassortment and genetic drift it is necessary to elucidate the coding assignments of the genome segments.

F. The Proteins

Structural Polypeptides. Verwoerd et al. (1972) demonstrated seven polypeptides in bluetongue virus type 10 capsids, four of which were major, and three minor. The instability of bluetongue virions in neutral and acidic caesium chloride gradients was utilized to demonstrate the presence of two capsid polypeptides in a diffuse layer surrounding a nucleocapsid or core (Verwoerd et al., 1972). In caesium chloride gradients these two poly-

peptides were removed, resulting in the activation of an RNA-dependent RNA polymerase. This is similar to the activation of RNA polymerase brought about by the removal of the reovirus outer capsid (Borsa & Graham, 1968; Shatkin & Sipe, 1968b; Skehel & Joklik, 1969). The loss of one of the two outer polypeptides of bluetongue virus led to loss of infectivity. Sucrose gradients did not disrupt the virus particle and produced virions with a particle count of 5 per p.f.u., whereas caesium chloride gradients at pH 7.0 produced particle counts of 4.7×10^5 per p.f.u. (Verwoerd et al., 1972).

When Martin and Zweerink (1972) purified bluetongue virus type 8 using caesium chloride gradients they obtained two particle types, both of which were infectious. However, the bluetongue virus "light" particles (BTV L) (buoyant density $1.36 g/cm^3$ in caesium chloride) had a specific infectivity 10-100 fold higher than the bluetongue virus "dense" particle (BTV D) (buoyant density $1.38 g/cm^3$ in caesium chloride). Analyses showed that BTV D contained two major and three minor protein species, while BTV L contained two additional major protein species. BTV D also exhibited a transcriptase activity whereas BTV L showed very little. They concluded that polypeptides P1, P3, P4, P6 and P7 comprised the core or nucleocapsid, and P2 and P5 formed the diffuse outer capsid. Martin et al. (1973) studied the topography of the bluetongue virus capsid proteins using ^{125}Iodine, dansyl chloride and fluorescein isothiocyanate. They found that BTV L had higher levels of labelling of P2 and P5 compared with the other polypeptides, suggesting that they were positioned on the outside of the virus particle. BTV D, which contained no P2 or P5, was labelled mainly on P1 and P3. These results supported the conclusions of Verwoerd et al. (1972) and

Martin and Zweerink (1972) on the arrangement of the polypeptides in the virion.

Bluetongue virus preparations were considered "pure" when no more than seven virus polypeptides were detected by polyacrylamide gel electrophoresis using the phosphate buffer system of Stone et al. (1974) (Martin & Zweerink, 1972; Huismans, 1979). However, Huismans and Bremer (1981), using a discontinuous stacking gel with a glycine buffer system (Laemmli, 1970), separated the minor capsid polypeptide P6 of bluetongue virus types 4 and 20 into two bands. Bremer (1976), in a comparison of African horsesickness virus with bluetongue virus, demonstrated a slightly different capsid structure, P3 replacing P2 in the outer capsid. He also detected an eighth capsid polypeptide, P4A (molecular weight 73 x 10^3). Huismans et al. (1979) purified epizootic haemorrhagic disease virus and identified eight polypeptides. The extra polypeptide P3A (molecular weight 92 x 10^3), was located on the outer capsid, possibly having a stabilizing function as the virus did not lose the outer capsid in caesium chloride gradients at pH 7.0.

The capsids of bluetongue, African horsesickness virus and epizootic haemorrhagic disease viruses were shown to be morphologically similar (Polson & Deeks, 1963; Oellerman et al., 1970; Thomas & Miller, 1971; Huismans et al., 1979); their constituent polypeptides, however, varied. The significance of some of these variations has not yet been determined. De Villiers (1974) compared the electrophoretic mobilities of the capsid polypeptides with their serological reactions and a relationship was found between the relative migration of P2 and serotype. The relationship between virus polypeptides and serological reactions is discussed in more detail below.

Intracellular viral polypeptides. Huismans (1979) identified seven structural and two non-structural polypeptides in BHK-21 cells infected with bluetongue virus type 10A. Sub-cellular fractions were prepared from infected cells, these included a cytoplasmic extract and a soluble fraction, the latter derived by centrifugation of the cytoplasmic extract. Most viral polypeptides were found in both fractions, the exception being a non-structural polypeptide, P5A, which was only detected in cytoplasmic extracts. P5A had a molecular weight of 54×10^3 and was synthesized in large amounts. The second non-structural polypeptide, P6A, had a molecular weight of 40×10^3 and co-migrated on gels with actin, a major cellular protein. Immunoprecipitation experiments showed P6A to be virus specified, as it was only precipitated from infected cell extracts by serum raised against partially purified bluetongue virus, and not by serum raised against uninfected cell extracts. In some cases P6A was also precipitated from infected cell extracts by serum raised against purified bluetongue virus. Huismans (1979) was undecided as to whether P6A was a true non-structural polypeptide or a modified form of one of the capsid polypeptides.

Gorman et al. (1981) detected ten virus specified polypeptides in BHK-21 cells infected with bluetongue virus type 20, the extra polypeptide being significantly smaller than P7, the lightest previously described (Huismans, 1979). Sangar and Mertens (1983), using an in vitro rabbit reticulocyte lysate system and the genome RNA of bluetongue virus type 1, demonstrated at least thirteen translation products which co-migrated with the polypeptides produced in infected cells. They suggested that the smaller polypeptides could be the result of either incomplete translation, possibly caused by premature

termination, or initiation at more than one site. Thirteen virus specified polypeptides have been identified in Vero cells infected with a Kemerovo group orbivirus (Fig. 1.4) [Spence et al., 1985b)]. Recently Gorman et al. (1984) have identified 11 polypeptides in BHK-21 cells infected with Nugget virus, a Kemerovo group orbivirus.

Processing of polypeptides was not demonstrated with bluetongue virus type 10A (Huismans, 1979) or bluetongue virus type 20 (Gorman et al., 1981) by pulse chase experiments, but the non-structural polypeptide, P6A, was shown to be phosphorylated (Huismans & Basson, 1983). It was the only ^{32}P-labelled polypeptide precipitated from infected cell cultures by antibodies that precipitated all the structural and non-structural proteins of bluetongue virus. P6A bound to mRNA in vitro and in vivo. This ability to bind single-stranded RNA was also shown by a non-structural polypeptide of reovirus (σNS) but this polypeptide does not appear to contain any phosphate groups (Huismans & Joklik, 1976). The function of this binding could be in morphogenesis, forming primary complexes with single stranded RNA, or in translation of mRNA. Gomatos et al. (1981) detected polymerase activity in a multimer of σNS. If P6A acts in the same way it may function during infection as a condensing agent, bringing together the ten single-stranded RNA templates in preparation for double-stranded RNA synthesis.

Huismans (1979) showed that the non-structural polypeptide P5A was synthesized in larger relative amounts than any other virus polypeptide. He also reported that it was rapidly transferred from the soluble cell fraction, being converted into a high molecular weight complex. Huismans and Els (1979) analysed tubular structures formed in cells during the

replication of bluetongue virus, African horsesickness virus and epizootic haemorrhagic disease virus. They identified the units of these structures as comprising a virus specific polypeptide, with a molecular weight 54 x 10^3 (the African horse-sickness virus tubule polypeptide was slightly larger), suggesting it was P5A. Microtubules were found in uninfected cells but they were smaller than virus specified tubules (29nm and 68nm, respectively) and differed in morphology. They were also sensitive to colchicine, whereas the virus tubules were not. However, the molecular weight of the microtubule component polypeptide, tubulin, was similar to that of the virus specified tubules (Snyder & McIntosh, 1976). The tubules were composed of a non-structural polypeptide, therefore they could not have been the product of aberrant assembly of viral capsids, as suggested by Holmes et al. (1975) for rotavirus associated tubules, but their function remains unclear. The tubule polypeptide was among the first virus specific polypeptides to appear in bluetongue virus infected cells (Huismans, 1979), closely associated with "virus factories" (Huismans & Els, 1979), but there was no evidence to suggest that they played a role in virus replication.

Serological interactions. Antigenic variation reflects differences in the capsid polypeptides, and the outer two, P2 and P5 would presumably be the most important. De Villiers (1974), using polyacrylamide gel electrophoresis, compared the molecular sizes of capsid polypeptides of different serotypes of bluetongue virus with those of serotype 10A. Eight of ten serotypes showed changes in the molecular weight of P2. Significantly, when P2 did not vary, P5 did. These results suggested that P2 and P5 were the polypeptides involved in serotype reactions (neutralization).

Huismans and Bremer (1981) infected cells with different serotypes of bluetongue virus and cross-immune precipitated the polypeptides from soluble cell fractions using immune guinea pig sera. Homologous sera precipitated P2, P3, P7 and in some cases, P6A. In cases involving bluetongue virus type 4, 10 and 20, P2 was precipitated by heterologous serum. Cross-immune precipitation of P2 did not indicate cross-neutralization in all cases. In immunoprecipitation tests polypeptide P2 of bluetongue virus types 4 and 20 cross-reacted. Furthermore P2 of bluetongue virus type 10 was precipitated by serum from types 4 and 20. Type 17 serum was also capable of precipitating P2 from types 4 and 20. It is possible that configurational homology at several antigenic sites is required for cross-neutralization, whereas cross-immune precipitation may require only one similar determinant (Huismans & Bremer, 1981; Spence et al., manuscript in preparation).

Huismans and Erasmus (1981) compared different host systems when raising antisera to bluetongue virus. Rabbit sera precipitated P5, but no evidence was produced to implicate P5 in serotype specificity. When sheep were inoculated with bluetongue virus isolates, antibodies to P2 and P7 developed rapidly. The levels of antibody to P7 declined faster than those to P2. A similar pattern of decline was seen with complement fixation and neutralization antibodies. Ascitic fluid from mice, inoculated with bluetongue virus, gave high complement fixation titres but low neutralization titres and only precipitated P7.

The outer capsid polypeptides of bluetongue virus were solubilized by treatment with magnesium chloride at varying pHs and used to raise antibodies in sheep. Neutralization was only detected in sera that contained antibodies to P2 as demonstrated

by immune precipitation. When a combination of P2 and P5 were used as the immunogen the neutralizing antibody titres were slightly raised (Huismans et al., 1983). Appleton and Letchworth (1983) raised monoclonal antibodies to bluetongue virus type 17. One antibody clone caused neutralization and haemagglutination inhibition, and precipitated P2 and P3, while another, non-neutralizing antibody clone precipitated P2 and P8. The authors suggested that P2 comprised two co-migrating polypeptides, only one of which was involved in neutralisation reactions.

These results indicate that the serotype specific antigen of bluetongue virus is located on P2, and the serogroup specific antigen is located on P7. Table 1.10 summarises the serological reactions of the capsid polypeptides of bluetongue viruses.

G. Coding assignments

The coding assignments of the genome segments of bluetongue virus type 10 were derived from molecular weight data by Verwoerd et al. (1972). They calculated the sizes of the polypeptides to be expected from translation of each genome segment and compared those with the size of the polypeptide found. Sangar and Mertens (1983) and Mertens et al. (1984) translated separately, in vitro, each genome segment of bluetongue virus type 1 (Fig. 1.2). Their data did not agree with Verwoerd et al. (1972). There was an incomplete relationship between molecular weight of protein and RNA, since segment 7 gave rise to a lower molecular weight protein than expected, and segments 6 and 10 each gave rise to two proteins (Sangar & Mertens, 1983; Mertens et al., 1984). Partial assignments for bluetongue virus type 4 have also been determined and the coding assignments for segments 2 and 3 and segments 5 and 6 were the reverse for those for bluetongue virus

type 1 (Mertens et al., 1984). Grubman et al. (1983) determined the coding assignments for bluetongue virus type 17, and also designated the genome segments coding for the products involved in neutralization and intergroup reactions. Segment 2 coded for the protein involved in neutralization reactions, segment 8 the protein involved in intergroup reactions (shared between 20 bluetongue virus serotypes and three other orbiviruses) and segment 7 the protein involved in intragroup reactions (complement fixation tests). These results demonstrate that, although there may be considerable similarity in coding assignments between different bluetongue virus types, the results cannot be extrapolated from one virus type to another.

H. Replication

Electron microscopic studies. Figure 1.5 represents a schematic interpretation of the major events in orbivirus replication. Replication of orbiviruses occurs in the cytoplasm, although one report described African horsesickness virus particles within the nucleolus of infected cells (Ozawa et al., 1965). Lecatsas and Erasmus (1967) reported that replication of African horsesickness virus occurred in the perinuclear region of the cytoplasm and there was no nuclear involvement. Lecatsas (1968) studied the formation of bluetongue virus in BHK-21 cells. Adsorption and penetration into the cell occurred within 5-10 minutes of infection, the virus particles entering within phagocytic vesicles. After 30 minutes dense nucleoid-containing bodies were visible which gave rise to small, dense lysosome-like inclusion bodies in the cytoplasm at about 2 hours post infection. At this time the rough endoplasmic reticulum formed vesicles lined with ribosomes. Tubules, often seen in cells infected by members of

the Reoviridae, were visible at about 8 hours post infection and these were followed 8 hours later by fine filaments, either in bundles or dispersed within the cytoplasm. Mature progeny virions were seen from about 8 hours, but most were visible at 12-20 hours post infection, usually clustered near the nucleus. There was little evidence of virus release from infected cells by budding. Murphy et al. (1971) suggested that the predominant mode of virus release from orbivirus infected cells was by cell lysis. Lecatsas et al. (1969) found Corriparta virus morphologically similar to bluetongue virus but they detected no virus associated tubules in infected cells. Lecatsas et al. (1973) showed the cytopathology of equine encephalosis virus, bluetongue and African horsesickness viruses to be similar.

The tubules apparent in most orbivirus infections are distinct from cellular microtubules (Section F) although they have features in common and are constructed from proteins of a similar size (Huismans & Els, 1979). Their function in orbivirus replication is unknown. The fine filaments in infected cells appear to be involved in the production of the viral core (Lecatsas et al., 1969; Lecatsas et al., 1973). Lecatsas (1968) suggested that they could be involved in genomic nucleic acid packaging into the core after capsid synthesis.

Virus release from infected cells probably occurs through lysis. Lewanczuk and Yamamoto (1982) observed the replication of epizootic haemorrhagic disease virus in BHK-21 cells and noted that virus particles were not released from the cell individually but egressed in aggregates. Infected cells did not disintegrate in a generalized manner, but instead, localized cell membrane breakdown occurred in proximity to the virus aggregates.

The growth cycle. Growth cycles for bluetongue virus type 10 (Howell et al., 1967) and epizootic haemorrhagic disease of deer virus have been determined (Lewanczuk & Yamamoto, 1982). Infectious virus appeared in the medium 5-6 hours after infection, the levels rising rapidly to a maximum 12-13 hours after infection, where they remained relatively constant for the subsequent 24 hours. Cell-associated virus also followed this pattern although the relative levels differed. When material obtained from the cell and supernatant fractions was sonicated, 80% of the infectivity was found in the cell-free supernatant, while in unsonicated preparations 97% of infectivity was associated with the cellular fractions.

Synthesis of RNA. Synthesis of single-stranded RNA began almost immediately after infection, even when inhibitors of protein synthesis were present (Verwoerd, 1970a). This suggested that in the early stages of infection the parental virus genome was used for transcription, and no protein synthesis was required. From 5 hours after infection the rate of single-stranded RNA synthesis increased, probably due to the transcription of progeny virus templates (Huismans, 1970). Huismans and Verwoerd (1973) showed that the single-stranded RNA in cells infected with bluetongue virus type 10 were complementary to one of the two strands of the ten genome segments. The strands were not present in equimolar amounts, but their proportions remained constant during the first eleven hours of infection. By comparing the control of transcription in vivo and in vitro and showing them to be the same, they demonstrated that control of transcription was an integral part of the activated virus particle or core. The genome segments appeared to be transcribed at the same rate, with the exception of segments five and ten, which were transcribed at

twice and half the expected rates, respectively. Bluetongue virus cores inhibited transcription; this may play a role in the regulation of virus RNA synthesis (Van Dijk & Huismans, 1980). The attachment to ribosomes and subsequent translation of the RNA did not appear to be regulated, except for segment 1 (Huismans, 1979; Huismans & Verwoerd, 1973). This data contrasted to that for reoviruses reported by Zweerink and Joklik (1970) who demonstrated that transcription of all genome segments was inversely proportional to their molecular weights in vitro, hence transcription was unregulated. In vivo the rates of transcription were altered by varying amounts, suggesting a cell-associated mode of control.

Synthesis of proteins. Bluetongue virus type 10 infection of L-cells caused inhibition of cellular protein synthesis shortly after infection, the degree of inhibition relating to the multiplicity of infection (Huismans, 1971). Studies on Mill Door/79 virus, an orbivirus belonging to the Kemerovo serological group, showed similar results [Spence et al., 1985b). In contrast, a reovirus failed to inhibit cellular protein synthesis even at very high multiplicities of infection (Huismans, 1971). Huismans (1971) demonstrated that inhibition of cellular protein synthesis was directly related to polyribosome degradation, possibly caused by the protein coat of the infecting virions. Inhibition was not attributable to macromolecular synthesis, since cycloheximide, which prevents ribosome breakdown due to normal translation, failed to inhibit polyribosome degradation (Huismans, 1971).

Synthesis of bluetongue type 10A proteins in BHK-21 cells was studied by Huismans (1979). The first virus specific proteins were detected 2-4 hours post-infection, the rate of

synthesis increasing to 11 hours post-infection, where it remained constant for a further 15 hours. The non-structural polypeptide P5A was synthesized at a higher rate than the other viral polypeptides.

VII. AIMS OF THESIS

Studies on the genome and intracellular proteins of orbiviruses have been mostly confined to members of the bluetongue and epizootic haemorrhagic disease serogroups and little is known of the groups such as the Kemerovo serogroup. Viruses belonging to the Kemerovo serogroup have been isolated from ticks at several sites in the UK (Main *et al.*, 1976; Nuttall *et al.*, 1981). The aim of this thesis was to isolate orbiviruses from ticks collected in seabird colonies on the Isle of May in Scotland, to characterize them serologically and then to examine the viruses at a molecular level. The latter studies aimed to identify and enumerate virus-specified polypeptides in cell culture and determine which were structural, study the processing or modification of the virus-specified polypeptides, determine the nature of the viral dsRNA, then translate it in an *in vitro* protein synthesizing system to determine the virus genome coding assignments and to compare the virus isolates from the Isle of May by their genomes and protein products.

Table 1.1

Members of the family Reoviridae

Genus	Type species	Host range
Orthoreovirus	Mammalian Reovirus	Vertebrates
Orbivirus	Bluetongue Virus	Vertebrates/ Insects
Rotavirus	Human Rotavirus	Mammals
Cypovirus	Cytoplasmic Polyhedrosis Virus of Silkworm	Insects
Phytoreovirus	Wound Tumour Virus	Plants/Insects
Fijivirus	Fijivirus	Plants/Insects

Adapted from Joklik, W.K. The Members of the Family Reoviridae, in: The Reoviridae (W.K. Joklik, ed), Plenum Press, London, 1983.

Table 1.2

Reovirus Proteins

Protein	Molecular weight x 10^{-3} daltons	Location	Function
Structural			
λ1	155	Core	-‡
λ2	140	Core	Spike protein
λ3	135	Core	Transcription/capping
λ2c*	120	-	-
μ1	80	Core	Precursor of μ1c/Transcription/capping
μ1c	72	Outer shell	Susceptibility to digestion
μ2	70	Core	Transcription/capping
σ1	42	Outer shell	Cell attachment
σ2	38	Core	-
σ3	34	Outer shell	RNA binding
"component viii"+	5-10	Outer shell	Cleavage product of μ1
Non-structural			
μNS	75		-
μNSC*	70		-
σNS	36		RNA polymerase

Expanded from Joklik, W.K. The Reovirus Particle in: The Reoviridae (W.K. Joklik, ed), Plenum Press, London, 1983.

* From Lee et al. (1981)

+ From Smith et al. (1969)

▲ Location or function unknown

‡ unassigned.

Table 1.3

Molecular weight estimates and nomenclature of rotavirus polypeptides found in infected cells

SA 11[a]	SA 11[b]	UK bovine[c]	Capsid Position	Thouless[d]
p113	VP1	VP1	Inner	I_1
p96	VP2	VP2	Inner	I_2
p91	NCVP1	VP3	Inner/non-structural	I_{3a}
p84	VP3	VP4	Inner/outer	I_{3b}
p57	NCVP2	VP5	Outer/non-structural	(O_1)
p42	VP6	VP6	Inner	(I_4)
gp34	VP7	VP7---┐e	Outer	(O_2)
		VP7c←┘	Outer	
p33	NCVP3	VP8	Non-structural	NS_1
p31	NCVP4	VP9	Non-structural	NS_2
p26		→VP10---┐	Outer	O_3
gp25	NCVP5	VP10c←	Outer/non-structural	O_4
	NCVP6	VP11→VP11c	Non-structural	
p21	pNCVP5	└-VP12	Non-stuctural	NS_3
	pNCVP6			

(a) Simian rotavirus SA 11, Dyall-Smith & Holmes (1981b).

(b) Arias et al. (1982).

(c) McCrae & Faulkner-Valle (1981).

(d) Thouless (1979). General terminology I = inner capsid, O = outer capsid, NS = non-structural.

(e) Arrows indicate direction of protein processing.

Table 1.4

Capsid and Polyhedral Polypeptides of Cytoplasmic Polyhedrosis

Virus type 1 (Bombyx mori)

Genome segment	Polypeptides molecular weight ($\times 10^{-3}$)				Position[a,b,c]
		a	b	c	
I	(VI[a,b,c])	151	126	144	Capsid (outer)
II	(V2[a,b,c])	142	121	138	Capsid (inner)
III	(V3[a,b,c])	130	109	125	Capsid (outer)
IV		122	-[d]	-	Non-structural/polyhedral matrix
V		100	-	-	Non-structural/polyhedral matrix
VI	(V4[a,b,c])	67	75	70	Capsid (inner)
VII	(P4[b,c])		58	57	Polyhedrin dimer?
VIII		48			Non-structural/polyhedral matrix
IX	(V5[a,b,c])	33	33	31	Capsid (inner)
X	(P5[b,c])	29	27	27	Polyhedrin
XI		19			Polyhedrin degeneration?

(a) Lewandowski & Traynor (1972)

(b) Payne & Kalmakoff (1974)

(c) Payne & Rivers (1976)

(d) Protein of this size not recognized

Table 1.5

Sizes of Phytoreoviruses and Fijiviruses from electron microscopic studies

Particle	Diameter (nm)			
	WTV[1]	RDV[2]	FDV[3]	MRDV[4]
Intact	73	70	71	63–67
Sub-viral	59–63	–[5]	54	50–57

[1] Wound tumour virus - Reddy and MacLeod (1976).

[2] Rice dwarf virus - Kimura and Shikata (1968).

[3] Fiji disease virus - Hatta and Francki (1977).

[4] Maize rough dwarf virus - Milne et al. (1973).

[5] No value reported.

Table 1.6

Structural polypeptides of phytoreoviruses and Fijiviruses

Polypeptides	Molecular weights ($x10^{-3}$)			
	WTV (a)	WTV (b)	MRDVc	FDVd
V1	156	160	139	145-150
V2		131	126	125-129
V3	122	118	123	
V4		96	111	
V5	63	58	97	
V6	44	36	64	36-39
V7		35		

(a) Wound tumour virus - Lewandowski & Traynor (1972)

(b) Wound tumour virus - Reddy & MacLeod (1976)

(c) Maize rough dwarf virus - Boccardo & Milne (1975)

(d) Fiji disease virus - Van der Lubbe et al. (1979)

Table 1.7

Serological Groups of Orbiviruses

Serogroup	Number of serotypes
African horsesickness	9
Bluetongue	21
Changuinola	12
Colorado tick fever	2
Corriparta	4
Epizootic haemorrhagic disease	7
Equine encephalosis	5
Eubenangee	3
Kemerovo	21
Palyam	6
Umatilla	2
Warrego	2
Wallal	3

Orbiviruses as yet ungrouped:

Ieri	TRVL 8762
Ife	Ib Ar 57245
Japanaut	Mk 6357
Lebombo	Ar 316
Orungo	Ug Mp 359
Paroo River	GG 668

From Gorman, B.M. (1983). Intervirology 20, 169-180.

Table 1.8

Orbivirus serological groups and serotypes[a]

Subgroup/subtype	Vector	Gorman Classification[b] Group	Type
Bluetongue subgroup		B	1-20
Bluetongue virus (21 serotypes	Culicoides		
Eubenangee subgroup		B	
Eubenangee	Mosquitoes		29
Pata	"		30
Tilligerry (NB 7080)	Not known		31
Corriparta subgroup		F	
Acado	Mosquitoes		2
Bambari	Not known		3
Corriparta	Mosquitoes		1
Jacareacanga	Not known		
Changuinola subgroup		E	
Be Ar 35646	Phlebotomines		
Be Ar 41067	"		
Be Ar 54342	"		
Changuinola	"		1
Irituia	Not known		2
Colorado tick fever subgroup		C	
Colorado tick fever	Ticks		1
Eyach	"		2
Kemerovo subgroup		G	
Baku	Ticks		2
Bauline	"		3
Cape Wrath	"		4
Chenuda	"		5
Fin isolate	"		
Great Island	"		6
Huacho	"		7
Kemerovo	"		1
Kenai	"		
Lipovnik	"		8
Mono Lake	"		9
Mykines	"		
Nugget	"		10
Okhotskiy	"		11

Subgroup/subtype	Vector	Gorman Classification[b] Group	Type
Kemerovo subgroup (cont)			
Poovoot	Ticks		
Seletar	"		12
Sixgun City	"		13
Tindholmur	"		
Tribec	"		14
Wad Medani	"		16
Yaquina Head	"		15
Palyam subgroup		D	
Abadina	Culicoides		5
D'Aguilar	"		4
Kasba	Mosquitoes		2
Nyabira	Not known		6
Palyam	Mosquitoes		1
Vellore	"		3
Epizootic disease of deer subgroup		B	21-28
EHD, New Jersey	Not known		
EHD, Can Alberta	Not known		
Ib Ar 22619	Not known		
Ib Ar 33853	Not known		
Ibaraki	Not known		32
Warrego subgroup		H	
Mitchell River (MRM 10434)	Culicoides		2
Warrego (CH 9935)	Not knonw		1
Wallal subgroup		I	
Mudginbarry	Not known		2
Wallal (CH 10248)	Culicoides		1
African horsesickness subgroup		A	
African horsesickness virus (9 serotypes)	Culicoides		1-9
Equine encephalosis subgroup		J	
EEV (5 serotypes)	Not known		1-5
Umatilla subgroup		K	
(2 serotypes)	Culex pipiens		1-2

Subgroup/subtype	Vector	Gorman Classification[b] Group	Type
Ungrouped			
Ife	Not known		
Japanant	Culicoides		
Lebombo	Aedes circumluteolus		
Llano Seco	Not known		
Orunga	Not known		
Paroo River	Not known		
T-50616 (Skunk isolate)	Not known		

[a] Matthews, R.E.F. (1982). Intervirology 17, 82-84.

[b] Gorman, B.M. (1979). Journal of General Virology 44, 1-15.

Table 1.9

Size and Structure of Orbiviruses

Virus	Diameter (nm)		No. of capsomers	References
	Complete Particle	Core		
Bluetongue	68-70	54	32-42	Els & Verwoerd (1969); Verwoerd et al. (1972)
Corriparta	61[a]	-	92	Carley & Standfast, (1969)
Eubenangee	62[a]	-	92	Schnagl et al. (1969)
African horsesickness virus	70-80	-	92	Polson & Deeks, 1963)
	55[a]	-	32	Oellermann et al. (1970)
Ibaraki virus	55[a]	-	32	Ito et al. (1973)

(a) These values may be for the core.

Table 1.10

Serological Reactions of the Capsid Polypeptides of Bluetongue Viruses

Polypeptide	Serological tests
P1	-[a]
P2 (external)	Neutralization, Immune precipitation
P3	Immune precipitation
P4	-
P5 (external)	Immune precipitation (rabbit)
P6	-
P7	Complement fixation, Immune precipitation

[a] No reaction.

From Spence et al. (1984). Archives of Virology (in press).

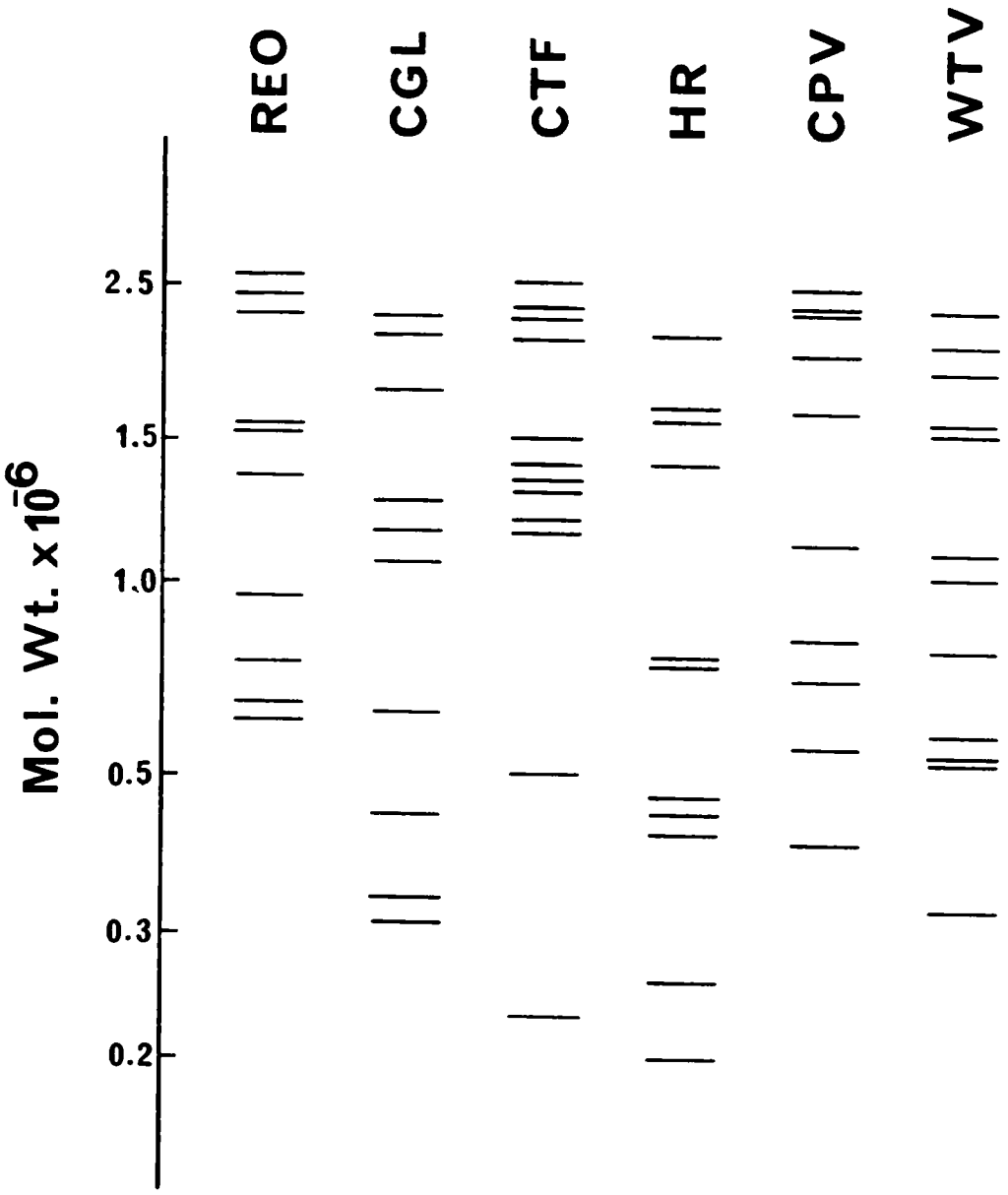
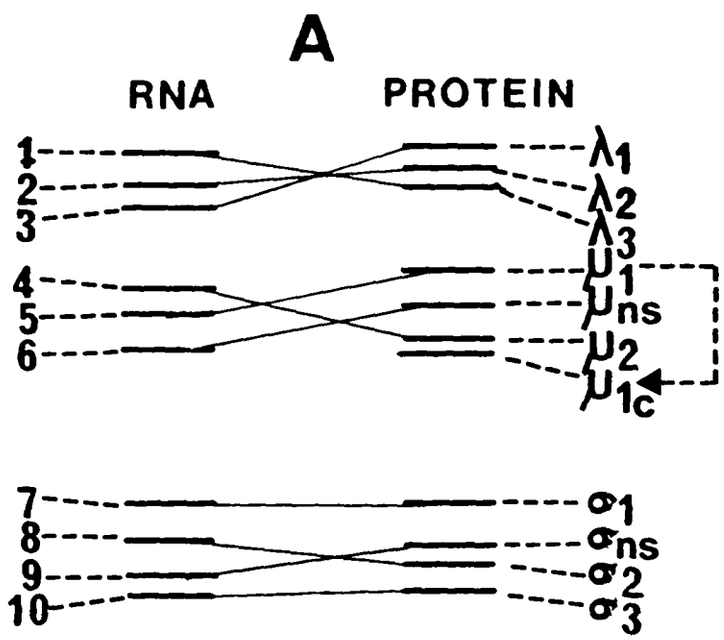
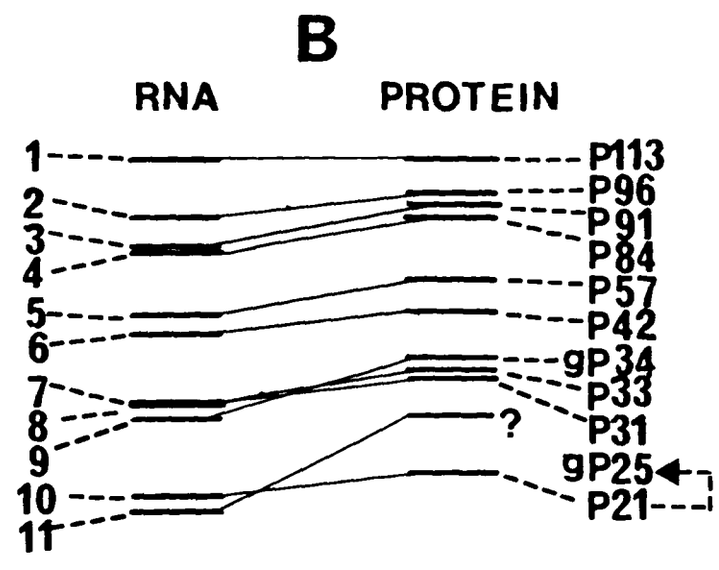
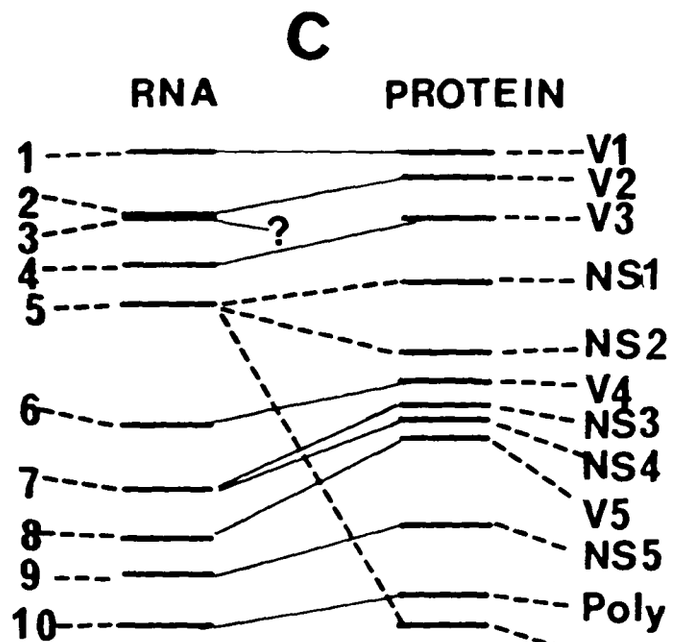


Figure 1.1. Relative mobilities of the genome segments of members of the Reoviridae. The genera represented are Reovirus (Reovirus type 3 Dearing strain - REO), Orbivirus (Changuinola strain BT 766 - CGL, and Colorado tick fever Florio strain - CTF), Rotavirus (human rotavirus - HR), Cytoplasmic polyhedrosis virus (Bombyx mori cytoplasmic polyhedrosis virus - CPV), and Phytoreovirus (wound tumour virus - WTV).

Figure 1.2. Genome coding assignments of members of the Reoviridae. A, Reovirus type 3 Dearing strain (McCrae & Joklik, 1978); B, Simian rotavirus SA 11 (Dyall-Smith & Holmes, 1981b); C, Cytoplasmic polyhedrosis virus of Bombyx mori (McCrae & Mertens, 1983); D, Bluetongue virus type 1 (Mertens et al., 1984).


A
RNA
PROTEIN
1
2
3
4
5
6
7
8
9
10
λ1
λ2
λ3
μ1
μns
μ2
μ1c
σ1
σns
σ2
σ3


B
RNA
PROTEIN
1
2
3
4
5
6
7
8
9
10
11
P113
P96
P91
P84
P57
P42
gP34
P33
P31
?
gP25
P21


C
RNA
PROTEIN
1
2
3
4
5
6
7
8
9
10
?
V1
V2
V3
NS1
NS2
V4
NS3
NS4
V5
NS5
Poly
NS6

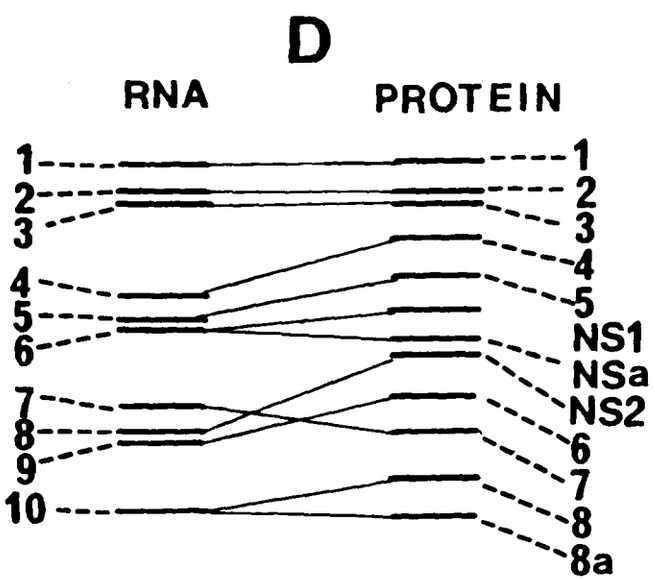

D
RNA
PROTEIN
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
NS1
NSa
NS2
6
7
8
8a



Figure 1.3. Genome of North Clett/81 virus. The dsRNA was separated on a 10% PAG, stained with ethidium bromide and visualized under UV light.

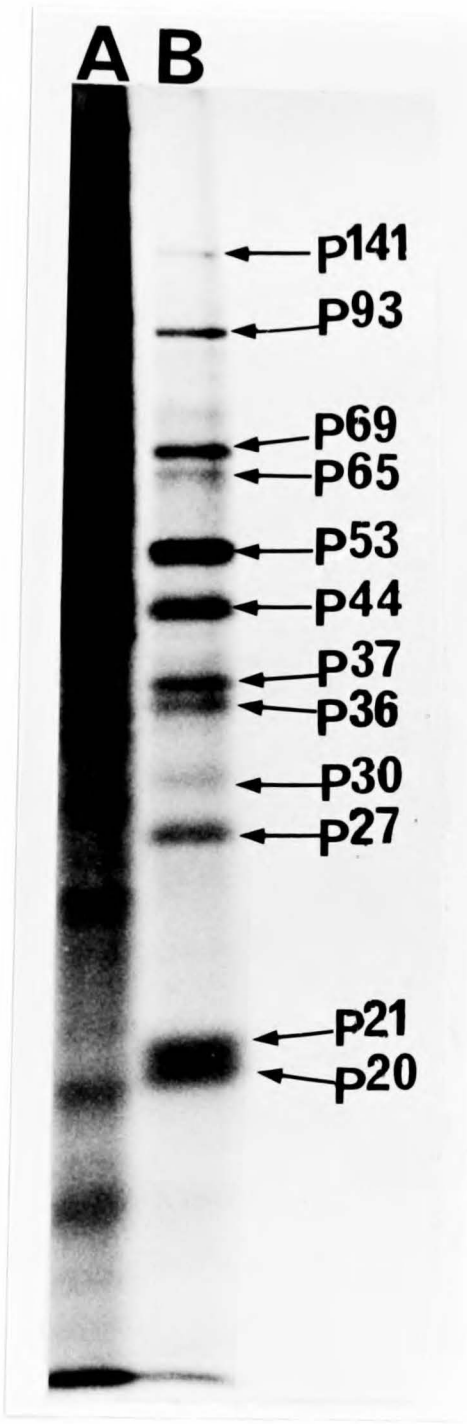


Figure 1.4. Proteins induced in Vero cells by Mill Door/79 virus. Separation was carried out on a 12.5% PAG, the proteins labelled with [^{35}S] methionine and visualized by autoradiography.

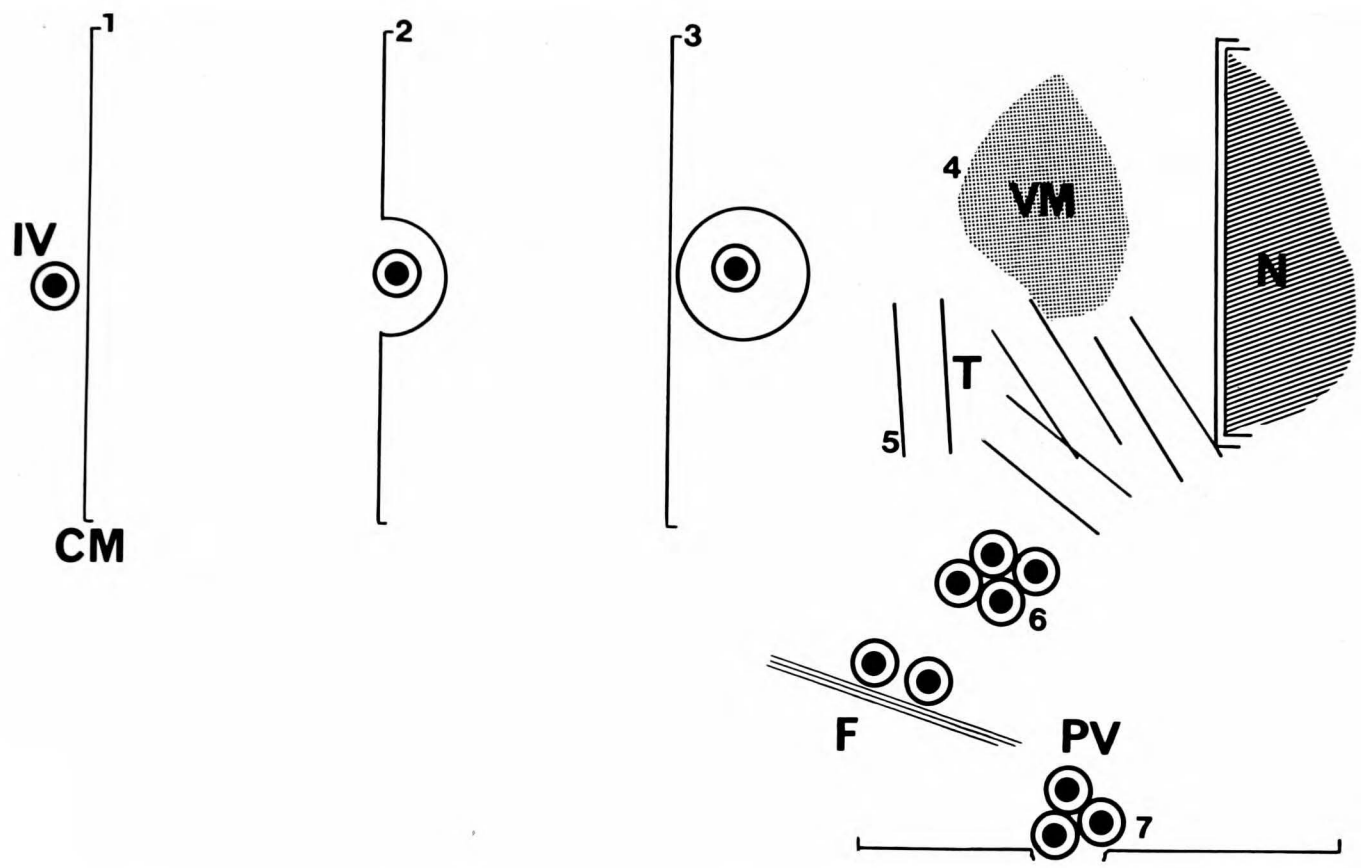


Figure 1.5. Schematic diagram representing the major events in orbivirus replication: (1) virion adsorbs onto cell membrane; (2) penetration at 5-10 minutes p.i.; (3) particle enclosed in phagocytic vesicle which gives rise to an inclusion body in the cytoplasm at 2 hours p.i.; (4) virus matrix becomes visible in the perinuclear region of the cytoplasm; (5) the formation of tubules at about 8 hours p.i.; (6) mature, progeny virus visible from about 12-20 hours p.i.; (7) virus released from cells by lysis.

IV - infecting virion; CM - cell membrane; VM - viral matrix; T - tubules; F - filaments; N - nucleus; PV - progeny virus.

CHAPTER 2

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I. MATERIALS

A. Reagents

Most reagents were supplied by BDH Chemicals Ltd, Poole, England. Media, penicillin, streptomycin, kanamycin, tryptose phosphate broth, fetal calf serum, L-glutamine, trypsin, trypsin-EDTA, bovine albumin and HEPES were obtained from Gibco Biocult, Paisley, Scotland. Tissue culture flasks were obtained from Nunc Plastics, Roskilde, Denmark. Trypan blue vital stain and Linbro plates were supplied by Flow Laboratories, Irvine, Scotland. All radioisotopes (including [^{14}C] labelled molecular weight markers) and rabbit reticulocyte lysates were provided by Amersham International plc, Amersham, England. Actinomycin D, L-methionine, α-chymotrypsin [N-tosyl-L-lysine chloromethyl ketone (TLCK) treated], trypsin [N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated], Tris (hydroxymethyl)-aminomethane, lithium chloride, crystal violet, Freund's adjuvant, Sigmacote, deoxyribonuclease 1, Staphylococcus aureus protein A and 2-deoxy-D-glucose were purchased from Sigma London Chemical Company Ltd, Poole, England. Sigma Ltd also supplied the protease inhibitors: iodoacetamide, phenylmethylsulphonylfluoride, TLCK, TPCK and zinc acetate. Staphylococcus aureus V8 protease and rabbit anti-mouse IgG were obtained from Miles Laboratories Ltd, Stoke Poges, England. Phosphate buffered saline and complement fixation test buffer tablets were supplied by Oxoid Ltd, England. Methyl mercury hydroxide was obtained from Lancaster Synthesis Ltd, Lancaster, England. EN^{3}Hance autoradiography enhancer was obtained from New England Nuclear, Boston, USA. Proteinase K was supplied by Boehringer Mannheim GmbH, West Germany, and tunicamycin was supplied by Calbiochem-Behring, La Jolla, USA.

B. Media

Eagle's minimum essential medium (EMEM). Batches of 9dm^3 were prepared by dissolving 95g of powdered medium and 15g of $NaHCO_3$ in deionized water, followed by addition of 100ml of penicillin-streptomycin solution (5,000 units penicillin and 5,000mcg streptomycin/ml). The pH was adjusted to 7.2 and the medium was filter sterilized and dispensed in 450ml aliquots and stored at 4°C until required. For growth media, fetal calf serum (FCS) and L-glutamine were added to the stock solution to final concentrations of 10% (v/v) and 1% (v/v) respectively. For maintenance media FCS was reduced to a final concentration of 1 to 3%. Labelling media were prepared as listed in Table 2.1.

Glasgow's modification of minimum essential medium (GMEM). Batches of 9dm^3 were prepared from 142g powdered medium, 47.6g N-2-hydroxyethyl piperazine N'2-ethanesulphonic acid (HEPES) and 8.5g $NaHCO_3$, as above. Growth and maintenance media were prepared by the addition of FCS and L-glutamine, as above. Labelling media was prepared as listed in Table 2.1.

Leibovitz (L-15) medium. Batches of 9dm^3 were prepared from 140g powdered medium and 29.5g tryptose phosphate broth, as above. Growth and maintenance media were prepared by the addition of FCS and L-glutamine, as above. Overlay medium was prepared by adding carboxymethyl cellulose (CMC) to L-15 stock media to a final concentration of 0.75% (v/v).

C. Cells

All cells used were mycoplasma free.

African green monkey (Vero) cells. These cells were obtained from Flow Laboratories Ltd (Irvine, Scotland). They were grown in EMEM at 37°C.

Baby hamster kidney (BHK-21) cells. This cell line was initiated by Macpherson and Stoker (1962) and cell stocks were obtained from Flow Laboratories Ltd. They were grown in GMEM at 37°C.

Xenopus laevis cells. These were initially derived from the African clawed toad (Pudney et al., 1973) and were provided by Dr M. Pudney, London School of Hygiene and Tropical Medicine. They were grown in L-15 medium at 28°C.

Chick embryo fibroblasts (CEF) primary cell cultures. These were prepared aseptically from 8 day-old fertile hens' eggs incubated at 40°C. The embryos were removed, divested of their yolk sacs and decapitated. The torsos were washed in phosphate buffered saline, pH 7.3 (PBS) then roughly chopped and added to a flask containing 5ml of 10% trypsin solution for each embryo. This was sealed and stirred at 37°C for 10 minutes, then left to settle and the supernatant decanted into an equal volume of EMEM, containing 20% FCS at 4°C. Fresh trypsin was added to the residual embryo tissue and the flask re-incubated at 37°C. After 10 minutes the resulting cell suspension was gently triturated and washed twice in EMEM by gentle centrifugation (1 x 10^3 rpm, 5 minutes). The cells were stained and counted as above and diluted to a final concentration of 2 x 10^6/ml in EMEM. 2ml of this suspension was added to the individual 25cm^2 flasks which were incubated overnight at 37°C before use.

D. Buffers

Electrophoresis buffer

Tris	0.025M
Glycine	0.192M
Sodium dodecyl sulphate (SDS)	0.1% (w/v)

Made up as a 10% stock solution

NTEP buffer

NaCl	0.15M
Tris	0.05M
EDTA	0.005M
Nonidet P40	0.05% (w/v)

The NaCl, Tris and EDTA were dissolved in deionized water then HCl was added to adjust the pH to 7.9 and the solution autoclaved before the addition of Nonidet P40.

pH buffer

NaCl	0.85% (w/v)
HEPES	0.2% (w/v)
Albumin	1.0% (v/v)

The pH was adjusted to the desired values before filter sterilization.

Resolving buffer

Tris	1.5M

The pH was adjusted to 8.7 with HCl

RNA extraction buffer

Tris	0.01M

Adjusted to pH 8.0 with HCl

Solubilization buffer

Tris	0.0025M
Glycine	0.02M
2-mercaptoethanol	2% (v/v)
Glycerol	15% (v/v)
SDS	2% (w/v)
Bromophenol blue	0.001% (w/v)

Stacking buffer

Tris	1.0M

Adjusted to pH 6.8 with HCl

TE buffer

Tris	0.01M
EDTA	0.001M

Adjusted to pH 7.6 with HCl before autoclaving

TE-20 buffer

Tris	0.02M
EDTA	0.001M

Adjusted to pH 8.0 with HCl before autoclaving

TES buffer

Tris	0.04M
EDTA	0.002M
Sodium acetate	0.02M

Adjusted to pH 7.8 with glacial acetic acid before autoclaving.

TNE buffer

Tris	0.01M
NaCl	0.1M
EDTA	0.001M

Adjusted to pH 7.6 with HCl before autoclaving.

TSE buffer

Tris	0.125M
SDS	0.1% (w/v)
EDTA	0.001M
Glycerol	18% (v/v)
Bromophenol blue	0.001% (w/v)

E. Equipment

Centrifugation steps were performed on the following machines: MSE-Fisons Chilspin benchtop centrifuge, Beckman L8-55 ultracentrifuge (using Beckman or Kontron rotors as described

under individual methods), and an MSE Centaur microfuge. Liquid scintillation counting was performed in Beckman Ready-Solve EP scintillation fluid using an LKB-Wallac 1217 Rack Beta liquid scintillation counter and programs supplied by LKB. Spectrophotometric analysis of RNA was performed using a Pye Unicam SP8-400 uv/vis spectrophotometer. The viruses studied during the course of this work were class group B pathogens (Howie, 1980), and consequently all work using infectious material was carried out in an isolation laboratory, under negative pressure. Infectious material was manipulated in Class III cabinets (Porton Hoods) and autoclaved before disposal.

II. METHODS

A. Cell Culture

Vero cells were routinely subcultured every 7 days. This was done by rinsing the monolayers with PBS and incubating with 0.25% trypsin and 0.02% EDTA for 5-10 minutes at 37°C to detach the cells. Fresh medium was added and the cells gently shaken to produce a uniform suspension. A 1ml sample of the suspension was removed and added to 1ml 0.5% (w/v) trypan blue stain and the cells counted in a Neubauer haemocytometer. Cells were seeded at about 2 x 10^6/75cm^2 flask or 1 x 10^6/25cm^2 flask and incubated at 37°C in 20ml or 5ml of medium respectively.

BHK-21 cells were subcultured as above, as were *Xenopus* cells except that they were incubated at 28°C.

B. Isolation of Viruses

Ticks collected from nesting sites on the Isle of May during

the summers of 1979, 1980 and 1981 were pooled according to the year of collection and sex. The pools were each homogenized in PBS (1ml/tick), containing 0.4% bovine plasma albumin (BPA), 200U/ml penicillin, 2000mcg/ml streptomycin and 100mcg/ml kanamycin (PBSA). The suspensions were clarified by centrifugation at 4 x 10^3 rpm at 4°C for 5 minutes in a benchtop centrifuge. The supernatant was stored in 0.25ml aliquots at -70°C. For inoculation of suckling mice the tick homogenate was diluted 1/5 in PBS containing 0.4% BPA (PBSa). The mice [2 day-old (Pathology Oxford)] were inoculated intracerebrally (i.c.), into the right hemisphere, with 0.01ml, using a 26G needle and 1ml syringe. For each tick pool homogenate a complete litter (approximately 10 mice) was used, including a control litter inoculated with PBSA only. The litters were examined daily for clinical signs, such as wasting and disorientation, at which time the mice were harvested by decapitation, and the heads stored at -70°C until required. The brains were aseptically removed by dissection, homogenized as a 20% suspension in PBSA and clarified by centrifugation at 2 x 10^3 rpm for 5 minutes at 4°C. The supernatant was either stored as above or diluted 1/10 in PBSa and inoculated into a litter of 2 day old mice. These mice were harvested after signs of encephalitis appeared and the brains prepared as above.

For virus isolation using cell cultures, CEF monolayers in 25cm^2 flasks were washed in PBS and inoculated with 0.5ml of either 1/4 or 1/10 dilutions of pooled tick homogenate in PBSa. After adsorbing for 1 hour, the inoculum was removed and the cells washed in PBS, before the addition of 5ml EMEM containing 1% FCS, and incubated at 37°C. Control, mock infected cells were also prepared in parallel. The cell cultures were examined twice

daily for cytopathic effects (cpe), and the flasks frozen at -70°C when the cells showed 75-90% cpe. After thawing, the cell debris was pelleted at 4 x 10^3 rpm for 20 minutes and the supernatant stored at -70°C in 0.25ml aliquots. The material from the first cell passage was inoculated into Vero and Xenopus cells as above, at a dilution of 1/5. Xenopus cells were incubated in L-15 medium containing 3% FCS at 28°C. When a cpe developed the cells were harvested, as above.

C. Titration of viruses

Virus isolates were titrated in either Vero, BHK-21 or Xenopus cells using the method of De Madrid and Porterfield (1969) adapted to Linbro plates. Ten-fold dilutions of virus were made in PBSa and transferred, in duplicate, in 0.1ml amounts to the wells of a Linbro plate, followed by the addition of 0.5ml of a cell suspension (3 x 10^5 cells/ml) in L-15 maintenance medium. After gentle mixing the plate was left for 3-4 hours at 37°C (28°C for Xenopus cells) after which the cells were overlaid with 0.5ml well L-15 medium containing 0.75% (v/v) CMC. Vero and BHK-21 cell titrations were then incubated at 37°C for 3 days, and Xenopus cell titrations at 28°C for 5 days. After incubation, the cells were fixed in formal saline (10% v/v formaldehyde in PBS) for 15 minutes and visualized by staining with 0.2% crystal violet in 95% ethanol for 5 minutes.

D. Plaque Purification

Viruses isolated using suckling mice (two passes) were grown in Vero cells before plaque purification; virus isolates from CEF and Vero cells were plaque purified from their 2nd passage.

The virus, diluted such that 0.1ml contained a single infectious, or plaque forming unit (pfu) was mixed with 0.5ml of Vero cell suspension (3×10^5 cells/ml) in each well of a Linbro plate, and left for 4 hours at 37°C before being overlaid with L-15 CMC medium. The plate was incubated at 37°C for 5 days, after which time the cell monolayers were examined under a microscope. Medium from wells that appeared to contain no plaques or a single plaque was removed and stored separately at 4°C. The cell monolayers were then stained with crystal violet, to identify the wells containing single plaques. The medium from these wells was diluted from 1/2 to 1/1024 in PBSa and incubated in Linbro plates with cells as before. The location and re-plaquing of single plaques was repeated, the single plaques from the third passage being amplified in 75cm^2 flasks containing Vero cell monolayers.

E. Preparation of Stock Virus

High titred stock virus was prepared by inoculating Vero cell monolayers of 1.2×10^7 cells in 175cm^2 flasks at a multiplicity of 0.1-1 pfu/ml, in 2.5ml, at 37°C. After adsorption 20ml of EMEM containing 1% FCS was added and the cells incubated at 37°C for 22-24 hours. The cells were scraped into the medium with a rubber policeman and disrupted using a 30ml dounce homogenizer. The cell debris was removed from the homogenate by centrifugation at 4×10^3 rpm for 20 minutes at 4°C. The supernatant was stored in 1.5ml aliquots at -70°C. Inocula prepared in this manner normally titred at $4-6 \times 10^7$ pfu/ml.

F. Physicochemical Tests

pH Stability. The effect of a range of pHs was studied using a modification of the method of Borden et al. (1971). The virus was diluted 1/10 in sterile pH buffer solution at pH 3, pH 5, pH 7.5 and pH 9 and held at 4°C for 3 hours. The various buffers were adjusted to near neutrality by diluting them in an equal volume of pH buffer at pH 7.5 (for pH 9.0 and pH 7.5) and in pH buffer at pH 9 (for pH 3 and pH 5.0). Each sample was then titrated using Vero cells.

Sodium deoxycholate (SDC) sensitivity. The sensitivity to SDC was determined using a modification of the method of Borden et al. (1971). Virus, diluted 1/10 in PBSa, was mixed with an equal volume of SDC (made to concentrations of 1.0%, 0.1% and 0.01% in deionized water and filter sterilized) and incubated for 1 hour at 37°C. The samples were then cooled on ice and titrated in Vero cells.

Ether and Chloroform sensitivity. Ether and chloroform sensitivities were determined essentially by the method of Borden et al. (1971). A 1/10 dilution of the virus in PBSa was mixed with an equal volume of ether or chloroform in 5ml bijoux which were sealed and held at 4°C, for 3 hours and 18 hours for chloroform, and 18 hours for ether. The aqueous phase was separated from the chloroform by centrifugation at 1×10^3 rpm for 5 minutes and the ether was removed by evaporation, before titration using Vero cells.

G. Serological Tests

Immune ascitic fluid preparation. These were prepared using the method of Shope and Sather (1979). A 2% suspension of infectious suckling mouse brain homogenate, or a 1/10 dilution of cell

culture supernatant, was inoculated i.c. into 1-2 litters of 2 day old mice to provide enough infected mouse brain to immunize 4 mice. The litters were harvested when they showed clinical signs of infection, the brains removed aseptically and homogenized in 0.85% (w/v) sterile NaCl, 1.8ml/brain. The homogenate was clarified by centrifugation at 4×10^3 rpm for 5 minutes at 4°C and stored in 1ml volumes. The first inoculum was prepared by mixing the homogenate with an equal volume of Freund's complete adjuvant and the remaining three inocula were prepared by mixing the homogenate with Freund's incomplete adjuvant.

The mice used for immune ascitic fluid production were six week old females (Pathology Oxford Strain). They were anaesthetized with ether and each inoculated with 0.5ml of adjuvant-brain emulsion into the peritoneal cavity. The following inoculation regime was adopted:

Day 0 1st inoculation - complete adjuvant

Day 7 2nd inoculation - incomplete adjuvant

Day 14 3rd inoculation - incomplete adjuvant

Day 21 4th inoculation - incomplete adjuvant

On day 18, 0.5ml of Landschult's strain of Erlich's ascites cells (2×10^6 cell/ml) were injected into the peritoneum of each mouse. Ascitic fluid was harvested from the mice between days 28 and 31. Cells were pelleted from the ascitic fluid by centrifugation at 4×10^3 rpm for 10 minutes before storage of the supernatant in 0.5ml aliquots at -20°C.

Antigen preparation for complement fixation tests. Antigens were prepared from suckling mouse brains by the sucrose-acetone extraction method of Clarke and Casals (1958). Infected brains, collected from 5-6 litters, were homogenized in 4ml/gram of chilled 8.5% (w/v) sucrose solution, followed by dropwise

addition to acetone (20ml/ml of homogenate) with stirring. The resulting mixture was centrifuged in a type 19 rotor at 15 x 10^3 rpm for 5 minutes. The supernatant was discarded, and replaced with an equal volume of fresh acetone and the mixture held on ice for one hour. After this time the sediment was dispersed with a teflon plunger and pelleted by centrifugation as above. The supernatant was discarded and the sediment air dried for 4 hours before being rehydrated overnight in 40% of the volume of the original homogenate of sterile 0.9% NaCl. The rehydrated extract was then centrifuged at 4°C in a 50 Ti rotor at 10 x 10^3 rpm for 1 hour. The supernatant, containing the antigen, was stored at -20°C in 0.5ml aliquots.

Complement fixation tests. Complement fixation tests (CFT) were carried out exactly as described by Grist et al. (1979). The ascitic fluid was diluted 1/4 in CFT buffer and heat inactivated for 0.5 hour before use. Complement and haemolysin were obtained from Wellcome Reagents Ltd, Beckenham, England, and prepared to the manufacturers directions. Immune ascitic fluid, when not prepared in this laboratory, was obtained from the American Type Culture Collection, Rockville, Maryland, USA.

Neutralization tests. Neutralization tests (NT) were performed using the plaque reduction method of De Madrid and Porterfield (1969) adapted to Linbro plates. 0.01ml dilutions of virus containing 75-100 pfu were mixed with equal volumes of two-fold dilutions of heat inactivated ascitic fluid (1/4-1/512) in PBSa, or equal volumes of PBSa as controls. The mixtures were incubated at 4°C overnight when Vero cell suspensions were added, and the normal titration procedure followed. After fixing and staining the plaques were counted. The ascitic fluid dilution neutralizing 50% of the plaques, by reference to the control

wells, was taken as the neutralizing titre.

H. Electron microscopy

Infected cells were pelleted at 1 x 10^3 rpm for 5 minutes in conical polypropylene centrifuge tubes (Medfor Products, Fleet, England) and the supernatants discarded. The cell pellets were fixed for one hour in 2% glutaraldehyde made up in 0.2M phosphate buffer (pH 7.2), and washed twice for 20 minutes in buffer. Further fixation was carried out for 1.5 hours in 1% osmium tetroxide, followed by an overnight wash in buffer. The pellets were dehydrated through 50%, 75% and 100% ethanol and stored in acetone for 48 hours. Each pellet was embedded in Emix resin and cured at 60°C for 24 hours. Thin sections were cut on a Reichert-Jung Ultracut ultramicrotome, collected onto 200 mesh copper grids and stained with 2% uranyl acetate for 15 minutes, followed by 2% lead citrate for 15 minutes. Stained sections were examined on a JEOL 100S electron microscope at 100kV. Electron micrographs were taken on Kodak Electron Microscope Film 4489, and processed as directed by Kodak.

I. Single and Multiple Phase Replication Cycles

Monolayers of 1 x 10^6 Vero cells in 25cm^2 flasks were inoculated at a multiplicity of 3 pfu/cell in 0.5ml of EMEM containing 1% FCS. The virus was adsorbed for one hour at 37°C, the inoculum removed and stored at -70°C. The cells were washed twice with PBS, followed by the addition of 4ml EMEM maintenance medium, 1% FCS and incubated at 37°C. At appropriate time intervals the cells were carefully scraped into the medium and pelleted at 1 x 10^3 rpm for 5 minutes. The supernatant was removed and the cells resuspended in an equal volume of EMEM

maintenance medium before both samples were stored at -70°C. The samples were thawed at 37°C and the cell fraction sample clarified by centrifugation at 1 x 10^3 rpm for 5 minutes before titration in Vero cells. Samples of the cell fractions and supernatant fractions were also sonicated separately for 1 minute, either in a Dawe Sonicleaner waterbath or using a Dawe Soniprobe with a 60mm long, 3mm diameter probe adjusted to setting No. 7. The sample vessel in each case was a 30ml capacity disposable bottle having thin glass walls.

Vero cell monolayers were inoculated as above at multiplicities of either 0.1, 1.0 or 10 pfu/cell. At appropriate time intervals the flasks were frozen at -70°C, thawed, and the cell debris removed by centrifugation at 1 x 10^3 rpm for 5 minutes before titration in Vero cells.

J. Virus Purification

Production of [^{35}S] labelled virus. Virus was inoculated into 3 to 6 0.5 gallon roller bottles (surface area 760cm^2) containing 5 x 10^7 cells/roller, at a multiplicity of 0.1 pfu/cell in 10ml EMEM maintenance medium. The inoculum was adsorbed for 1.5 hours at 37°C, then removed, 40ml of EMEM maintenance added and the cells incubated for a further 6 hours. The medium was then changed to EMEM containing 1% dialysed FCS and 10% of the normal methionine concentration. [^{35}S] methionine was added to a specific activity of 5µCi/ml and the cells incubated for a further 18-24 hours at 37°C. The cells were then scraped into the medium, which was frozen and thawed once to disrupt the cells.

Polyethylene glycol method of purification. Polyethylene glycol 6000 and NaCl were added to the cell suspension at 5% (w/v) and

2.2% (w/v) respectively and the mixture stirred at 4°C overnight. Precipitated material was pelleted at 14 x 10^3 rpm (23 x 10^3g) for 1 hour at 4°C in a type 30 rotor. The supernatant was discarded and the pellet resuspended in 3% of the original volume in TNE buffer containing 1% Nonidet P40. The suspension was disrupted by 40 strokes in a Dounce homogenizer, layered onto a 20-60% (w/v) sucrose gradient (all sucrose solutions were made in TE buffer) and centrifuged at 30 x 10^3 rpm (100 x 10^3g) for 3 hours at 4°C in an SW40 rotor. The gradient was harvested from the bottom of the tube through a pin-hole, either by gravity or by applying pressure to the top of the gradient through a glass syringe. Fractions of 1ml were collected, from which 10µl samples were taken for scintillation counting and titration, diluted in TNE buffer and pelleted through a 20% (w/v) sucrose cushion at 30 x 10^3 rpm (100 x 10^3g) for 1.5 hours at 4°C in an SW40 rotor. The pellets were resuspended in 100µl of solubilization buffer, heated to 100°C for 2 minutes and stored at 4°C until they were used for electrophoresis. 10µl samples were taken from the solubilized pellets for scintillation counting.

Ether extraction method. Virus was inoculated and labelled in 0.5 gallon roller bottles and the cells harvested at 24 hours post infection (p.i.) by scraping into the medium and pelleting at 4 x 10^3 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 1/100 the volume of TE-20 buffer containing 1% Nonidet P40. The suspension was disrupted by 40 strokes in a Dounce homogenizer at 4°C, then centrifuged at 1 x 10^3 rpm for 5 minutes at 4°C, to remove cell nuclei and debris. The supernatant was decanted and extracted with an equal volume of ether by shaking for 5 minutes. The phases were separated by centrifugation at 1 x 10^3 rpm for 1 minute and the

aqueous phase re-extracted with an equal volume of ether. Once separated from the ether the final aqueous phase was pelleted through a 20% sucrose cushion (all sucrose solutions were made in TE-20 buffer) at 25 x 10^3 rpm (105 x 10^3g) for 1.5 hours at 4°C in an SW40 rotor. The supernatant was discarded, the pellet resuspended in 1ml TE-20 buffer and homogenized again before being layered onto a 10-50% (w/v) sucrose gradient. This was centrifuged at 20 x 10^3 rpm (78 x 10^3g) for 1 hour at 4°C in an SW40 rotor. The gradient was harvested, as described previously, into 0.5ml fractions, from which 10µl samples were taken for scintillation counting and titration. Pooled fractions (2 x 0.5ml) were diluted in TE-20 buffer before being pelleted through a 20% (w/v) sucrose cushion at 25 x 10^3 rpm (105 x 10^3g) for 1.5 hours at 4°C in an SW40 rotor. The supernatants were discarded, the pellets resuspended in 100µl solubilization buffer, boiled at 100°C for 2 minutes and stored at 4°C until required for electrophoresis. 10µl samples were taken from the solubilized pelleted for scintillation counting. Pellets were also resuspended in TE-20 buffer and prepared for electron microscopy. Immediately before electrophoresis the samples were boiled for 30 seconds to disrupt protein aggregates.

K. Radiolabelling of cells for analysis of polypeptides

Vero or BHK-21 cell monolayers, containing 1 x 10^6 cell/ 25cm^2 flask, were inoculated at a multiplicity of 20-30 pfu/cell in 0.5ml at 37°C. The virus was adsorbed for 1 hour when the inoculum was removed, the cells washed in PBS and 2ml of maintenance medium added, followed by incubation at 37°C. Zero time p.i. was taken as the time the medium was added. At appropriate times p.i. (which varied depending on experimental

aims) the medium was removed and the cells washed twice in 2ml of labelling medium (the composition of which varied, depending on the cells used and the radiolabel to be added, see Table 2.1), followed by incubation for 0.5 hour at 37°C in 450µl of labelling medium. The radiolabel was added in 50µl labelling medium and incubation continued for a period depending on experimental aims. At the end of the labelling period the cell monolayers were harvested into ice cold PBS by scraping, and pelleted at 1.5-3 x 10^3 rpm for 5 minutes at 4°C. The supernatant was discarded, the pellet resuspended in solubilization buffer (250µl/10^6 cells), boiled immediately for 2 minutes and stored at 4°C until required.

L. Immune precipitation of intracellular virus products

Cells inoculated and labelled as described above were harvested and solubilized in 1ml/25cm^2 flask of NTEP buffer. Samples were frozen and thawed 6 times to disrupt cell membranes before insoluble cell debris was removed by centrifugation at 4 x 10^3 rpm for 10 minutes in a benchtop centrifuge. The supernatant was adsorbed, under constant agitation, with 1% (w/v) Staphylococcus aureus protein A for 1 hour at room temperature. The protein A was removed by pelleting in a microfuge at 11.6 x 10^3g and the supernatant was treated sequentially with immune ascitic fluid (10µl), rabbit anti-mouse IgG (10µl) and protein A (100µl/ml of 10% stock suspension), each for 1 hour with constant agitation. The protein A-antibody conjugate was pelleted at 11.6 x 10^3g in a microfuge and washed 6 times before solubilization by heating at 100°C for 5 minutes in solubilization buffer (150µl/ml NTEP). The insoluble material was removed by centrifugation and the supernatant stored at 4°C until required for electrophoresis.

M. Polyacrylamide gel electrophoresis of proteins

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (PAGE) on 1mm thick, 125mm long slab gels. For resolving gels the mixture consisted of 0.374M resolving buffer, 0.1% (w/v) SDS, 0.034% (w/v) ammonium persulphate, 0.033% (v/v) N-N-N'-N'tetramethylethylenediamine (TEMED) and varying amounts of acrylamide and N-N'methylenebisacrylamide (bis). 12.5% (w/v) acrylamide gels contained 0.1% (w/v) bis, and 17.5% (w/v) acrylamide gels contained 0.074% (w/v) bis. The gel mixtures were made by dilution of stock solutions of 1.5M resolving buffer, 10% (w/v) SDS, 10% (w/v) ammonium persulphate (made fresh on each occasion), 30% (w/v) acrylamide, 1% (w/v) bis and 100% TEMED, made up to 35.3ml with deionised water. Resolving gels were overlaid with stacking gels containing 5% (w/v) acrylamide, 0.15% (w/v) bis, 0.143M stacking buffer, 0.1% (w/v) SDS, 11% (v/v) glycerol, 0.2% (w/v) ammonium persulphate and 0.1% (v/v) TEMED prepared from stock solutions of 30% (w/v) acrylamide, 1% (w/v) bis, 10% (w/v) SDS, 50% (v/v) glycerol, 10% (w/v) ammonium persulphate, 1M stacking buffer and 100% TEMED in a volume of 26ml.

10-20% gradient gels were made in a gradient maker from 20% (w/v) acrylamide gel solutions (0.065% w/v bis) and 10% (w/v) acrylamide gel solutions (0.13% bis) using a 5% stacking gel. Electrophoresis was performed using the discontinuous tris-glycine system of Laemmli (1970), containing 0.025M tris, 0.192M glycine and 0.1% (w/v) SDS, pH 8.5, for 15 hours at 10mA (100V). Processing of polyacrylamide gels. After electrophoresis, stacking gels were removed to avoid distortion during processing. The gels were fixed for 1 hour in 40% (v/v) methanol, 10% (v/v) glacial acetic acid in water before drying. If the proteins were

required for partial proteolysis the gels were washed for 10 minutes in 3 changes of water before drying. Gels containing samples labelled with [^{14}C] mannose and [^{14}C] glucosamine were processed for fluorography by impregnating with "EN^{3}HANCE" according to the manufacturers instructions. Gels were dried under vacuum and exposed to Fuji X-ray film at -20°C, or at -70°C for fluorography. Densitometer scans of autoradiograms were made using a Joyce-Loebl microdensitometer with a thin layer scanning attachment.

N. Partial Proteolysis

Peptide mapping of proteins separated by PAGE was performed by partial proteolysis with S.aureus V8 protease, α chymotrypsin and trypsin (Cleveland et al., 1977). Following autoradiography, using the autoradiograph as a template, bands were excised from the gel and placed in the wells of a 5% stacking gel overlaid on a 17.5% resolving gel. The bands were swollen in elecrophoresis buffer for 2 hours and were then overlaid with 70µl of S.aureus V8 protease in TSE buffer. Electrophoresis was performed at 5mA (60V). Simultaneous analysis of several proteins was achieved by excising strips, corresponding to the length of the gel track, and placing them horizontally on top of a 5% stacking gel overlaid on top of a 17.5% separating gel (Crump & Moore, 1981). The strips were swollen in electrophoresis buffer for 2 hours, treated with 1ml of S.aureus V8 protease in TSE buffer and electrophoresed as above.

O. Molecular Weight Determinations

Samples from virus infected cells labelled with [^{14}C] protein hydrolysate were electrophoresed on a 12.5% PAG with a

mixture of [^{14}C] labelled molecular weight marker proteins, comprising cytochrome C, 12,300; β lactoglobulin, 18,400; α-chymotrypsinogen, 25,700; ovalbumin, 43,000; bovine serum albumin, 68,000; phosphorylase B, 92,500; and myosin (H-chain), 200,000. A densitometer scan was made of the autoradiograph from which a mobility curve was constructed, according to the method of Hames (1982), from which the virus polypeptide molecular weights were derived.

P. Extraction of double-stranded RNA from infected cells

All apparatus and solutions used for dsRNA preparation was sterilized, either by autoclaving at a pressure of 15 lb/in^2 for 20 minutes, or by baking in an oven at 180°C overnight. Glass tubes, pasteur pipettes and microfuge tubes were coated with "Sigmacote" before use.

dsRNA was extracted from infected cells by a modification of the method of Travassos da Rosa et al. (1984). Vero cell monolayers in 175cm^2 flasks were inoculated at a multiplicity of 0.1-0.3 pfu/cell and were incubated for 24 hours at 37°C before being harvested by scraping into the medium, and pelleted at 1 x 10^3 rpm for 20 minutes at 4°C. The cell pellet was resuspended in 10mM EDTA pH 7.6 (10ml/10^8 cells) and solubilized by the addition of 20% (w/v) SDS (2ml/10^8 cells). After gentle shaking for 20 minutes 5M NaCl (3ml/10^8 cells) was added and the mixture stored at 4°C overnight. The precipitated DNA was pelleted by centrifugation at 15 x 10^3 rpm (55 x 10^{3}g) for 45 minutes at 4°C in an SW40 rotor. The supernatant was decanted and digested with 200μg/ml Proteinase K for 3 hours at 37°C before extraction with buffer equilibrated phenol. This was prepared in the following manner: fresh solid phenol was shaken

with RNA-extraction buffer, then warmed to 37°C to aid solution. The phases were separated by centrifugation at 3 x 10^3 rpm in a benchtop centrifuge and the top, aqueous, phase removed. The phenol was shaken twice more with fresh buffer, the final phenol phase being used for the extraction. The proteinase-treated infected cell suspension was mixed with an equal volume of buffer-equilibrated phenol by shaking for 5 minutes and the phases separated by centrifugation as described above. The aqueous phase was removed and the phenolic phase and interface were re-extracted with an equal volume of fresh buffer. The aqueous phase was re-extracted with an equal volume of fresh buffer-equilibrated phenol. The aqueous phases from these extractions were pooled and extracted a third time with an equal volume of buffer-equilibrated phenol. The aqueous phase was then washed 3 times in water saturated ether to remove phenol, and the ether, removed by evaporation, either at 37°C for 1 hour or by gently bubbling N_2 gas through the solution. Nucleic acid was precipitated by adding 2.2 volumes of ethanol and storing at -50°C for 16 hours. The precipitate was pelleted at 3 x 10^3 rpm for 30 minutes at 4°C, the supernatant mostly removed, the nucleic acid resuspended by vortexing and the suspension transferred to a 1.5ml microfuge tube. The nucleic acid was washed 3 times in 75% ethanol and twice in absolute ethanol by resuspending and pelleting at 11.6 x 10^3g for 4 minutes each time in a microfuge. It was dried under vacuum for 1 hour. Dried nucleic acid was dissolved in Analar water, diluted 5-fold in 10mM Tris-HCl pH 8, 10mM MgCl, and incubated at 37°C for 1 hour with 1µg/ml DNase 1. The digest was then extracted once with phenol-chloroform (50% v/v water saturated phenol, 50% v/v chloroform) and washed twice with ether. Single-stranded RNA

(ssRNA) was precipitated by adding LiCl to 2M final concentration, storing at 4°C for 16 hours and pelleting at 11.6 x 10^3g for 10 minutes. The dsRNA was precipitated by adding 2.2 volumes of ethanol and 0.1 volumes of 3M sodium acetate to the supernatant followed by storage at -50°C for 16 hours. It was pelleted as above and washed in 75% and absolute ethanol before being dried under vacuum. The dried dsRNA was dissolved in Analar water and its concentration determined spectrophotometrically. All dsRNA solutions were stored at -50°C.

Q. Polyacrylamide gel electrophoresis of dsRNA

Electrophoresis of dsRNA was carried out essentially as described under electrophoresis of proteins. The gel concentrations used were either 5% (0.26% bis) or 10% (0.13% bis) resolving gels with 3% (0.15% bis) stacking gels. SDS was not used in the gel solutions or electrophoresis buffer when the gels were to be silver stained. Electrophoresis was performed at 15mA (160V) for 16 hours.

The dsRNA was visualized either by ethidium bromide or silver staining. For ethidium bromide staining, gels were soaked in 3 changes of 0.5M ammonium acetate for 10 minutes then in ammonium acetate containing 0.5µg/ml ethidium bromide for 30 minutes. The stained gel was viewed by ultra-violet light on a transilluminator and photographed through an R (25A) red filter. Silver staining was carried out using a modification of the method of Herring et al. (1982). Gels were fixed in 10% ethanol, 0.5% glacial acetic acid for 30 minutes and then soaked in 150ml/gel 0.011M silver nitrate (freshly made up) in the dark for 2 hours. They were then rinsed briefly in water and the impregnated silver ions were reduced to solid silver using

250ml/gel of 0.75M NaOH, 0.1M formaldehyde for 5-10 minutes. When the image was dark enough the reduction was stopped by removing the reducer and adding 100ml/gel 2.7M citric acid. If necessary the gels were bleached, by the addition of about 5ml of (0.1M) potassium ferricyanide in 1M sodium thiosulphate, to the citric acid (C. Reinganum, personal communication). Care was taken throughout the staining procedure to avoid touching the gel, so solutions were removed by vacuum pump.

R. In vitro protein synthesis

dsRNA was dried onto the bottom of a sterile 0.75ml microfuge tube and denatured with 10mM methyl mercury hydroxide (1µl/10µl assay) at room temperature for 15 minutes. Translation of the denatured dsRNA was performed in a commercially available micrococcal nuclease treated rabbit reticulocyte lysate (Pelham & Jackson, 1976). Immediately prior to starting the assay the lysate was rapidly thawed from liquid nitrogen and placed on ice. For 10µl assays (larger assays contained proportionately more of each component), 8µl of lysate was mixed with 1µl [^{35}S] methionine and this added to the RNA and methyl mercury hydroxide. The translation mixture was incubated at 30°C for 1 hour and stopped by the addition of 30µl solubilization buffer followed by boiling for 2 minutes. Samples were stored at 4°C until required for electrophoresis. When the incorporation of [^{35}S] methionine into protein was to be followed 1µl samples were removed from the translation mixture at appropriate time intervals throughout the incubation period for up to 90 minutes. These were spotted onto 1cm^2 squares of Whatman 3MM-filter paper which was boiled for 10 minutes in 10% (w/v) trichloroacetic acid (TCA). This was cooled by the addition of ice, decanted and the

filter paper washed twice in water, ethanol and acetone before air drying. The squares were each placed in scintillation vials with 5ml scintillation fluid and counted.

S. Preparation of individual genome segments

Throughout this procedure sterile "Sigmacoted" apparatus was used and all solutions were autoclaved. 1.5mg of dsRNA, in solution, was mixed with 40% (w/v) sucrose in Analar water containing 0.001% (w/v) bromophenol blue to give a final volume of 1.5ml. This solution was carefully layered onto a 3% stacking gel placed on top of a 5% resolving gel, both containing 0.1% (w/v) SDS. Electrophoresis was performed at 20mA (100-200V) for 18 hours when the gel was stained with ethidium bromide. The dsRNA genome segments, visualised on a transilluminator, were cut from the gel with a sterile knife and stored separately at -50°C. Bands which could not be separated on the gel were removed together and subjected to further electrophoresis at 30mA for 24 hours in 5% gels. Once thawed, the segments were placed in dialysis tubing (previously boiled in EDTA) that was long enough to hold the gel strips lengthways. About 2ml of TES buffer was added to the dialysis tubing and the air carefully expelled before the ends were clipped. The dsRNA was then electroeluted by placing the tubing in a flat-bed gel tank filled with enough TES buffer to cover the tubing by about 1mm. Electroelution was performed at 100mA (60V) at 4°C for 22 hours. Before the gel was removed from the tank the current was reversed for 5 minutes to release the eluted dsRNA from the tubing. The liquid contents of the dialysis tube were removed and the tube washed out with about 2ml of Analar water. The combined solutions were then centrifuged at 1 x 10^3 rpm for 5 minutes in a

benchtop centrifuge to remove small pieces of acrylamide. The supernatant was extracted 3 times with an equal volume of butan-1-ol to reduce the aqueous volume and remove ethidium bromide. The aqueous phase was transferred to a microfuge tube and extracted once, for 2 minutes, with an equal volume of phenol-chloroform (50% v/v Analar water saturated phenol and 50% v/v chloroform) and twice with an equal volume of water saturated ether. The ether was removed using nitrogen and the dsRNA precipitated by adding 2.2 volumes of ethanol and 0.1 volumes of sodium acetate, pH 5.7, and storing overnight at -50°C. The dsRNA was pelleted at 11.6 x 10^3g for 10 minutes in a microfuge, washed twice in 75% ethanol and absolute ethanol, dried under vacuum and redissolved in 25µl of Analar water. Insoluble material was removed by centrifugation at 11.6 x 10^3g for 2 minutes and the dsRNA solution precipitated as above. The final dsRNA precipitate was washed and dried as above and redissolved in 10µl Analar water. It was stored at -50°C until required for electrophoresis or translation.

Table 2.1

A Summary of Labelling Media and Radiolabels

Cells	Medium	Radiolabels
Vero	EMEM without methionine containing 1% dialysed FCS*	[^{35}S] methionine 100-400μCi/ml
	EMEM without amino acids, containing 1% dialysed FCS	[^{14}C] protein hydrolysate 4-10μCi/ml
	EMEM with 10% normal sucrose levels containing 1% dialysed FCS	[^{14}C] mannose 10μCi/ml or [^{14}C] glucosamine 10μlCi/ml
BHK-21	GMEM without methionine containing 1% dialysed FCS	[^{35}S] methionine 100-400μCi/ml

* dialysed against 0.11M NaCl

CHAPTER 3

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ISOLATION AND PRELIMINARY CHARACTERISATION OF ORBIVIRUSES FROM THE ISLE OF MAY

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I. INTRODUCTION

This chapter reports the isolation and physicochemical, morphological and serological characterisation of 3 virus isolates from the Isle of May in Scotland.

II. RESULTS

A. Tick Collection

The Isle of May (1°57'W, 56°2'N) lies in the Firth of Forth, off the eastern coast of Scotland. The island is about 4.8km long and 1.2km wide and is composed of granite with covering vegetation mainly of grass and heather. Many species of seabird nest on the island during their summer breeding seasons. During visits to the island in the summers of 1979, 1980 and 1981, several species of bird and their nesting sites were examined for the presence of ticks. The chicks and nests of two species, kittiwakes (Rissa tridactyla) and guillemots (Uria aalge), both cliff nesting birds, were commonly found to be infested with hard ticks of the species Ixodes uriae White 1852 (Fig. 3.1). Female and male ticks were found in and around the nests, and females were found on the chicks themselves, usually under the wings, around the cloaca, or on the body. Occasionally, by chipping away the rock around the nests, ticks were found in crevices. Some ticks were found in crevices far removed from occupied nesting sites, but these were never engorged. Most of the ticks examined were collected from 2 nesting sites that contained numerous infested nests (Mill Door and North Clett), and from a smaller site (South Plateau).

North Clett, on the eastern, sloping side of the island (Fig. 3.2), contained colonies of kittiwakes nesting on cliffs 6-20m high (Fig. 3.3). Mill Door, on the western side of the island (Fig. 3.2) contained colonies of kittiwakes, guillemots and razorbills (Alca torda) nesting along narrow ledges on cliffs 90-100m high (Fig. 3.4). South Plateau (Fig. 3.2) contained a small colony of kittiwakes on low cliffs near sea level. Ticks collected from these sites were stored in plastic tubes and kept moist with damp cotton wool during transit to the laboratory, where they were stored at -70°C.

B. Virus isolation and cultivation

The ticks collected in 1979 were pooled according to collection site and sex, homogenized in PBSA and inoculated into suckling mice. The ticks collected in 1980 and 1981 were pooled in a similar manner and inoculated directly onto CEF cultures. Viruses were isolated from 3 of the 6 tick pools prepared (Table 3.1) and were re-isolated 1 year later.

Mill Door/79 virus, when first inoculated into mice, produced signs of encephalitis in 9-10 days, which was reduced to 7-8 days on the second passage and 7 days on subsequent passage (Table 3.2). When material from the second mouse passage was titrated in Vero cells it produced clear, well defined plaques of 1-2mm in diameter, 4 days after inoculation (Fig. 3.5) and had a titre of 3.5 $\log_{10}$ pfu/ml. When passed into cell culture this same material produced cpe in CEF, Vero and BHK-21 cells but not Xenopus cells. The cpe was characterised by cell rounding, followed by detachment from the flask and eventual lysis. The titre of Mill Door/79 virus, when grown in Vero and BHK-21 cells was 6.9-7.0 $\log_{10}$ pfu/ml, while that grown and titrated in

Xenopus cells produced no plaques. After inoculation of Mill Door/79 virus into Xenopus cells followed by titration in either Vero or BHK-21 cells no plaques were visible (Table 3.3).

Although originally isolated in CEF, North Clett/81 and Mill Door/81 viruses were also passaged in suckling mice (Table 3.2). North Clett/81 virus produced signs of encephalitis in 8 days on first passage, and 4 days on the second passage. Subsequent passage of North Clett/81 virus reduced its incubation period in mice to 3 days. Mill Door/81 virus produced signs of encephalitis on first passage in suckling mice in 11-13 days, reduced to 6-7 days on second and subsequent passages. Although Mill Door/81 virus was inoculated into suckling mice at a higher titre than North Clett/81 virus (Table 3.3, Vero cells) it took considerably longer for the mice to become sick. On subsequent passage the incubation periods of all the viruses were reduced, but North Clett/81 virus still maintained the shortest. In common with Mill Door/79 virus, both North Clett/81 and Mill Door/81 viruses caused cpe in CEF, Vero and BHK-21 cells, but not Xenopus cells. North Clett/81 and Mill Door/81 viruses, when titrated in Vero cells, produced an identical plaque morphology to that described for Mill Door/79 virus (Fig. 3.5). After a single passage in Vero cells the titres of the two 1981 isolates were 1.5-2.5 $\log_{10}$ less than Mill Door/79 virus, but subsequent passage in Vero cells produced higher titres.

Virus isolation from the tick pools collected in 1980 was unsuccessful (Table 3.1).

C. Plaque purification

Before studies on virus replication and biochemistry were initiated (see Chapters 4, 5, 6 and 7), Mill Door/79, North

Clett/81 and Mill Door/81 viruses were plaque purified. Five clones were produced from Mill Door/79 virus and 2 each from North Clett/81 and Mill Door/81 viruses. The clones were all capable of growth to titres of $\log_{10}$ 7.7 in Vero cells, and none replicated in Xenopus cells.

D. Electron microscopy

Infected Vero and BHK-21 cells were examined by thin section electron microscopy. Virus was readily visualized when titres were 6 $\log_{10}$ pfu/ml or greater. Virus particles were observed in both cell lines and their morphology and the morphology of associated structures were indistinguishable. Figures 3.6 and 3.7 show thin section electron micrographs of BHK-21 cells, infected with Mill Door/79 virus (North Clett/81 and Mill Door/81 viruses are not shown as they were morphologically indistinguishable from Mill Door/79 virus). Virus particles were found in the cytoplasm, either in loose clusters (Fig. 3.7b) or in regularly arranged matrices of up to 1.2µm in diameter, having no boundary structure and containing 200 or more virus particles (Fig. 3.6a, b). The matrices were normally located in the perinuclear region of the cytoplasm, whereas the virus clusters were located near to the cell membranes. The virus particles themselves consisted of uniformly round, electron-dense, double shells with a highly electron dense core. The diameter of the complete structure was 66 ± 4nm, while the core measured 37 ± 4nm (mean of 25 particles). The particles appeared to be unenveloped. Commonly found in infected cells were what appeared to be the outer capsids of virus particles without the cores (Fig. 3.6b). They were between 65-69nm in diameter and were often irregular in shape.

Prominent tubular and filamentous structures in the cytoplasm were commonly seen in cells infected with all three virus isolates, and in both cell lines. Figure 3.7a shows the cytoplasm of a cell containing numerous tubular structures (T) and "empty", capsid-like, structures (EC). The "empty" capsid-like structures were commonly associated with the tubules. Tubules varied in length from 150nm to 1µm and were 70 ± 5nm in diameter. Also present, although less numerous, were filaments. Figure 3.7b shows a filament (F) associated with virus particles (V). The filaments comprised a densely stained centre 31 ± 4nm in diameter (mean of 10 measurements), surrounded by a diffuse layer of variable diameter.

E. Physicochemical properties

Table 3.4 shows the results of physicochemical tests. All 3 isolates were moderately resistant to ether. The titre of Mill Door/79 was reduced by 1.6 $\log_{10}$ pfu/ml, while the other two isolates were reduced by 0.5 and 1.0 $\log_{10}$ respectively. Treatment with chloroform for 18 hours completely inactivated Mill Door/79 virus while reducing the titre of the other isolates by 3 $\log_{10}$. Conversely, a 3 hour treatment with chloroform led to a reduction of only 1 $\log_{10}$ for Mill Door/79 virus, while the other isolates were reduced by about 2 $\log_{10}$. Treatment with SDC at 0.5% completely inactivated all three isolates. Reduced concentrations of SDC (0.05% and 0.005%) had little effect.

Each isolate was subjected to a range of pH treatments. Mill Door/79 virus was completely inactivated by pH 3.0 and moderately sensitive to pH 5.0. North Clett/81 and Mill Door/81 viruses were both inactivated at pH 3.0 but remained unaffected at pH 5.0. All three isolates were unaffected at pH 9.0.

F. Serological Studies

(i) Complement fixation tests. Complement fixation tests were carried out on each of the isolates (Table 3.5). Apart from testing the immune ascitic fluids raised against each isolate, ascitic fluids against other serotypes were also used. The three isolates each cross-reacted to varying degrees, but gave higher homologous titres. Each isolate was tested against ascitic fluids from other serological groups of orbiviruses. Cape Wrath ascitic fluid was the only one to give a positive reaction.

(ii) Neutralization tests. Table 3.6 shows the neutralization reactions of the Isle of May isolates. Ascitic fluid raised against Mill Door/79 virus failed to neutralize the other two isolates, and ascitic fluid raised against North Clett/81 virus failed to neutralize Mill Door/79 virus. Both Mill Door/79 and North Clett/81 viruses were neutralized by ascitic fluid raised against Mill Door/81 virus. Mill Door/81 virus was also neutralized by ascitic fluid raised against North Clett/81 virus. Mill Door/81 and North Clett/81 viruses therefore cross neutralize, whereas Mill Door/79 virus only shows one way neutralization with Mill Door/81 virus.

Ascitic fluid was raised against 2 of the 5 clones of Mill Door/79 virus and these were used, together with the uncloned virus ascitic fluid, to determine whether the clones were serologically distinct. Table 3.7 shows that the ascitic fluid raised against the uncloned virus neutralized all the clones with equal efficiency. Ascitic fluid raised against clones No. 2 and No. 3 neutralized the uncloned virus and the other clones, except No. 1, at equal titres.

III. DISCUSSION

Viruses were isolated from 3 of the 6 tick pools collected from the Isle of May. Neither of the 2 pools of ticks collected at South Plateau produced virus on inoculation onto CEF. This does not mean that virus was not present in the kittiwake colony on South Plateau. Their presence in the ticks may have been at such a low level that they were unable to initiate infection successfully. It is also worth noting that the methods of isolation employed were in themselves selective, and viruses present in the tick pools may not all have been able to replicate in CEF or suckling mice. In the future a cell line derived from the virus host, in this case I.uriae, may allow the isolation of viruses unable to grow in vertebrate culture systems.

The 3 successful isolations, Mill Door/79, North Clett/81 and Mill Door/81 viruses, were isolated from pools of both male and female ticks. Since adult male I.uriae are not known to take blood meals, the presence of virus in males is evidence of transtadial survival in the tick. Each of the isolates displayed the same growth restrictions. They replicated in CEF, suckling mice, Vero and BHK-21 cells but not in Xenopus cells. The failure of the viruses to replicate in an amphibian cell line may have been due to the lack of suitable attachment sites for the virus on the cell surface, or possibly a temperature restriction (Xenopus cells were incubated at 28°C whereas the other lines were incubated at 37°C), although this is unlikely since the viruses presumably replicate in the tick vector at ambient temperature. After a single passage in Vero cells, the two 1981 isolates titred at 1.5-2.5 $\log_{10}$ less than Mill Door/79 virus, although on subsequent passages the titres were similar. These

lower titres were probably a function of the multiplicity of infection or incubation period (this is examined in more detail in Chapter 4). The virus isolates produced different responses in suckling mice. Mill Door/79 and Mill Door/81 viruses produced morbidity in 6-7 days in suckling mice while infection with North Clett/81 virus resulted in deaths after only 3 days. The brains of these mice yielded similar amounts of virus, the titres differing by 1 $\log_{10}$. These data suggest that North Clett/81 virus was more virulent for suckling mice than the other 2 isolates.

The morphology of Mill Door/79 virus (and North Clett/81 and Mill Door/81 viruses) seen in thin section preparations was very similar to bluetongue virus (Bowne & Jones, 1966; Cromack et al., 1971; Murphy et al., 1971). Murphy et al. (1971) compared different orbivirus serotypes, grown in cell culture and mouse brain, by thin section electron microscopy. All were similar and each was associated with tubules and filaments. The tubules seen in Mill Door/79 virus infected cells were about 70nm in diameter, while those of bluetongue virus were 68nm (Huismans & Els, 1979). [All size determinations, during the electron microscope studies on Mill Door/79 virus, were made using the magnification given by the electron microscope. This had been calibrated using catalase crystals as a standard, but errors may have arisen due to variations in high tension voltage, the precise location of specimen plane and lens current variation due to drift in reference voltages (Meek, 1976). The size data presented must therefore only represent an estimate and not an absolute value]. The tubule length varied quite substantially and it was possible that this was due to their orientation when the sections were

cut. The filaments also varied in length, possibly for the same reason. It was suggested by Murphy et al. (1971) that the tubules were virus capsomers assembled in an anomalous manner. Recent biochemical studies have shown that the tubules are, in fact, composed of virus-specified non-structural protein (Huismans & Els, 1979). At present no information is available to explain the function of the tubules and filaments in virus replication, although their appearance is a characteristic feature of all orbiviruses studied so far. The size of the Mill Door/79 virus particle was 66nm and this is in agreement with the estimate of 65-75nm reported for Tribec virus, a member of the Kemerovo antigenic complex (Murphy et al., 1971).

Borden et al. (1971) investigated the physicochemical properties of several orbivirus serotypes. Their results were broadly similar to those reported for the Isle of May viruses. However, the effects of chloroform and SDS differed. The titres of the Isle of May viruses were significantly reduced, whereas Borden et al. (1971) found only small reductions in the titres of most viruses examined. Only Wad Medani infectivity was reduced by 3 LD_{50}/ml. 0.5% SDC completely inactivated the Isle of May viruses, whereas Borden et al. (1971) found less than 2 LD_{50}/ml reductions using the same methods. Borden et al. (1971) demonstrated the acid lability of orbiviruses; the Isle of May viruses were also inactivated at pH 3.0. Reoviruses, which are similar to orbiviruses in many of their physicochemical and morphological properties (Borden et al., 1971; Murphy et al., 1971) are not inactivated by acid pH. Owen (1964) reported that bluetongue virus was acid sensitive and Verwoerd (1969) showed that bluetongue virus was more stable in alkaline conditions.

The physicochemical properties of the Isle of May viruses are therefore very similar to those of orbiviruses. The only major difference is the inactivation by 0.5% SDC which may reflect differences in the virus capsid proteins, since the lack of ether sensitivity suggests these viruses are not enveloped.

The cross reactivity of the Isle of May viruses in CFT indicates that, although distinct, they can be placed within the same serogroup. Each virus antigen reacted with ascitic fluid raised against Cape Wrath virus. This virus was isolated from I.uriae ticks collected at Cape Wrath in Scotland during 1973, and is a member of the Kemerovo serogroup, Great Island Complex (Main et al., 1976). The ascitic fluids which failed to react with the Isle of May isolates represented other serogroups of the orbivirus genus, and members of the same serogroup (Kemerovo) but different antigenic complexes (Kemerovo, Chenuda, Mono Lake, Wad Medani, Tribec and Huacho viruses). These results demonstrate that the Isle of May viruses are all members of the Great Island Complex, one of 4 antigenic complexes of the Kemerovo serogroup. The Great Island viruses are quite closely related to one another and to a lesser extent to the Kemerovo-Tribec complex by CFT. No member of the Great Island complex is recognised as being of public health significance.

The Isle of May viruses showed either no or varying degrees of cross reactivity in neutralization tests. The lack of reaction between Mill Door/79 and North Clett/81 viruses indicates that they are different serotypes. However, Mill Door/81 virus ascitic fluid neutralized both the other isolates. These contradictory results can be explained if one does not regard viruses as being members of discrete serotypes. Thomas et al. (1979) showed that bluetongue viruses formed a continuum from

clear antigenic differences to virtually identical. If this is the case for the Isle of May viruses, they can be placed in an order of relationship: Mill Door/79 - Mill Door/81 - North Clett/81.

The original isolations of Mill Door/79, North Clett/81 and Mill Door/81 viruses were made from tick pools which may have contained more than one strain of orbivirus. This would not necessarily have been detected during serological studies, but could possibly have led to confusion when analysing biochemical data relating to these viruses. The clones of Mill Door/79 virus were found to be serologically identical with the possible exception of clone 1.

Table 3.1

Details of tick (*Ixodes uriae*) pools examined for the presence of viruses

Tick pool	Collection date	Collection site	Isolate name
10 female engorged *I.uriae*	2.7.79	North Clett	
5 male *I.uriae*	2.7.79	Mill Door	Mill Door/79
5 female engorged *I.uriae*	22.6.80	South Plateau	
5 male *I.uriae*	25.5.80	South Plateau	
5 female engorged *I.uriae*	28.6.81	North Clett	North Clett/81
5 male *I.uriae*	2.7.81	Mill Door	Mill Door/81

Table 3.2

Virus yield from infected mice

Inoculation	Incubation Period (days)		Titre[3] Log_{10} pfu/ml
	Pass 1	Pass 2	
Mill Door/79[1]	9-10	7-8	3.5
North Clett/81[2]	8	4	4.3
Mill Door/81[2]	11-13	6-7	4.6

[1] Inoculum, tick homogenate

[2] Inoculum was second passage cell culture

[3] Titrated in Vero cells

Table 3.3

Results of inoculating different cell lines with virus isolates

Virus inoculum	Cell lines			
	CEF[1]	Vero[2]	Xenopus[3]	BHK-21[4]
Mill Door/79	cpe[5]	6.9	<1.0	7.0
North Clett/81	cpe	4.6	<1.0	cpe
Mill Door/81	cpe	5.4	<1.0	cpe

[1] No titrations performed in CEF

[2] Virus grown and titrated in Vero cells

[3] Virus grown and titrated in Xenopus cells

[4] Virus grown and titrated in BHK-21 cells (North Clett/81 and Mill Door/81 not titrated)

[5] Cytopathic effect visible (sample not titrated)

Table 3.4

Sensitivity to physicochemical treatment

Treatment[1]			Virus isolate		
			Mill Door/79	North Clett/81	Mill Door/81
Ether			1.6[4]	0.5	1.0
Chloroform	18h		>5.2	3.1	3.0
	3h		1.0	2.4	1.8
Sodium deoxycholate[2]		0.5%	>4.5	>6.4	>5.6
		0.05%	0.9	0.2	0.3
		0.005%	0.1	0	0
Acid/Base[3]	pH 3.0		>5.5	>7.2	>6.7
	pH 5.0		1.5	0	0
	pH 9.0		0	0	0

[1] Mean of two experiments; titrated in Vero cells

[2] 0.5% SDC was toxic for cells at highest concentration when titrated

[3] Titres compared to HEPES at pH 7.4

[4] Log_{10} pfu/ml reduction in titre relative to a control diluted in either PBSa, for ether, chloroform and sodium deoxycholate, or pH buffer pH 7.5 for acid/base tests

Table 3.5

Comparison of the isolates by complement fixation tests

Immune ascitic fluid	Virus antigens[1]		
	Mill Door/79	North Clett/81	Mill Door/79
Mill Door/79	64[6]	16	8
North Clett/81	32	128	32
Mill Door/81	32	16	64
Cape Wrath[2]	32	32	16
Palyam[3]	<4	<8	ND[7]
Kemerovo[4]	<4	<8	ND
Polyvalent 8[5]	<4	<8	ND

[1] Antigen titres of Mill Door/81 and North Clett/81 - 1/8, antigen titre of Mill Door/79 - 1/16

[2-5] Reference ascitic fluids obtained from the American Type Culture Collection. (2) Kemerovo serogroup, Great Island Complex; (3) Palyam serogroup containing: Palyam, Vellore, Kasba, Corriparta, Acado, Eubenangee, Pata and D'Aguilar; (4) Kemerovo serogroup containing: Kemerovo, Chenuda, Mono Lake, Wad Medani, Tribec and Huacho; (5) Polyvalent 8 containing: Epizootic haemorrhagic disease virus, Changuinola, Irituia, Colorado tick fever, Bluetongue, IB Ar 22619

[6] Reciprocal of ascitic fluid titre

[7] Not done

Table 3.6

Comparison of the isolates by neutralization tests

Immune ascitic fluid	Virus		
	Mill Door/79	North Clett/81	Mill Door/81
Mill Door/79	64[1]	<8	<4
North Clett/81	<8	64	16
Mill Door/81	32	8	64

[1] Reciprocal of dilution of ascitic fluid which produced a 50% reduction in the number of plaques in Vero cells, compared with the control, untreated virus

Table 3.7

Serological comparison of the five plaque purified "clones" of Mill Door/79 virus

Virus	Immune ascitic fluid		
	Mill Door/79 "wild type"	Mill Door/79 "clone" 2	Mill Door/79 "clone" 3
Mill Door/79 "wild type"	64[1]	32	32
Mill Door/79 "clone" 1	64	16	<8
Mill Door/79 "clone" 2	64	32	32
Mill Door/79 "clone" 3	64	32	32
Mill Door/79 "clone" 4	64	32	32
Mill Door/79 "clone" 5	64	32	32

[1] Reciprocal of dilution of ascitic fluid which produced a 50% reduction in the number of plaques compared with the control, untreated virus in Vero cells



Figure 3.1. A typical tick collection site showing female *Ixodes uriae* ticks (arrowed). Finger indicates scale.

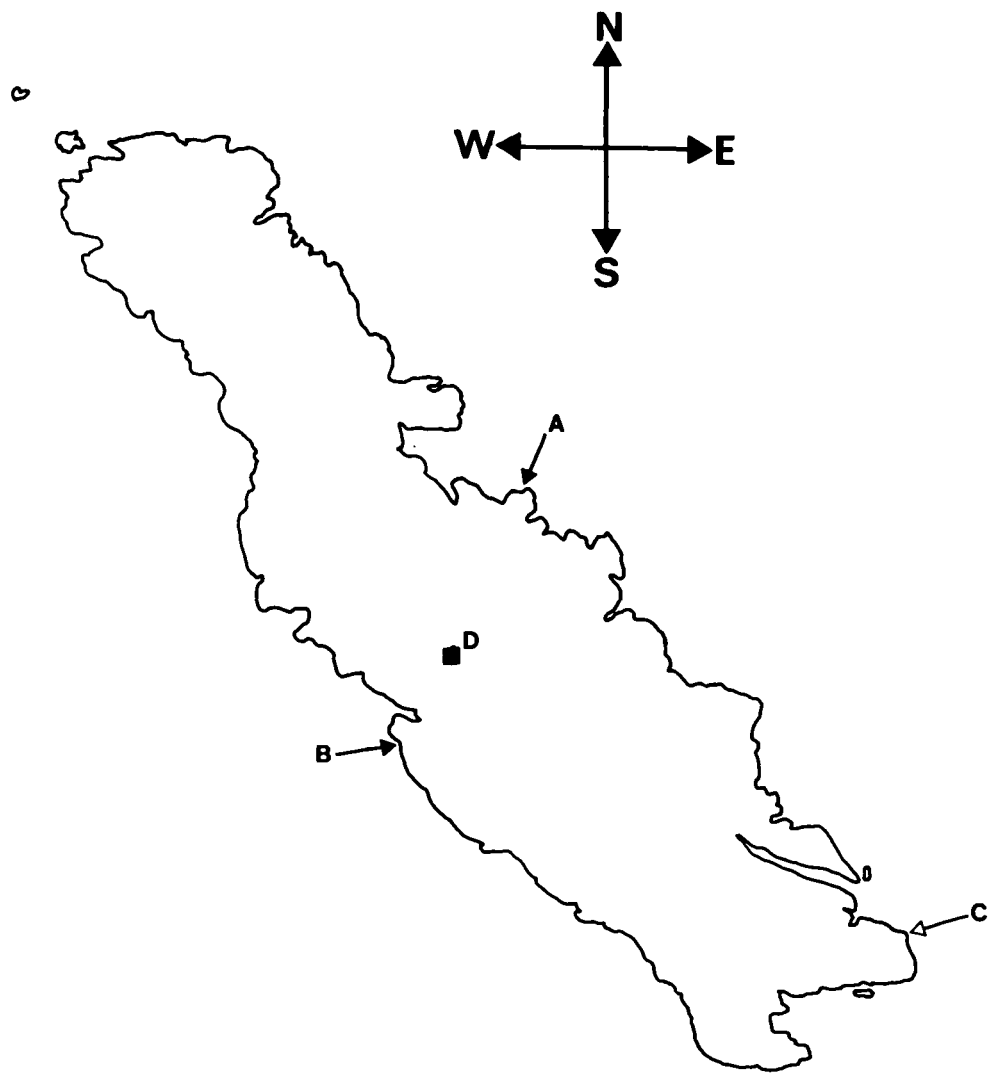


Figure 3.2. An outline map of the Isle of May. The bar represents 1.6km. A, North Clett; B, Mill Door; C, South Plateau; D, Light House (highest point of the Island).

◆ sites from which collected ticks contained viruses.

◇ sites from which collected ticks contained no virus.



Figure 3.3. North Clett, on the Isle of May, showing kittiwakes (Rissa tridactyla) on nests. Viruses were isolated from ticks collected at this site in 1981.



Figure 3.4. Mill Door, on the Isle of May showing nesting kittiwakes (Rissa tridactyla), guillemots (Uria aalge), and razorbills (Alca torda). Viruses were isolated from ticks collected at this site in 1979 and 1981.

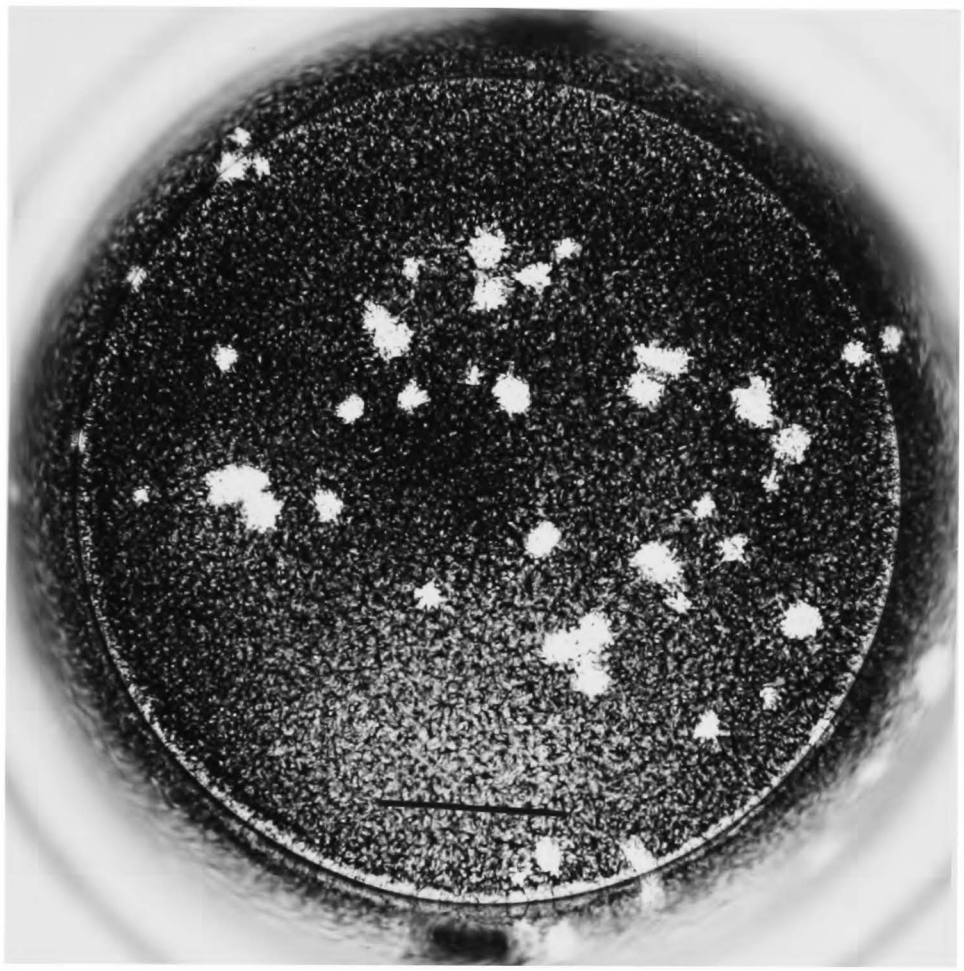
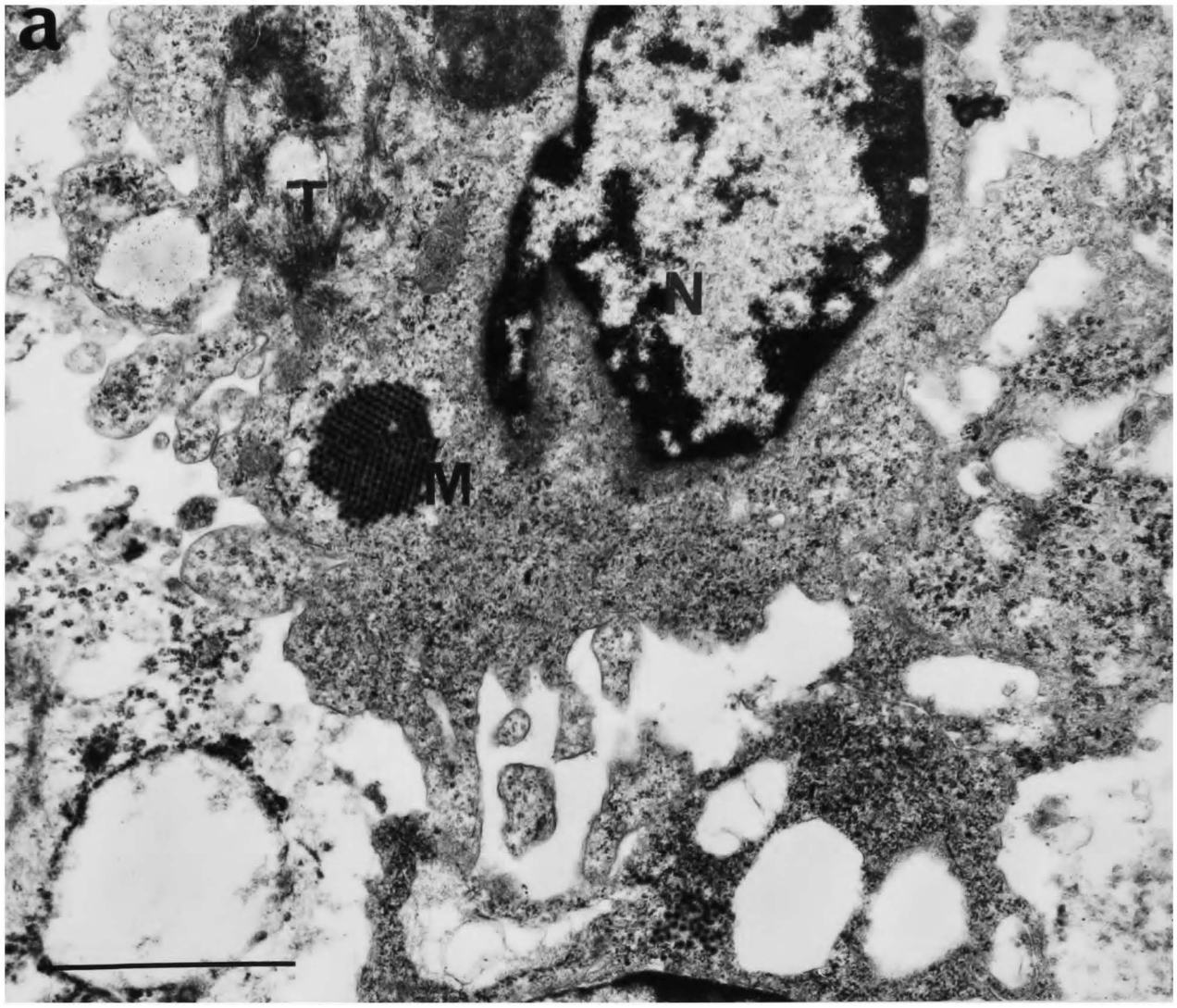


Figure 3.5. Plaque morphology of Mill Door/79 virus in Vero cell culture using L15-CMC overlay. Bar represents 5mm.

Figure 3.6. Thin section electron micrographs of Mill Door/79 virus infected BHK-21 cells. (a) 15,000x magnification of a cell containing a virus matrix (M) in the perinuclear region and virus associated tubules and "empty" capsids (T). N; nucleus. The bar represents 2μm. (b) 140,000x magnification of a virus matrix (M) showing the regular arrangement of particles. Some empty capsids (EC) are also shown in the region of the matrix. The bar represents 200nm.


a
T
N
M

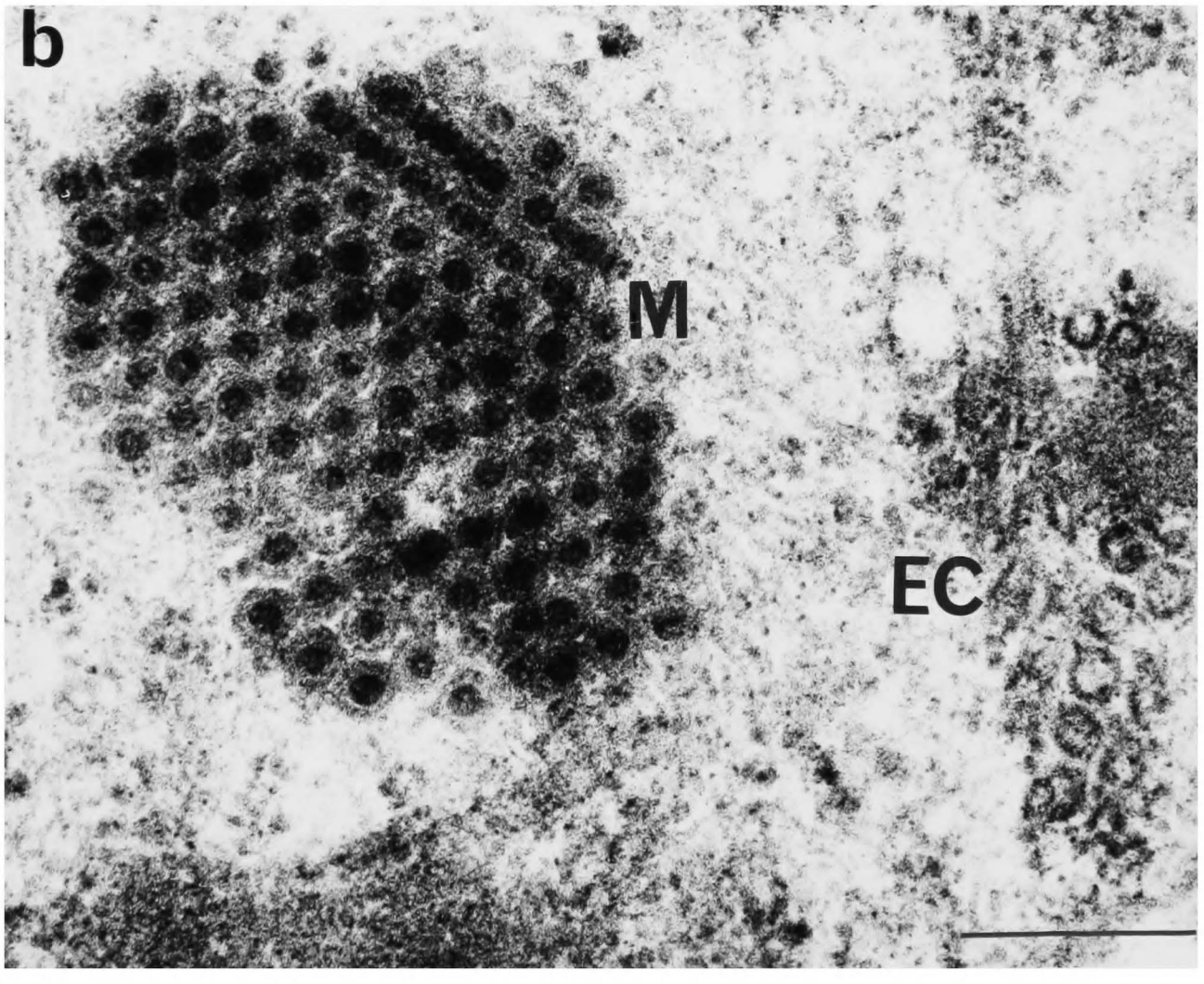
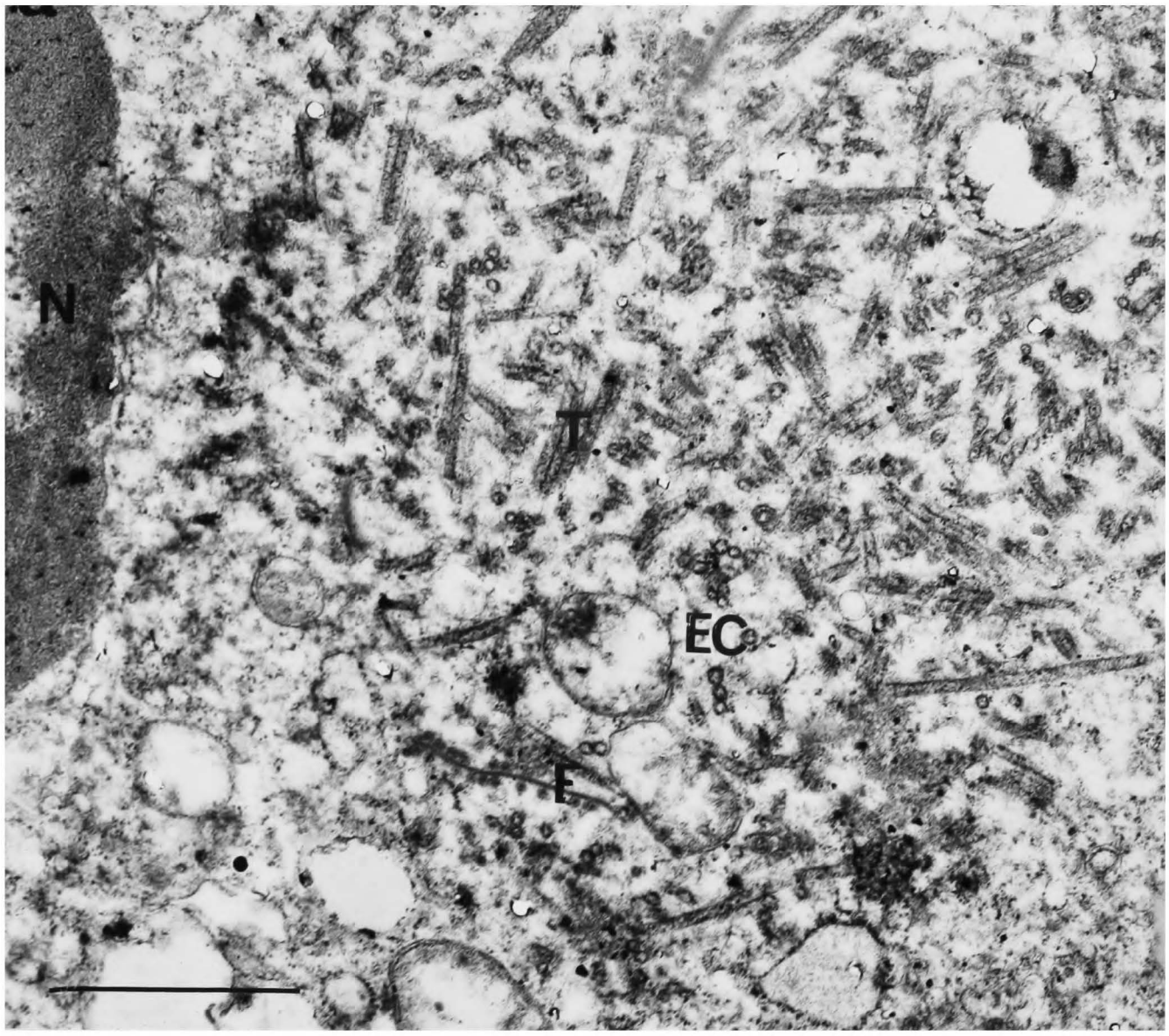
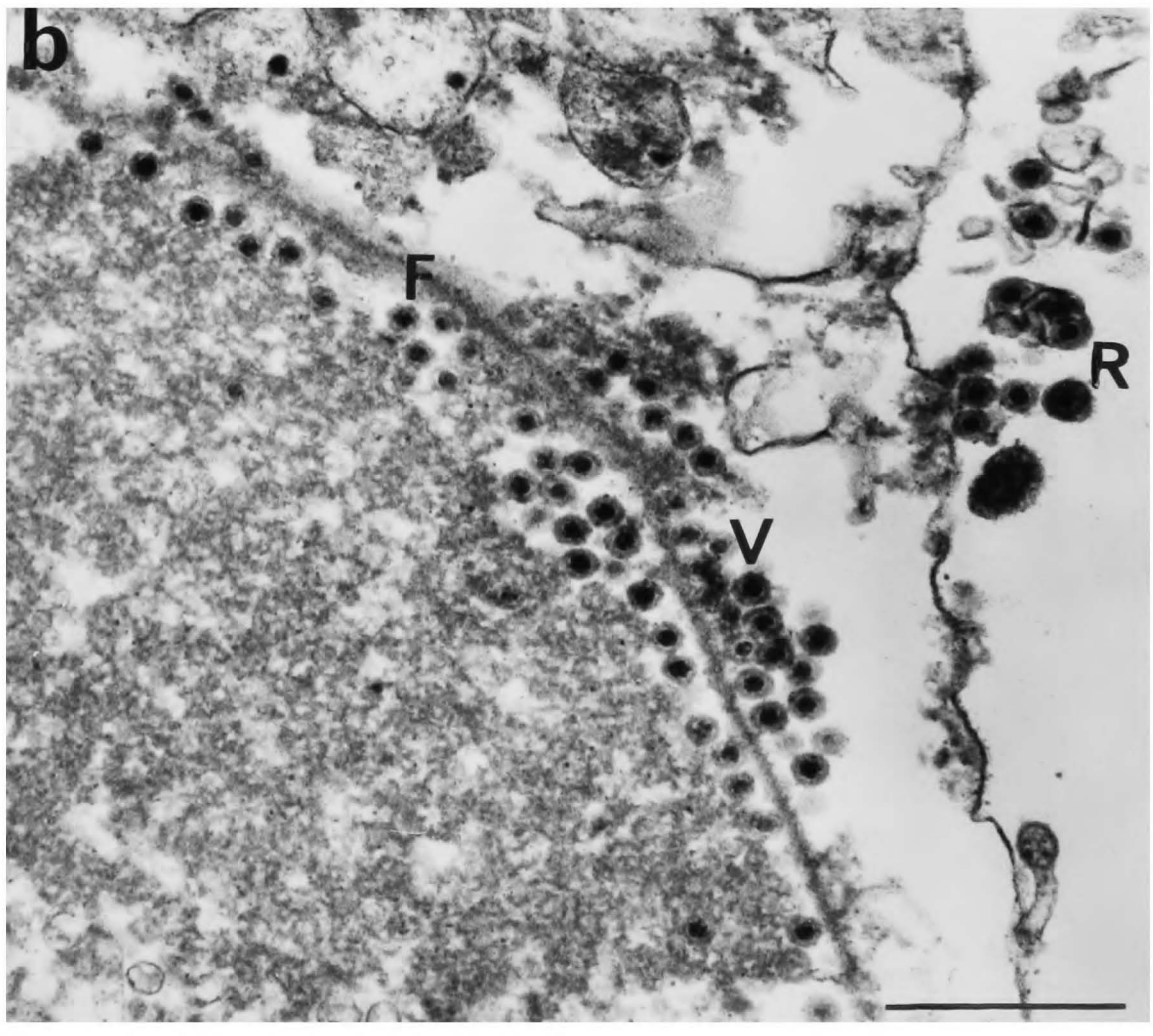

b
M
EC

Figure 3.7. Thin section electron micrographs of Mill Door/79 virus infected BHK-21 cells. (a) 14,500x magnification of cell cytoplasmic showing tubules (T), filaments (F) and empty capsids (EC). N; nucleus. The bar represents 2µm. (b) 615,000x magnification of a filament (F) with a cluster of virus particles (V). Virus particles are also shown released from the cells (R). The bar represents 440nm.


N
T
EC
F


b
F
V
R

CHAPTER 4

Page

THE REPLICATION AND PRODUCTION OF POLYPEPTIDES BY MILL DOOR/79 VIRUS

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I. INTRODUCTION

This chapter describes a study on the replication, and induction of protein synthesis by, Mill Door/79 virus, in both Vero and BHK-21 cells. Preliminary experiments were carried out to determine the time scale of a single replication cycle and the optimum multiplicity of infection. These experiments were followed by the use of radioactive precursors to identify the virus-induced polypeptides which were then characterized. A report on attempts to purify Mill Door/79 virus from cell culture is also presented. All experiments described below and in subsequent chapters were carried out on clone No. 2 of Mill Door/79 virus.

II. RESULTS

A. The Replication Cycle

Mill Door/79 virus was inoculated into Vero cell monolayers containing 1 x 10^6 cells at a multiplicity of 3 pfu/cell (6 x 10^6 pfu/ml). At intervals from 1 hour p.i. the cells were separated from the medium by centrifugation and disrupted by freeze-thawing to release virus. The titres of virus associated with the cells, and that released from the cells, were estimated separately. The titres given in Fig. 4.1 and quoted below are from a single experiment, although the pattern of replication was reproducible. At 1 hour p.i., the titre of cell-associated virus was 5.2 $\log_{10}$ pfu/ml (0.6 pfu/cell), which was 20% of the titre of virus originally adsorbed onto the cells. The titre rose steadily to a maximum of 7.1 $\log_{10}$ pfu/ml (50.4 pfu/cell) at 8-9 hours p.i., after which it remained relatively constant until 24 hours p.i.

At 40 hours p.i. the titre had fallen to 6.7 $\log_{10}$ pfu/ml (20.1 pfu/cell) (not shown); at this stage most of the cells had completely detached from the flask and were floating in the medium. From 2 hours p.i. the titre in the medium rose, reaching a maximum of 4.8 $\log_{10}$ pfu/ml at 11 hours p.i. Unlike the cell associated fraction, the titre in the extracellular fraction subsequently fell, dropping to less than 3.0 $\log_{10}$ pfu/ml at 40 hours p.i. (not shown). At 12 hours p.i. the first signs of cytopathic effects were visible under the light microscope. Comparison of the virus titres in the cell- associated and cell-free fractions at 5 and 10 hours p.i. indicated that only 0.1 and 0.2%, respectively, of virus was released from the cells. Samples of cell-associated and extra- cellular virus were each sonicated, using both a sonicating water bath and a soniprobe, with no change in titre in either sample compared to an unsonicated control. The total virus yield at 10 hours p.i. was 70 pfu/cell, 23 times the original infecting titre.

B. Effect of the multiplicity of infection on virus yield

The results below were used to determine the optimum multiplicity of infection for producing high yields of virus. Mill Door/79 virus was inoculated into Vero cells at multiplicities of 10, 1.0 and 0.1 pfu/cell and the cells and media harvested together at appropriate time intervals. When inoculated at 10 pfu/cell the titre reached a peak at 7-8 hours p.i.; cpe was observed from about 9 hours p.i. and the titre fell by 1 $\log_{10}$ pfu/ml by 24 hours p.i. (Fig. 4.2). When inoculated at 1 pfu/cell it took 12 hours for the titre to peak; cpe appeared between 16–24 hours p.i. At 24 hours p.i. the titre was

reduced from its maximum by 1 $\log_{10}$ pfu/ml. The titre, after inoculation at 0.1 pfu/cell, peaked at 24 hours p.i. when cpe was just evident. The yield of virus at each peak titre was 210 pfu/cell (at infecting multiplicities of 10 and 1.0 pfu/cell) and 84 pfu/cell (at an infecting multiplicity of 0.1 pfu/cell). Infecting cells at a multiplicity of 0.1 pfu/cell and harvesting at 24 hours p.i. was therefore 4-40 times more efficient than infecting cells at a multiplicity of either 1.0 or 10 pfu/cell and harvesting at 12 hours p.i.

C. Virus-induced polypeptides in infected Vero cells

Vero cell monolayers, inoculated at a multiplicity of 20-30 pfu/cell, were labelled with 100μCi/ml [^{35}S] methionine for 1 hour periods from 1-10 hours p.i. Figure 4.3 shows the protein profiles of the cell lysates harvested after each labelling period (the virus-induced polypeptides are identified according to molecular weight, see Section E). The production of polypeptides in uninfected cells did not change significantly during the period of the experiment (Fig. 4.3, tracks A and K). At 1-2 hours p.i. virus-induced polypeptides were not detected (Fig. 4.3, track B), whereas at 2-3 and 3-4 hours p.i. polypeptides p53, p51 and p21 could be seen (Fig. 4.3, tracks C and D). At 4-5 hours p.i. most of the virus-induced polypeptides were visible (Fig. 4.3, track E). Between 4 and 10 hours p.i. host-cell protein synthesis was reduced and by 9-10 hours p.i. nearly all of the polypeptides labelled were virus-induced (Fig. 4.3, tracks J and L).

A total of thirteen polypeptides were identified in infected cells, if p53 and p51 were regarded as a doublet (although it was not always resolved). The virus-induced polypeptides were

analysed on 10-20% gradient PAGs in order to determine whether polypeptides smaller than those resolved on 12.5% PAGs could be identified. Polypeptides were not seen below p20 (Fig. 4.4) and no additional virus-induced polypeptides were identified elsewhere. The resolution of the polypeptides between p141 and p36 was not as clear as on 12.5% PAG, hence further investigations on gradient gels were not performed.

Each virus-induced polypeptide remained throughout all subsequent labelling periods once it had appeared in infected cells. The relative amount of each polypeptide also remained constant between each labelling period (Fig. 4.5). A protein digest of host-cell polypeptides and virus-induced polypeptides is shown in Fig. 4.6. The oligopeptide patterns produced by uninfected cells (left) and infected cells (right) were distinct, indicating that the virus-induced polypeptides were unique to infected cells and were unlikely to be cell products which either failed to "shut-off" of were amplified by infection.

D. The suitability of [^{35}S] methionine as the radioactive precursor

The possibility that some of the virus-induced polypeptides were deficient in methionine, and were therefore not detected in cell lysates labelled with [^{35}S] methionine, was examined. Cells were labelled from 7-8 hours p.i. with [^{14}C] protein hydrolysate in amino acid deficient media. Figure 4.7 shows a densitometer scan of [^{14}C]-labelled proteins separated on a 12.5% PAG. The uninfected cell protein profile is at the bottom and the infected cell protein profile at the top. When compared with the densitometer scan of [^{35}S] methionine-labelled proteins (Fig. 4.5) no extra virus-induced proteins could be identified.

E. Determination of the molecular weights of the virus-induced polypeptides

[^{14}C] amino acid-labelled virus-induced proteins were co-run on a 12.5% PAG with [^{14}C]-labelled molecular weight marker proteins. Figure 4.8 shows a densitometer scan of their profiles after autoradiography. Using the method of Hames (1982) a calibration curve was drawn (Fig. 4.9) from which the molecular weights of the virus-induced polypeptides were derived (Table 4.1). These ranged between 141,300 and 20,000 daltons.

F. Determination of the relative amounts of virus-induced polypeptides

Assuming that the X-ray film used to make autoradiographs had a linear response to the level of radioactivity in each band, the amount of each protein present in infected cells was estimated. Peaks from densitometer scans of autoradiographs of [^{14}C] amino acid-labelled protein PAGs were cut out and weighed. The weight of each individual peak was taken to be directly proportional to the amount of each polypeptide it represented. Table 4.2 shows the relative amount of each virus-induced polypeptide in infected cells when labelled with either [^{14}C] amino acids or [^{35}S] methionine. Polypeptides p141, p30 and p27 were produced at a third to half the expected value (each of the 13 polypeptides, if produced in equal amounts, would comprise 7.7% of the total virus-induced protein) whereas p69 and p44 were produced at 1.5-2.5 times the expected values. When the relative amounts of [^{14}C] amino acid and [^{35}S] methionine-labelled polypeptides were compared differences were apparent. Only p65, p44, p36 and p20 gave similar values. p141 and p93 appeared to contain proportionately less methionine, while p37, p30, p27 and

p21 appeared to be methionine-rich. Because of the difficulty in separating the putative doublet, p53/51, it was not possible to calculate separate molecular amounts for these polypeptides.

G. Effect of Actinomycin D on virus replication

Vero cells were inoculated with Mill Door/79 virus at a multiplicity of 0.1 pfu/cell and incubated in the presence of several concentrations of actinomcycin D (AMD) for 24 hours, when the cells and media were harvested for titration. Table 4.3 shows the titre of virus at each AMD concentration. Even at concentrations of 10µg/ml no reduction of virus titre was observed.

Vero cells were also inoculated with Mill Door/79 virus at a multiplicity of 20-30 pfu/cell, treated with 10µg/ml AMD at 3 hours p.i., and pulse labelled with [^{35}S] methionine for 1 hour at 7 hours p.i. Host cell protein synthesis was not affected (Fig. 4.10, tracks A and B), although a slight, but reproducible, reduction was seen in the levels of virus-induced protein synthesis (Fig. 4.10, tracks C and D). Similar results were obtained with several samples of freshly prepared AMD using Vero, CEF and BHK-21 cells. Since Mill Door/79 virus was efficient at shutting off host cell protein synthesis, further treatment with AMD under these experimental conditions was abandoned.

H. Virus-induced polypeptides in BHK-21 cells

To confirm that the polypeptides produced in infected Vero cells were virus-specified, polypeptide synthesis in BHK-21 cells infected with Mill Door/79 virus was examined. Both BHK-21 and Vero cells were inoculated at a multiplicity of 20-30 pfu/cell and pulse labelled for 1 hour at 7 hours p.i. A similar pattern

of virus-induced polypeptides was produced from both cell lines, although there were some differences (Fig. 4.11). p65 (arrowed) was present in Vero, but not BHK-21 cells and p27 was present in reduced amounts in BHK-21 cells. The products of virus-infected Vero and BHK-21 cells, when subjected to partial proteolysis using S.aureus V8 protease, were found to be indistinguishable (Fig. 4.12). Digestion products of p27 were present in BHK-21 cells in small amounts.

I. Immune precipitation of virus-induced polypeptides

[^{35}S] methionine-labelled proteins from infected and uninfected cells were subjected to immune precipitation using both preimmune ascitic fluid and ascitic fluid raised against Mill Door/79 virus (clone No. 2). Of the 13 virus-induced polypeptides, only 4 reacted with the immune ascitic fluid (Fig. 4.13, track F). The putative doublet p53/51 also reacted slightly with the preimmune ascitic fluid (Fig. 4.13, track E). The specificity of these reactions was demonstrated by the failure of host cell polypeptides to react with either ascitic fluid (Fig. 4.13, tracks B and C).

J. Purification of Mill Door/79 virus

Two methods of purification were examined.

(i) Polyethylene glycol (PEG-6000) method. Before attempting to purify Mill Door/79 virus by this method, the effect of treatment with PEG-6000 and Nonidet P40 (a non-ionic detergent) was determined. Stock virus was precipitated with 5% (w/v) PEG-6000 and titrated. Approximately 90% of the infectivity was recovered from the precipitated fraction. 1% (v/v) Nonidet P40 had no effect on the titre of Mill Door/79 virus after 4 hours

incubation at 4°C.

Cells, inoculated at a multiplicity of 0.1 pfu/cell, were labelled with [^{35}S] methionine 6 hours p.i. in order to reduce the amount of host-cell protein labelling. The cells were disrupted by a single cycle of freeze-thawing and then mixed with PEG-6000 to precipitate the proteins. Cell membranes in the pelleted precipitate were broken down by the addition of Nonidet P40 followed by gentle homogenization. The components of this mixture were separated by centrifugation through a 20-60% sucrose gradient. Since no visible band was detected, the gradient was fractionated and each fraction (1ml) analysed for both radioactivity and infectivity. Figure 4.14A and B shows the radioactive (pre- and post-pelleting) and infectivity profiles of the gradient. A relatively high number of counts were present throughout the gradient, although more were present at the top (Fig. 4.14A). Due to the pelleting step after PEG-6000 precipitation, most of the radioactivity was probably associated with particulate matter, rather than being present as free [^{35}S] methionine. Fractions were therefore pelleted to concentrate the radioactive components. Before pelleting, samples were removed from each of the fractions, pooled and titrated. The maximum titre was located in fractions 5 and 6; no infectivity was present above fraction 7 and 8 (Fig. 4.14B). The pelleted fractions 4, 5 and 6 contained the highest levels of radioactivity (Fig. 4.14A). Each pelleted fraction was analysed by PAGE followed by autoradiography. Figure 4.15 shows the polypeptide profile of each fraction. Track A contains intracellular virus-induced polypeptides. Fractions 5 and 6 (tracks F and G), which contained the highest infectivity and levels of radioactivity contained a high background of host cell proteins, but

also contained virus polypeptides p93, p69, p53/51 and p37. The small virus polypeptides p21 and p20 (indicated by a single arrow) were also detected at low levels. Infectivity was detected throughout the bottom half of the gradient; these fractions (1-3) also contained the polypeptides listed above, with the exception of p21 and p20 (Fig. 4.15, tracks B-D). No infectivity was associated with fractions 9 and 10 and 11 and 12, although at the top of the gradient (fraction 11) polypeptides p93, p53/51 and p37 were clearly visible (Fig. 4.15, track L). p69 was not detected in fractions 7 to 11 (Fig. 4.15, tracks H to L).

(ii) Ether extraction method. Vero cells, inoculated and labelled as for the polyethylene glycol method, were harvested by scraping and centrifugation. The results from Section A showed Mill Door/79 virus to be highly cell associated, and this fact was exploited, to avoid an extra concentration step, by pelleting the intact cells. The supernatant, which only contained 0.1-0.2% infectious virus was discarded. The cell pellet was homogenized in a low ionic strength (20mM Tris, 1mM EDTA), high pH (pH 8.0) buffer (conditions that stabilize orbiviruses; Verwoerd, 1969) containing EDTA (to chelate divalent cations, reducing virus aggregation) and 1% (w/v) Nonidet P40 (to rupture the cell membranes and lyse the cells, while leaving the nuclear membranes intact). The cell debris and nuclei were pelleted and the supernatant, which contained 84% of the total infectivity, was extracted twice with ether at 4°C for a total period of 10 minutes. Ether treatment did not affect the virus titre. After concentration by pelleting, the extract was centrifuged through a sucrose gradient and, as no bands were visible, fractionated. Each fraction (0.5ml) was analysed for both radioactivity and

infectivity before being pooled and pelleted. Figure 4.16 shows the infectivity and radioactive profile of the gradient. The highest number of counts occurred near the bottom of the gradient, and steadily decreased towards the top (Fig. 4.16A). Infectious virus was present from the bottom of the gradient (fractions 1-3) to fractions 19-21, but none was detected above these fractions (Fig. 4.16B). Two slight peaks of infectivity were identified (fractions 10-12 and 16-18) (Fig. 4.16B). Before pelleting, the gradient fractions were pooled into groups of 2. The radioactive profile of the pellets reflected that of the original gradient fractions; no peaks could be detected. The low number of counts in the pelleted fractions necessitated further pooling before analysis by PAGE. Figure 4.17 shows the polypeptide profile of each fraction. Track A contains intracellular virus-induced polypeptides, tracks B and C contain samples from the infectivity peaks (fractions 9-12 and 17-20, respectively) and tracks D and E contain samples from the bottom of the gradient (fractions 1-4 and 5-8, respectively). Both the fractions containing the higher infectivity (9-12 and 17-20) contained polypeptides p93, p69 p53/51 and p37. The background of host polypeptides was reduced in the pooled fractions 9-12 compared to fractions 17-20. The fractions from the bottom of the gradient contained fewer polypeptides, with only p93, p53/51 and p37 being easily detected. The relative amounts of labelled polypeptides were calculated from the densitometer scan of the autoradiograph in Fig. 4.16 (Fig. 4.18). The doublet p53/51 was present in large quantities, comprising 50-80% of the protein in the PAG profile (Table 4.4).

No orbivirus-like particles were observed when the pelleted material was examined by electron microscopy.

III. DISCUSSION

In the previous chapter Mill Door/79, North Clett/81 and Mill Door/81 viruses were shown to belong to the Kemerovo serogroup. Although this is a large group containing 21 serotypes, some of which are human and animal pathogens, little information is available on the intracellular events occurring during the replication of these viruses. The aim of the study reported here was to identify the proteins produced in infected cells by one of the Isle of May isolates, Mill Door/79 virus, to characterize them by size and relative abundancy, and to purify the virus from cellular components in order to determine which virus-induced proteins were structural. The first two of these aims were achieved, but virus purification proved difficult to accomplish.

The replication of orbiviruses in cell culture has been studied, in relation to the production of infectious progeny, by Howell et al. (1967) and Lewanczuk and Yamamoto (1982). These workers, using bluetongue and epizootic haemorrhagic disease viruses respectively, constructed single step growth curves showing peak infectivity at 12 hours p.i., with titres remaining relatively constant afterwards. In contrast, Mill Door/79 virus reached maximum titres at 8-9 hours p.i. Orbiviruses are usually regarded as highly cell associated (Verwoerd, 1969; Huismans et al., 1979), but Lewanczuk and Yamamoto (1982) showed that sonication of the extracellular and cell-associated fractions of an epizootic haemorrhagic disease virus preparation before titration led to most infectivity being present in the extracellular fraction. In contrast, even after sonication of the cellular and extracellular fractions, Mill Door/79 virus appeared to be highly cell associated. [Lewanczuk and Yamamoto (1982) used a 3/8 inch

diameter sonicator probe and treated samples for 45 seconds at a maximum setting; the settings for sonication of Mill Door/79 virus were arranged to simulate these conditions]. The extracellular fraction of Mill Door/79 virus appeared to be significantly less stable than the cell-associated fraction, its titre falling by 99.5% between 12 and 40 hours p.i. It was possible that virus released into the medium was inactivated while that remaining within the cells was protected or stabilized. Extracellular titres of epizootic haemorrhagic disease virus were not reduced with time (Lewanczuk & Yamamoto, 1982) and this virus was relatively resistant to heat inactivation, losing 1.5 LD_{50}/ml after 24 hours at 37°C (Borden et al., 1971). However, other orbiviruses, including Kemerovo serogroup viruses, were more susceptible to heat inactivation (Borden et al., 1971) and this may have accounted for the instability of extracellular Mill Door/79 virus. The fall in extracellular titre after 12 hours p.i. was, therefore, probably due to a combination of inactivation and reduced virus release from cells. This reduction was probably the result of cell damage (cpe was visible from 12 hours p.i.) leading to reduced virus production (the titre of intracellular virus failed to increase from 8-9 hours p.i.).

The intracellular proteins of orbiviruses have received scant attention in the literature and consequently there is some confusion as to how many virus specified proteins are produced in infected cells (Huismans, 1979; Gorman et al., 1981; Sangar & Mertens, 1983; Gorman et al., 1984). Mill Door/79 virus produced 13 polypeptides in both Vero and BHK-21 cells. The polypeptide p53/51 was regarded as a doublet, although it was not always possible to separate the two components. The virus specificity

of the polypeptides was confirmed by (i) their presence in infected, but not uninfected cells, (ii) the stimulation of their synthesis following infection, (iii) similar infected cell polypeptide profiles in different cell lines and (iv) the specific precipitation of some of the polypeptides by antisera raised against the virus grown in suckling mice. This last observation only confirmed the virus specificity of four polypeptides. Huismans (1979) demonstrated the production of 9 viral polypeptides associated with bluetongue virus infections in cell culture at 31°C. The first polypeptides appeared 2-4 hours p.i. and P3, P4, P5 and P5A were present after 6-8 hours. At 37°C, Mill Door/79 virus polypeptides first appeared at 2-3 hours p.i.; the appearance of the other virus polypeptides followed rapidly, all being present at 5 hours p.i. The 9 virus-specified polypeptides of bluetongue virus ranged in molecular weight from 140,000 to 29,000 daltons (Huismans, 1979) (Table 4.5). Mill Door/79 virus polypeptides ranged from 141,000 to 20,000 daltons. Huismans (1979) used 7% PAGs and may therefore have failed to detect the smaller polypeptides seen in Mill Door/79 virus infected cells. Using 8% PAGs, Gorman et al. (1984) detected 11 virus-specific polypeptides in cells infected with Nugget virus, a member of the Kemerovo serogroup. These workers identified two small polypeptides (possibly comparable to p21 and p20 of Mill Door/79 virus), but did not detect a polypeptide comparable to p27 of Mill Door/79 virus. However, Gorman et al. (1984) used BHK-21 cells and it has been shown that the levels of p27 are reduced in these cells when infected with Mill Door/79 virus (Section II, H). Although Gorman et al. (1984) did not calculate the sizes of the polypeptides of Nugget virus the pattern they gave on PAGs was similar to that of Mill Door/79 virus.

In a comparison of the polypeptides of bluetongue and Mill Door/79 viruses, P1 of bluetongue virus and p141 of Mill Door/79 virus are approximately the same size, whereas P2-P4 are significantly larger than p93-p65, and P5-P7 have similar respective molecular weights to p53/51-p30 of Mill Door/79 virus. Comparing proteins by their size alone can be misleading, since proteins that co-migrate on PAGs may have completely different functions and amino acid compositions. Bluetongue and Mill Door/79 viruses are classified within the same genus and share many morphological properties in infected cells, such as capsid structure and the production of tubules and filaments (Murphy et al., 1971; Spence et al., 1985a). If their basic modes of replication and their structures are similar, the relative amounts of each virus specified protein, produced by either Mill Door/79 or bluetongue virus, should depend on their functions. Thus, proteins with the same function should be present in similar quantities. The relative molar amounts of Mill Door/79 virus polypeptides, when compared with those of bluetongue virus, could indicate which proteins are functionally or structurally similar. Using the values obtained by Huismans (1979) for bluetongue virus type 10A, the following polypeptides were matched (when making these matches some effort was made to pair polypeptides of similar size): P1 with p141, the group P2-P4 with the group p93-p65, P5 with p53/51, P5A with p44 and the group P6-P7 with the group p37-p27. Polypeptides displaying similar relative molecular amounts did not necessarily have the same molecular weights, for example, P5 and the doublet p53/51 comprised 10.9 and 11.1% respectively of the relative molar amounts of polypeptides in bluetongue and Mill Door/79 virus infected cells but the molecular weights varied by about 9,000

daltons. The interpretation of these data must be made with care as the calculations of the relative molar amounts were based on three assumptions. [^{14}C]-labelled protein hydrolysate contains a pool of all 20 amino acids required for protein synthesis. It was assumed that these were incorporated into the protein products in such a way that the level of radioactivity in each was proportional to the amount of protein produced. However, if the sizes of different amino acid pools within the cells varied, some labelled amino acids may have been incorporated to a greater or lesser extent than others. Hence, the level of radioactivity in each product would depend not only on the amount produced but also on the amino acid composition. By using a mixture of labelled amino acids these errors were minimised. It was also assumed that the response of the X-ray film to the amount of radioactivity in each band was linear and that the response of the densitometer to the exposed autoradiograph was also linear. These assumptions could lead to significant errors, since a high degree of radioactivity would turn the X-ray film black and any subsequent exposure would not be detected. Moreover, under exposure of the X-ray film, to avoid this problem may cause a failure to accurately quantitate faint bands.

Knowledge of the relative molar amounts of virus-specified polypeptides in infected cells can be used to determine whether protein production is under any form of control. If the transcription and translation rates of each virus specified mRNA species were equal, the virus polypeptides would be present in equal amounts as listed in Table 4.2, column 6. If these values are compared with those obtained from infected cells (column 5) it is clear that the proteins cannot all be translated at equal frequencies. p141, p30 and p27 were present in reduced amounts

while p53/51 and p44 were present in increased amounts. The amounts of the other virus-specified polypeptides did not deviate greatly from their expected values. This data does not identify the level at which control is occurring. However, Huismans (1979) showed that the rate of translation to transcription frequency did not deviate far from unity for bluetongue virus, hence control probably occurred only at the level of transcription (Huismans & Verwoerd, 1973).

Through its interaction with double-stranded DNA, AMD, from Actinomyces antibioticus, can block DNA-dependent RNA synthesis by blocking chain extension (Sobell, 1973). This property has been used to reduce host-cell protein synthesis and hence reveal the synthesis of proteins by RNA viruses (Reich et al., 1962). The effect of AMD on Mill Door/79 virus replication was investigated because Verwoerd (1969) had reported bluetongue virus to be sensitive at concentrations of 0.5µg/ml, leading to a reduction of infectious virus yield. Mill Door/79 virus was unaffected by AMD concentrations up to 10µg/ml, but because host cell protein synthesis was also unaffected doubt was raised as to the activity of the antibiotic. The repeated failure of fresh preparations of AMD to inhibit cell protein synthesis led to the conclusion that it was inactive under the experimental conditions employed. Mill Door/79 virus was able to efficiently reduce host-cell protein synthesis without AMD, so further investigations using AMD were not performed under normal conditions.

Mill Door/79 virus protein production in BHK-21 cells was investigated for two reasons. First, to demonstrate the virus specificity of the proteins produced, and secondly, because most previous investigations of orbiviruses had been performed using

BHK-21 cells (Verwoerd, 1969; Verwoerd et al., 1972; Martin & Zweerink, 1972; Gorman et al., 1981; Gorman et al., 1984). A polypeptide corresponding to p65 was not readily detected in BHK-21 cells and p27 was present in reduced amounts. The significance of this was not clear since the functions of these polypeptides are unknown. The production of proteins by Kemerovo serogroup orbiviruses in different cell lines has been studied by Eley et al. (manuscript in preparation). Some polypeptides were readily detected in CEF while being less apparent in BHK-21 and Vero cells, whereas another polypeptide, identified in CEF, was not found in Vero and BHK-21 cells. However, a polypeptide, VP7 (corresponding to p27 of Mill Door/79 virus), was present in reduced amounts in BHK-21 cells and CEF compared to Vero cells.

If ascitic fluid capable of neutralizing Mill Door/79 virus is mixed with virus-specified proteins from infected cells it should be possible to identify which of these proteins are involved in serological reactions, although this would depend somewhat on the quality or method of preparation of the antigen used to produce the ascitic fluid. Only 4 polypeptides (p93, p53/51, p37 and p36) were precipitated from infected cell lysates by homologous ascitic fluid. This contrasted with the data of Huismans (1979) in which 8 bluetongue virus polypeptides were precipitated from infected cells. The method of antibody preparation differed in each case: Huismans (1979) prepared anti-sera against cytoplasmic extracts of infected cells, whereas Mill Door/79 virus ascitic fluid was prepared against virus-infected suckling mouse brain. Since the ascitic fluid used to precipitate the virus-specified proteins was capable of neutralizing Mill Door/79 virus, one of the polypeptides precipitated must have been involved in type-specific serological reactions.

In attempts to purify Mill Door/79 virus from cell culture two main problems had to be overcome: the strong tendency of the virus to adhere to cellular components, and its instability following removal of this material during purification. Previously, purification methods employed for orbiviruses have included extractions with fluorocarbons and ether, and treatment with detergents such as Triton X-100 (Verwoerd, 1969; Verwoerd et al., 1972; Martin & Zweerink, 1972). These procedures proved unsuitable for Mill Door/79 virus since they reduced the infectivity by over 99%. For this reason a method of purification involving gentle procedures was carried out using PEG-6000 to precipitate virus particles, having first shown that PEG-6000 had little effect on the infectivity of Mill Door/79 virus. PEG-6000 also precipitated host cell and virus non-structural proteins. Attempts were therefore made, using Nonidet P40 and sucrose density gradient centrifugation, to remove this unwanted material. These attempts were not entirely successful, since infectivity was found spread across the gradient and samples contained many non-viral proteins, when analysed by PAGE. A less gentle method, involving ether extraction to remove some of the lipid from the samples, was also employed. After sucrose density centrifugation, infectivity was still present throughout most of the gradient, although the samples contained much less host cell protein contamination as judged by PAGE. The results indicate that Mill Door/79 virus was tightly bound to cellular material and that removal of this cellular material led to loss of infectivity. Whether this was due to the process of removal, or the instability of the virus after removal of cellular material, was not clear. The presence of infectivity throughout the gradients also suggested that the virus particles were attached

to a heterogeneous collection of cellular components, producing aggregates with different densities.

The problems discussed above were also encountered during attempts to purify Nugget virus (Gorman et al., 1984). These workers used chelating agents and a zwitterionic detergent together with fluorocarbon extractions to remove cellular contaminants. Infectious fractions from sucrose density gradients were analysed by PAGE. Some of the samples contained 7 polypeptides, the same number as that reported for purified bluetongue virus (Martin & Zweerink, 1972). Mill Door/79 virus contained 5-7 polypeptides (p93, p69, p53/51, p37 and possibly p21 and p20). This difference may have been due to fragmentation of Mill Door/79 virus during the final pelleting stage of purification, as negative stained preparations of the pellet did not appear to contain virus particles.

Three of the "structural" polypeptides, p93, p53/51 and p37, were precipitated from infected cells by ascitic fluid raised against infected suckling mouse brain, but it was unclear why the other "structural" polypeptides were not precipitated. The difference between the relative amounts of polypeptides present in infected cells and those present in "purified" virus preparations was marked. p53/51 was found in infected cells in nearly twice the expected amount but comprised over 50% of the "purified" virus (calculations made using [^{35}S] methionine labelled proteins). This data would suggest that the composition of the Mill Door/79 virus capsid is different from the bluetongue virus capsid, which contains 4 major and 3 minor proteins. However, until an improved purification procedure for Mill Door/79 virus is developed comparisons with other orbiviruses must be made with care.

Table 4.1

Molecular weights of Mill Door/79 virus induced polypeptides

Polypeptides[a]	Mol. wt. x 10^{-3}[b]
A (p141)	141.3
B (p93)	93.3
C (p69)	69.2
D (p65)	64.6
E (p53)	52.5
F (p51)	51.3
G (p44)	43.7
H (p37)	37.2
I (p36)	35.5
J (p30)	29.5
K (p27)	27.0
L (p21)	20.9
M (p20)	20.0

[a] refers to Fig. 4.3

[b] determined on a 12.5% polyacrylamide gel

Table 4.2

Relative amount of labelled Mill Door/79 virus polypeptides in infected cells

Polypeptide	Relative amount in cells		$[^{14}C]/[^{35}S]^{c}$	Adjusted relative molar amount[d]	Expected relative molar amounts[e]
	$[^{14}C]$ label[a]	$[^{35}S]$ label[b]			
p141	3.0	0.2	15.00	0.8	2.0
p93	7.3	2.3	3.30	3.4	3.0
p69	13.6	8.9	1.53	3.9	4.0
p65	7.6	8.0	0.95	5.1	4.3
p53/51	13.6	11.6	1.17	11.1	5.4
p44	18.8	13.1	1.44	18.3	6.4
p37	8.8	15.4	0.57	10.2	7.6
p36	6.3	4.0	1.26	7.6	7.8
p30	3.0	6.1	0.49	4.2	9.4
p27	1.9	5.2	0.37	3.0	10.4
p21	8.0	15.0	0.53	16.2	13.4
p20	7.6	8.9	0.88	16.2	14.0

[a] Relative amounts calculated from densitometer tracing in Fig. 4.7. Each peak was cut out and weighed and the results normalized to a percentage of the total.

[b] Calculated as (a) from densitometer tracing from Fig. 4.5.

[c] $[^{14}C]:[^{35}S]$ ratio.

[d] Relative amount from (a) divided by corresponding molecular weight and normalized to a percentage.

[e] Relative molar amounts if all polypeptides are translated at the same frequency.

Table 4.3

Effect of Actinomycin D (AMD) on the growth of Mill Door/79 virus

AMD concentration (µg/ml)	Titre of virus
0	7.3*
0.1	7.4
1.0	7.3
5.0	7.4
10.0	7.6

* Titres expressed as $\log_{10}$ pfu/ml in Vero cells.

Table 4.4

Relative amounts of labelled Mill Door/79 virus polypeptides in gradient fractions compared with infected cells

Polypeptide	Relative amount in [^{35}S] labelled cells[a]	Relative amount in gradient fractions [^{35}S] labelled[b]	
		Top fraction	Bottom fraction
p141	0.2		
p93	2.2	10.0	3.9
p69	8.9	6.9	0.1
p65	8.0		
p53/51	11.6	51.6	78.6
p44	13.1		
p37	15.4	19.1	12.7
p36	5.0		
p30	6.1		
p27	5.2		
p21	15.0	5.4	2.0
p20	8.9	9.9	2.6

[a] Taken from Table 4.2.

[b] Calculated as Table 4.2 from scan Fig. 4.18

Table 4.5

Molecular weights and relative molar amounts of bluetongue virus induced polypeptides[a]

Polypeptide	Molecular wt. x 10^{-3}	Relative molar amount
P1	140	1.1
P2	110	5.2
P3	101	4.7
P4	82	5.6
P5	61	10.9
P5A	54	25.2
P6	42	8.2
P6A	40	24.4
P7	29	14.6

(a) Data from Huismans (1979).

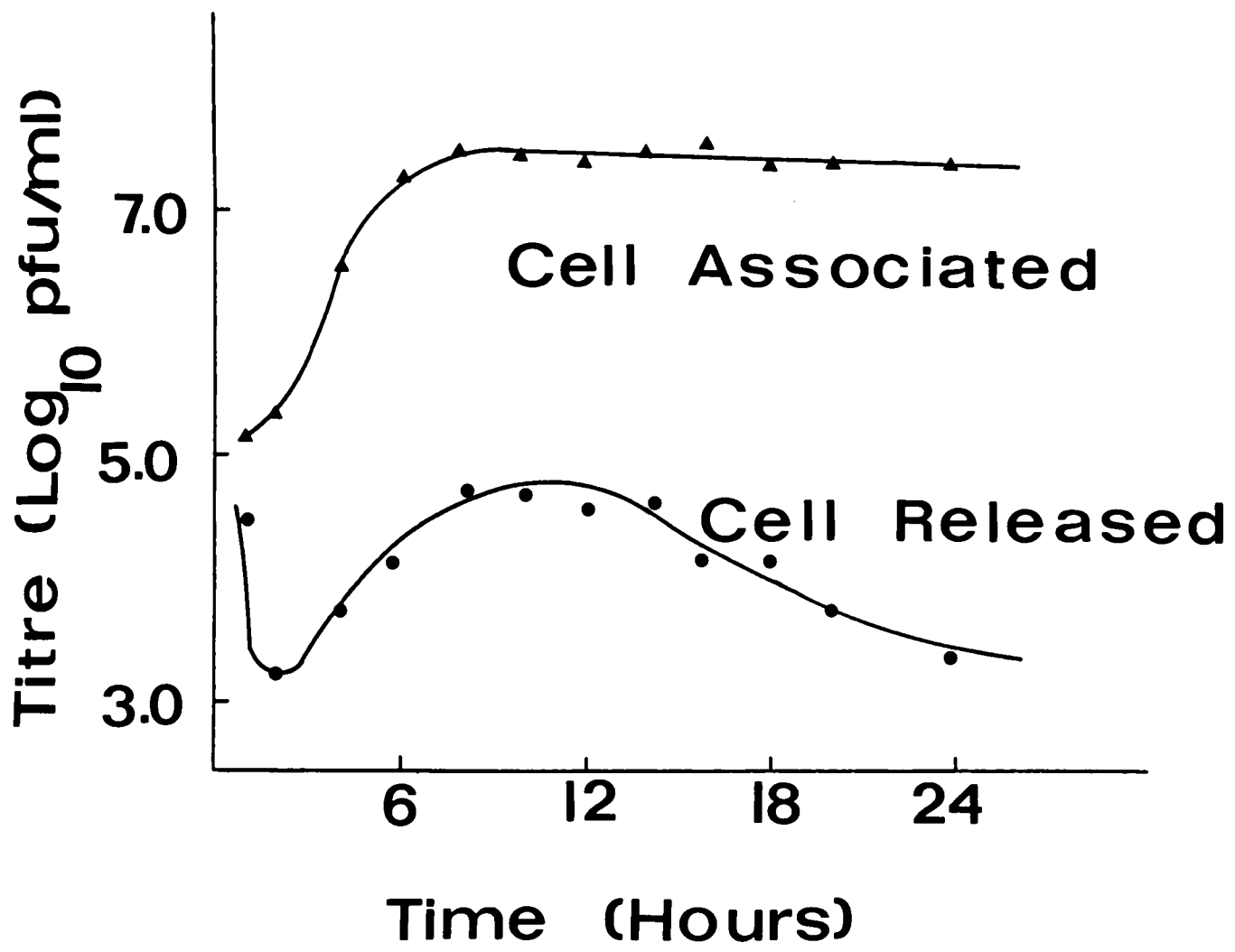


Figure 4.1. Single step replication cycle of Mill Door/79 virus in Vero cells incubated at 37°C.

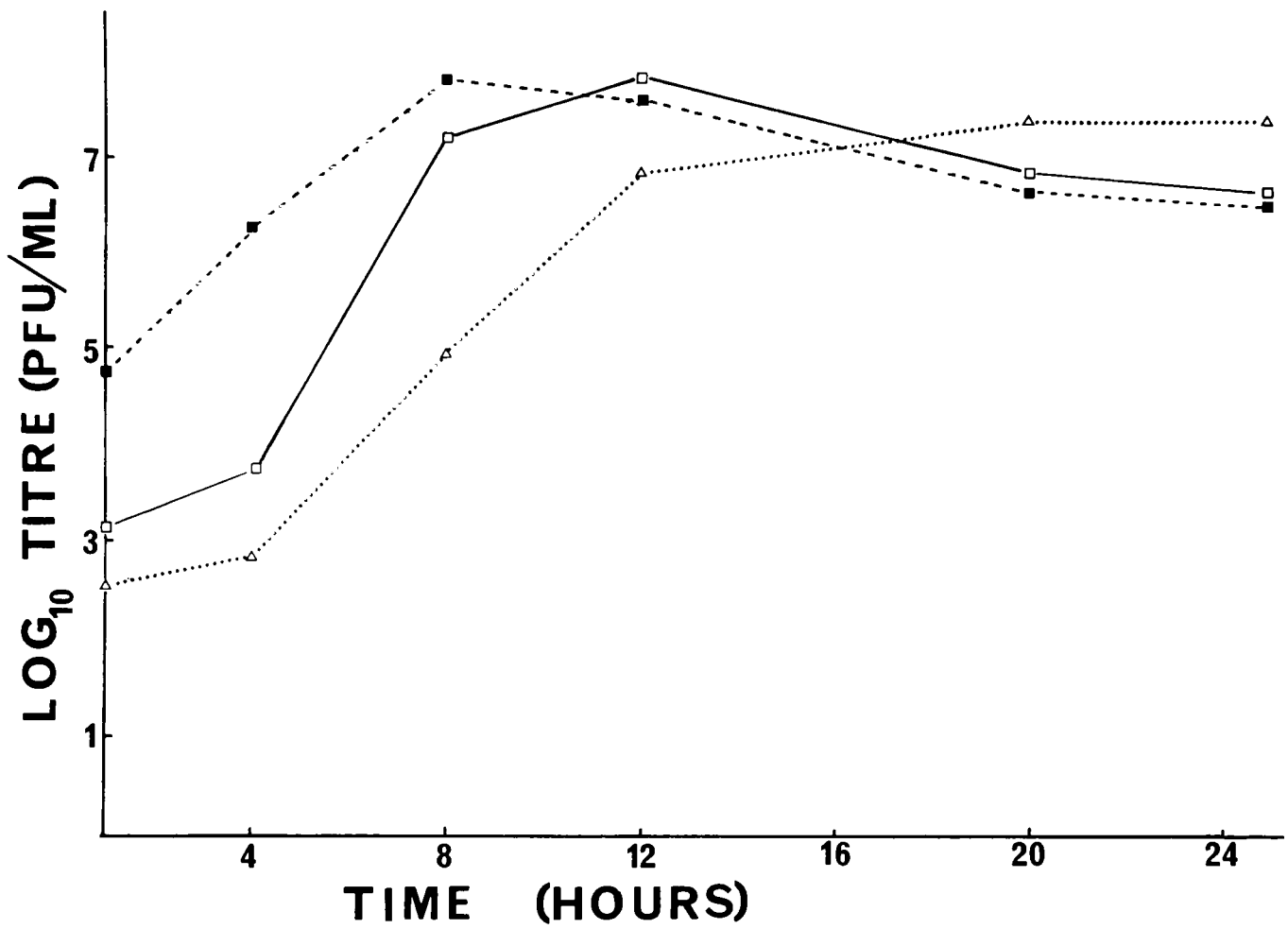


Figure 4.2. Total yield of Mill Door/79 virus from Vero cells after inoculation at multiplicities of infection of 10 pfu/cell (-■-), 1 pfu/cell (—□—) and 0.1 pfu/cell (-△-).

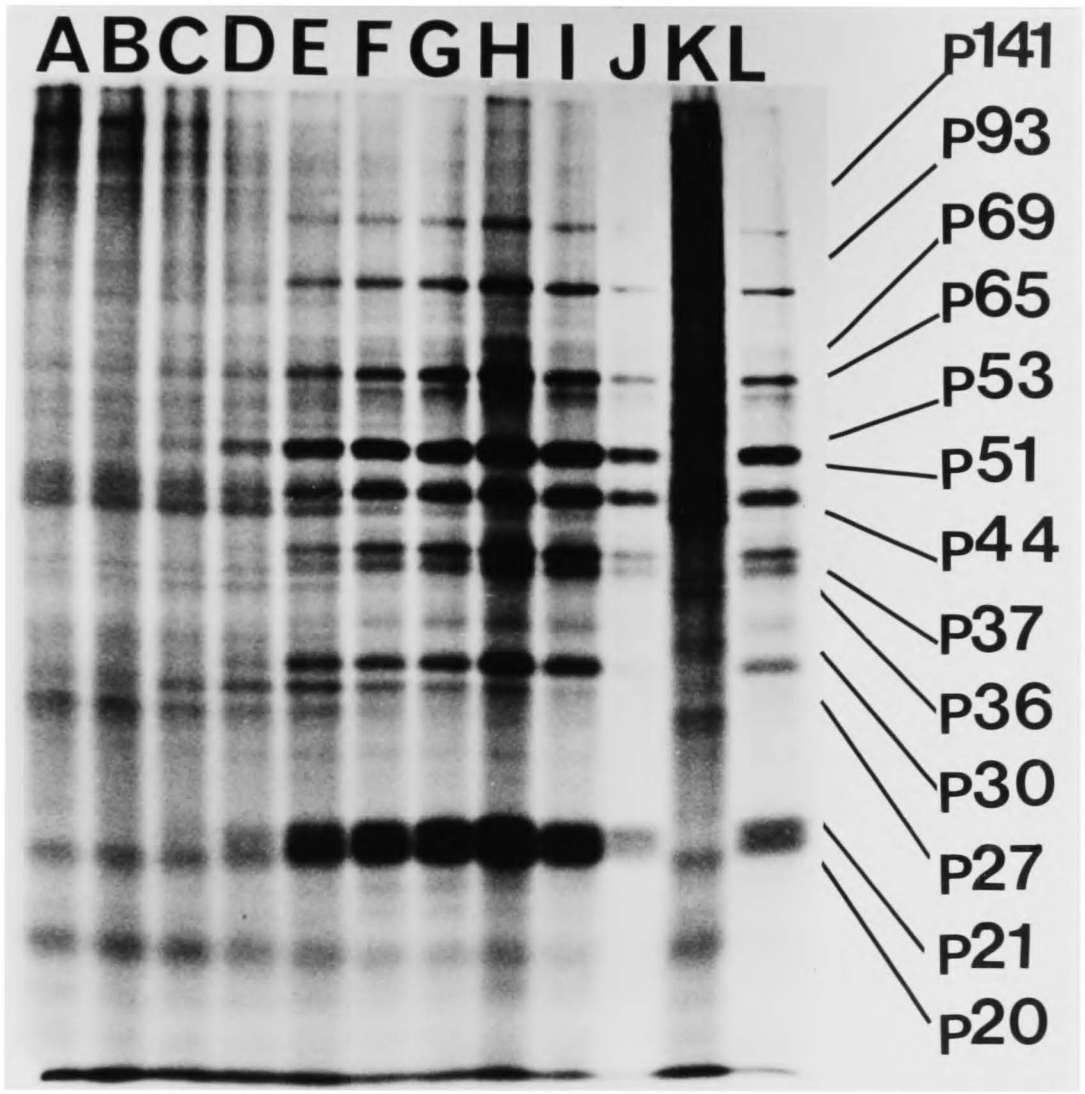


Figure 4.3. Vero cells infected with Mill Door/79 virus. An autoradiogram of a 12.5% PAG of cells labelled with [^{35}S] methionine (100µCi/ml). Track A, mock infected cells labelled for 1 hour at 1 hour p.i.; B-J and L, infected cells labelled for 1 hour at 1-2 hours, 2-3 hours etc until 10 hours p.i.; K, mock infected cells labelled for 1 hour at 10 hours p.i. The virus specific polypeptides are identified by molecular weight x 10^{-3}.

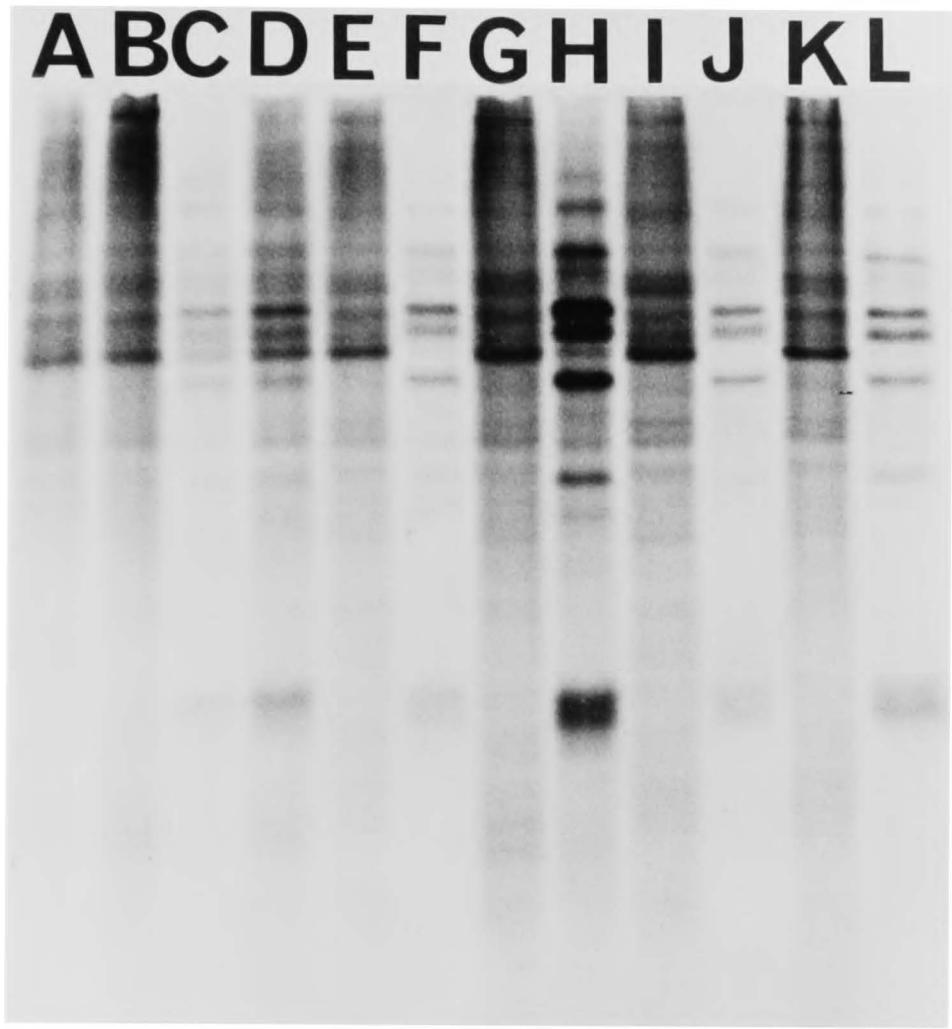


Figure 4.4. Virus-induced polypeptides. An autoradiograph of a 10–20% gradient PAG of Vero cells infected with Mill Door/79 virus and labelled with [^{35}S] methionine (100μCi/ml). Tracks A and B, mock infected cells labelled at 2–3 hours and 4–5 hours p.i. respectively; C and D, infected cells labelled as A and B; E, G, I and K, mock infected cells labelled at 6–7, 8–9, 10–11 and 12–13 hours p.i. respectively; F, H, J and L, infected cells labelled as mock infected cells E–K.

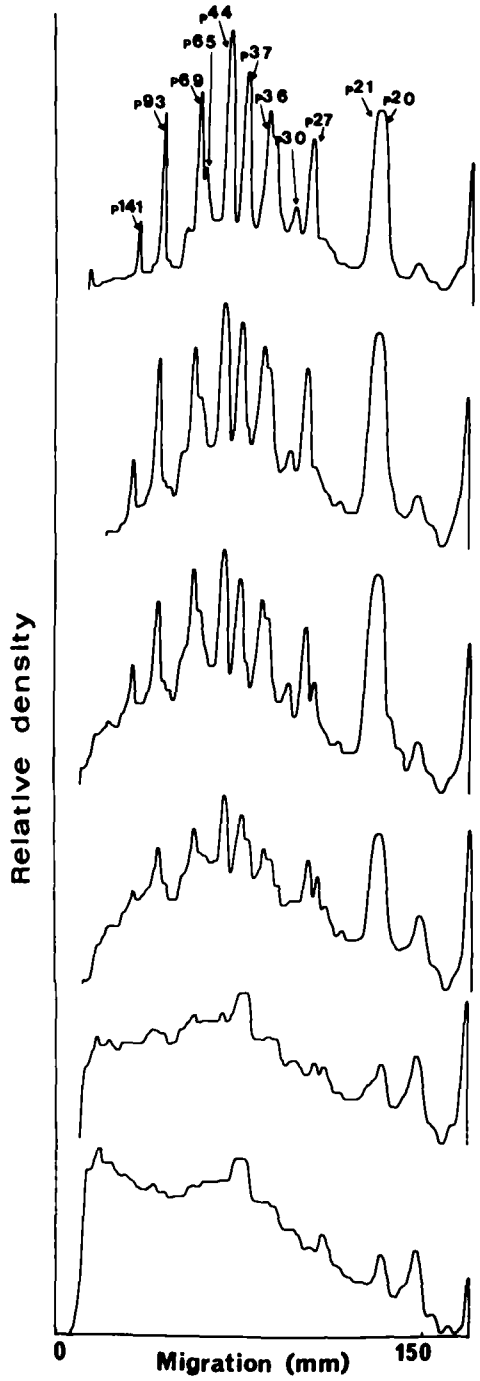


Figure 4.5. Electrophoretic profiles of the 12.5% PAG (Fig. 4.3). Bottom profile mock infected cells labelled at 1-2 hours p.i. The other profiles, going up, represent infected cells labelled at 2-3, 4-5, 6-7, 8-9 and 10-11 hours p.i. respectively.



Figure 4.6. Partial proteolysis of cell and virus-specified polypeptides. Both strips were digested with S.aureus V8 protease (50µg/ml). The uninfected cell digest is on the left, and the infected cell digest on the right of the figure.

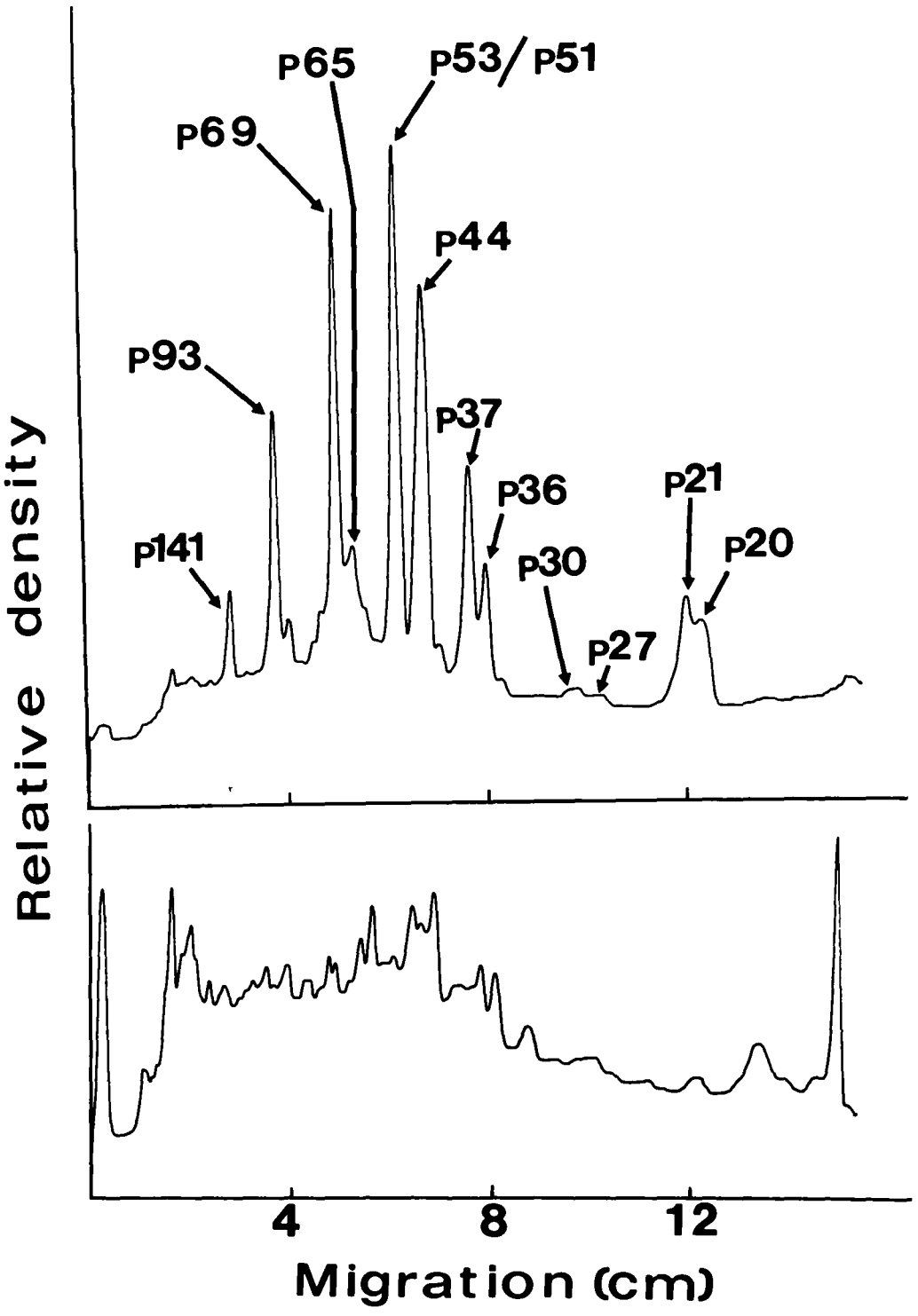
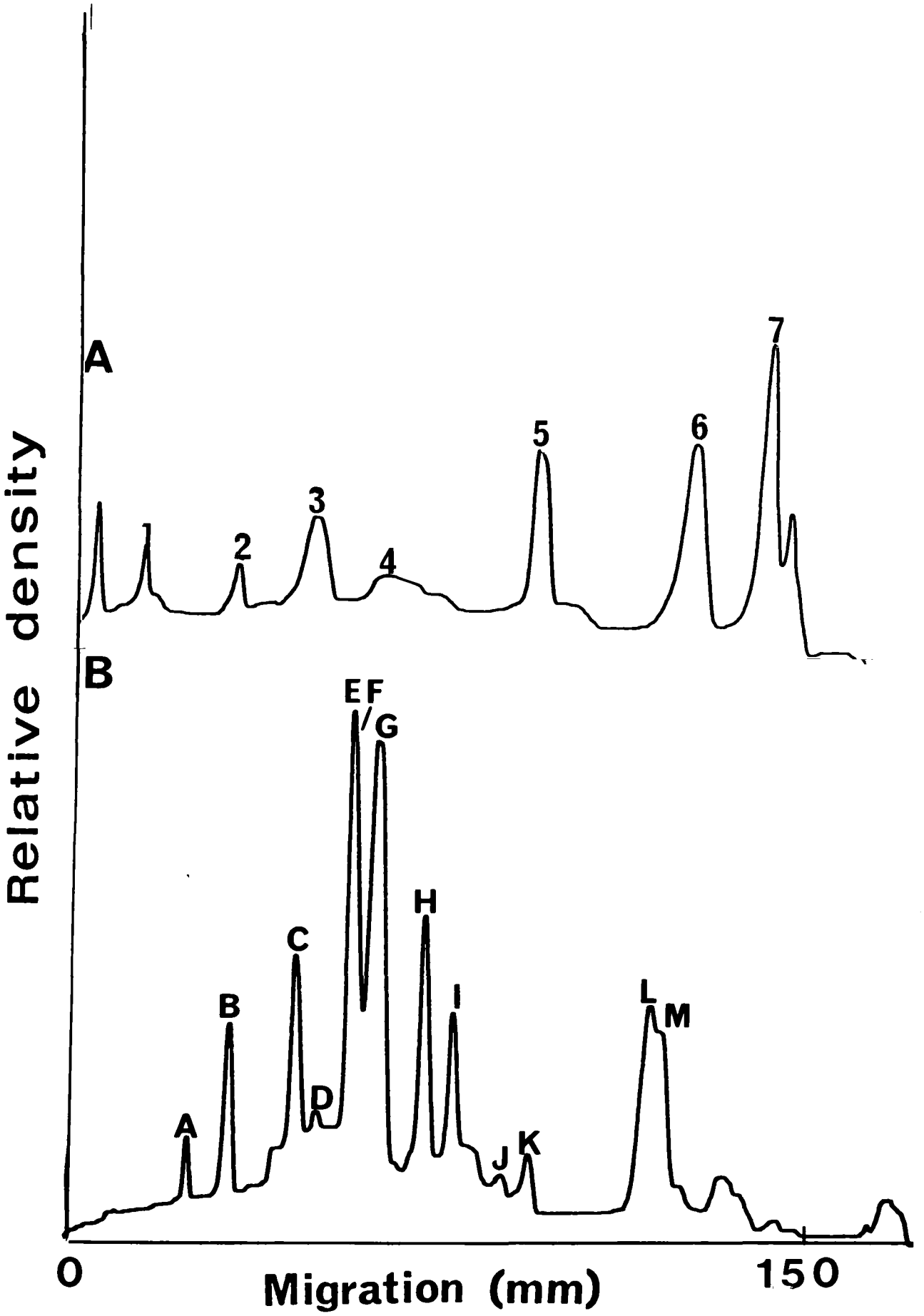


Figure 4.7. Electrophoretic profile of the 12.5% PAG of [^{14}C] protein hydrolysate labelled mock infected (bottom) and Mill Door/79 virus infected cells (top). Labelling was for 1 hour at 7 hours p.i. with 10µCi/ml.

Figure 4.8. Electrophoretic profile of a 12.5% PAG of [^{14}C] protein hydrolysate labelled Mill Door/79 virus infected cells (bottom) and [^{14}C]-labelled molecular weight markers (top). The letters A-M (bottom) represent the virus induced polypeptides p141, p93, p69, p65, p53, p51, p44, p37, p36, p30, p27, p21 and p20 respectively. The numbers 1-7 (top) represent, respectively, the molecular weight markers myosin (H chain), phosphorylase B, bovine serum albumin, ovalbumin, α chymotrypsinogen, β lacto-globulin and cytochrome c.


A
B
Relative density
1
2
3
4
5
6
7
A
B
C
D
E
F
G
H
I
J
K
L
M
0
Migration (mm)
150

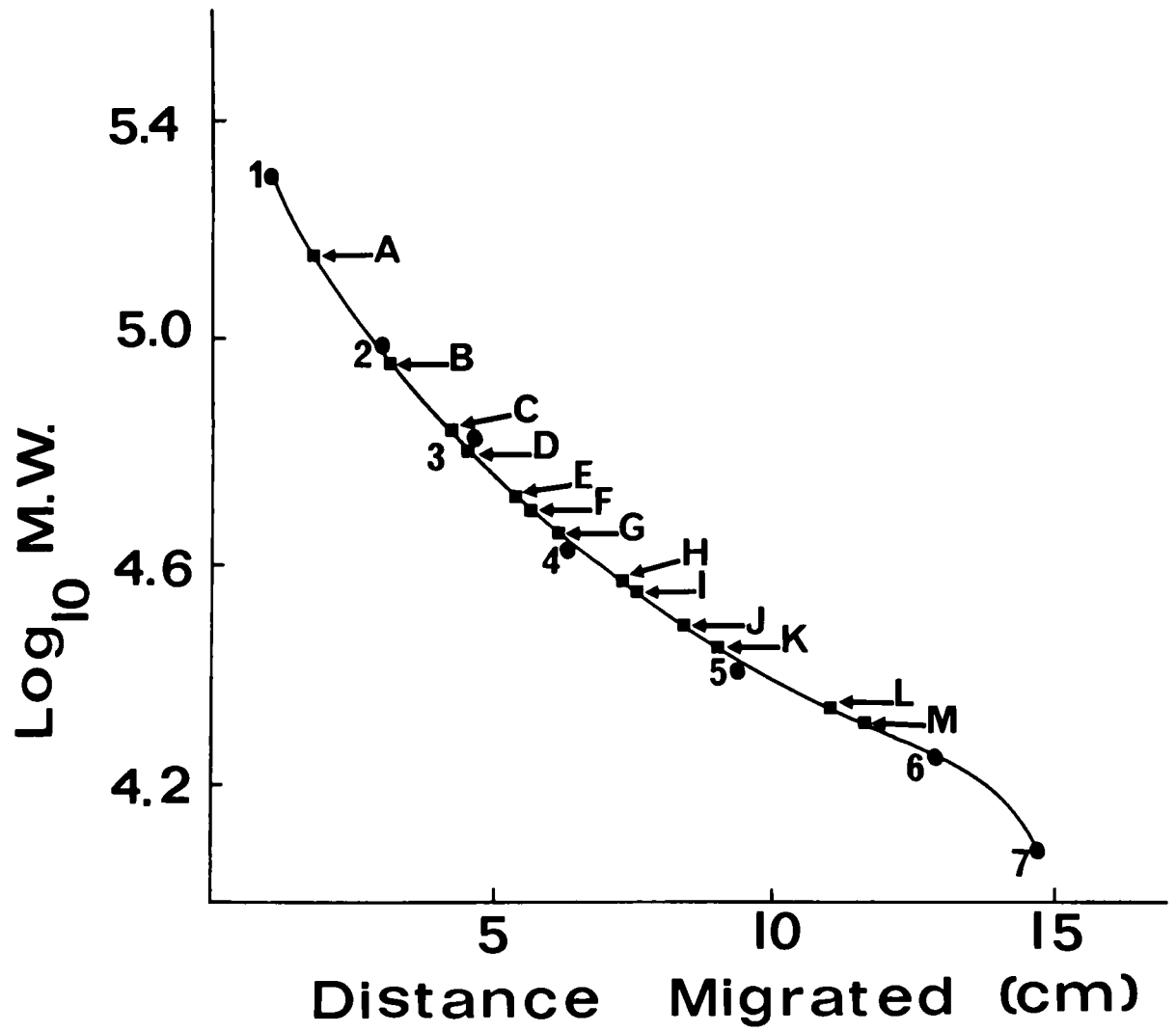


Figure 4.9. A calibration curve of standard molecular weight markers electrophoresed on a 12.5% PAG (each labelled as Fig. 4.8). The virus induced polypeptides are identified by letters (as Fig. 4.8).

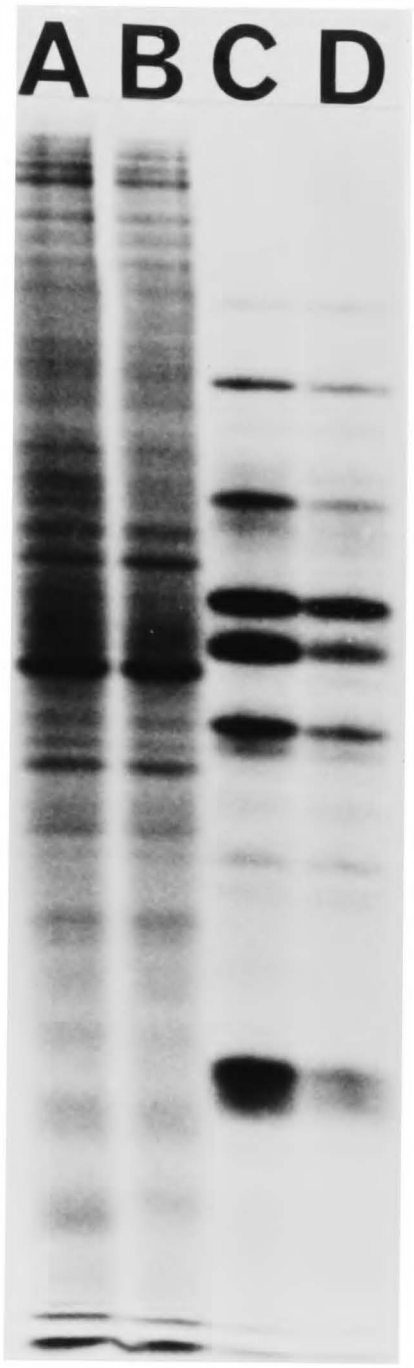


Figure 4.10. Effect of actinomycin D on infected and uninfected Vero cell protein synthesis. An autoradiograph of a 12.5% PAG of cells infected with Mill Door/79 virus and treated with 10µg/ml AMD at 3 hours p.i. All radiolabelling was carried out at 7-8 hours p.i. with 100µCi/ml [^{35}S] methionine. Track A, mock infected cells; B, mock infected cells treated with AMD; C, infected cells; D, infected cells treated with AMD.

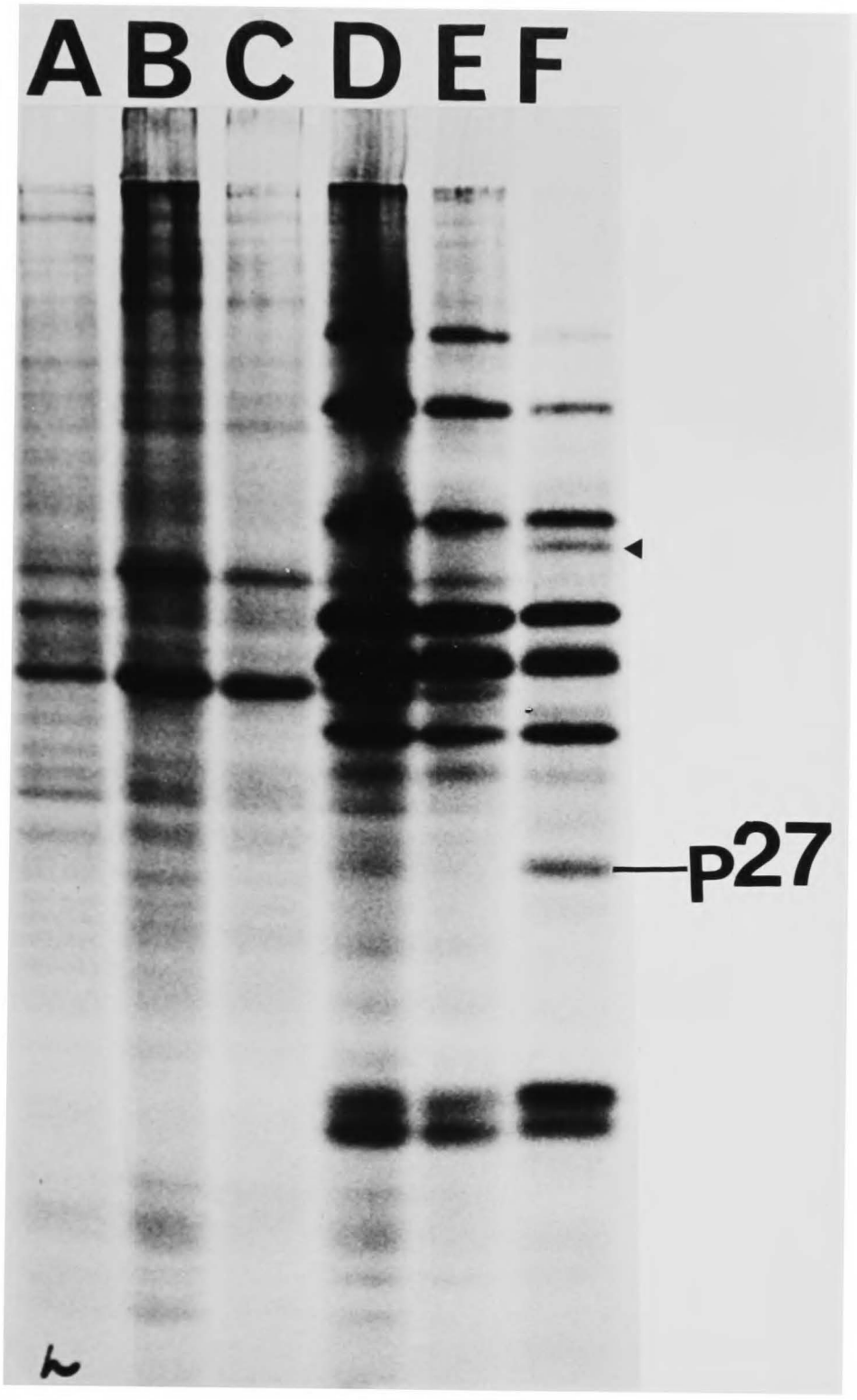


Figure 4.11. Virus-induced polypeptides in Vero and BHK-21 cells infected with Mill Door/79 virus. An autoradiograph of a 12.5% PAG. All cells were labelled at 7-8 hours p.i. with [^{35}S] methionine (100µCi/ml). Track A, mock infected BHK-21 cells; B, mock infected BHK-21 cells treated with 10µg/ml AMD; C, mock infected Vero cells; D, infected BHK-21 cells; E, infected BHK-21 cells treated with 10µg/ml AMD; F, infected Vero cells. p65 is indicated by an arrow.

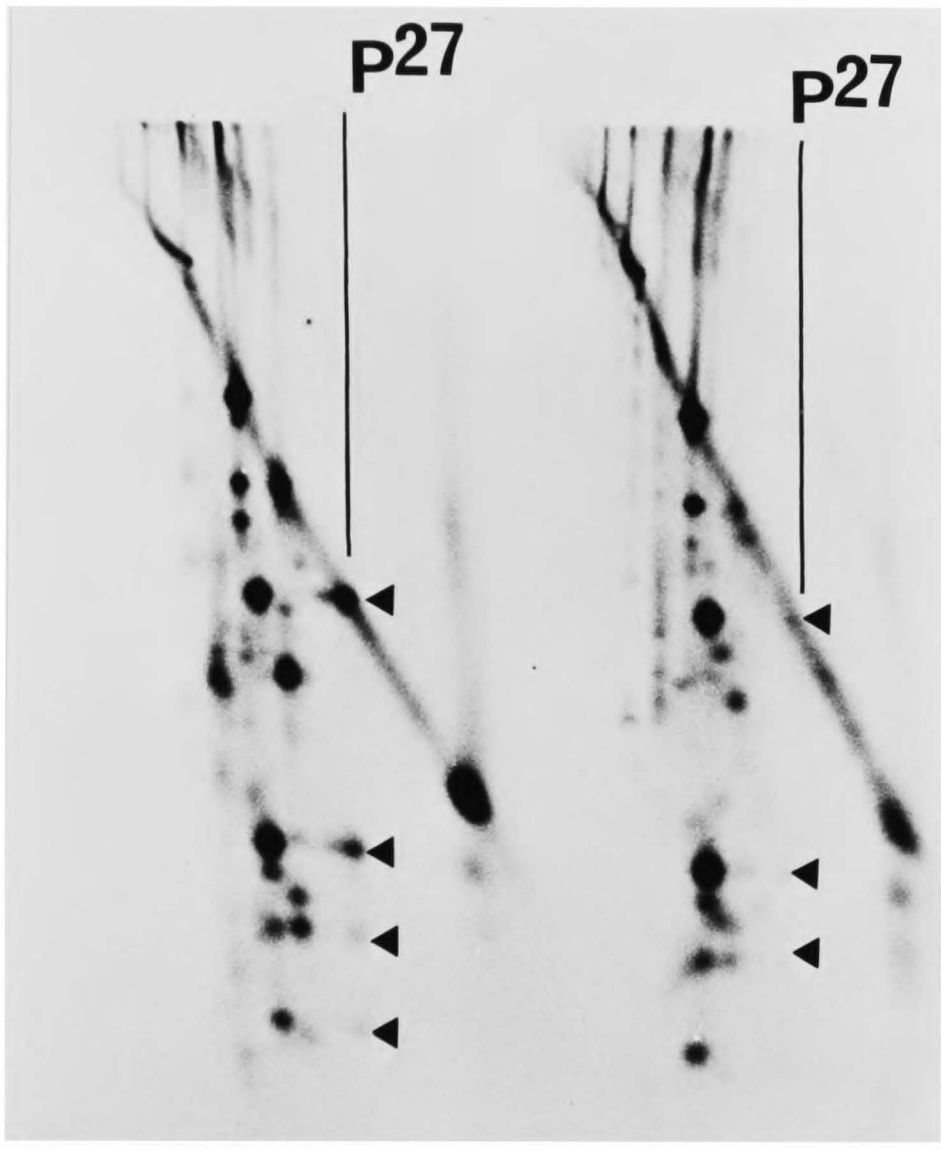


Figure 4.12. Partial proteolysis of Mill Door/79 virus induced polypeptides in Vero (left) and BHK-21 cells (right). Digestion was carried out using S.aureus V8 protease (50µg/ml). The products of p27 are indicated in each cell line by arrows.

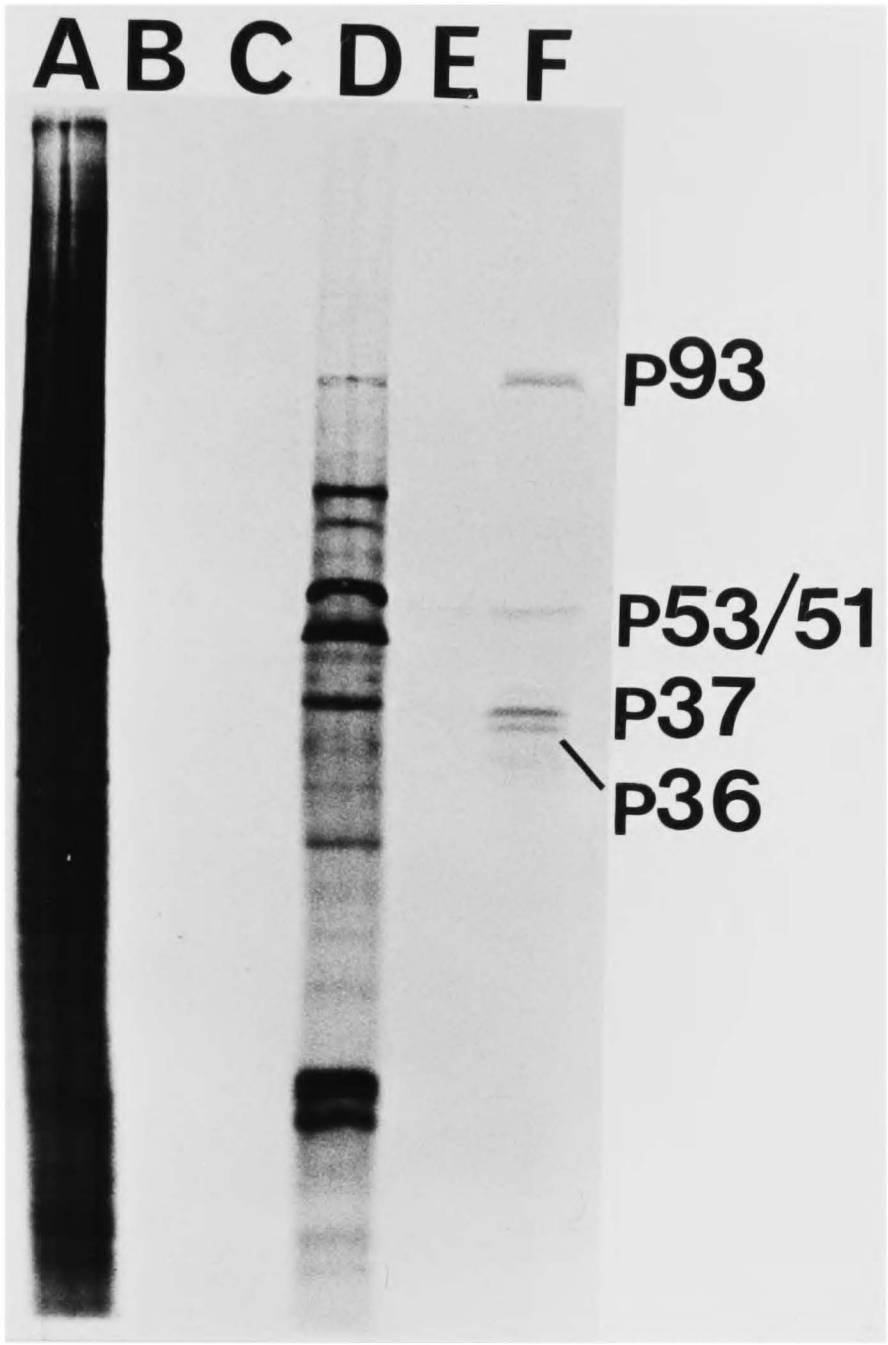
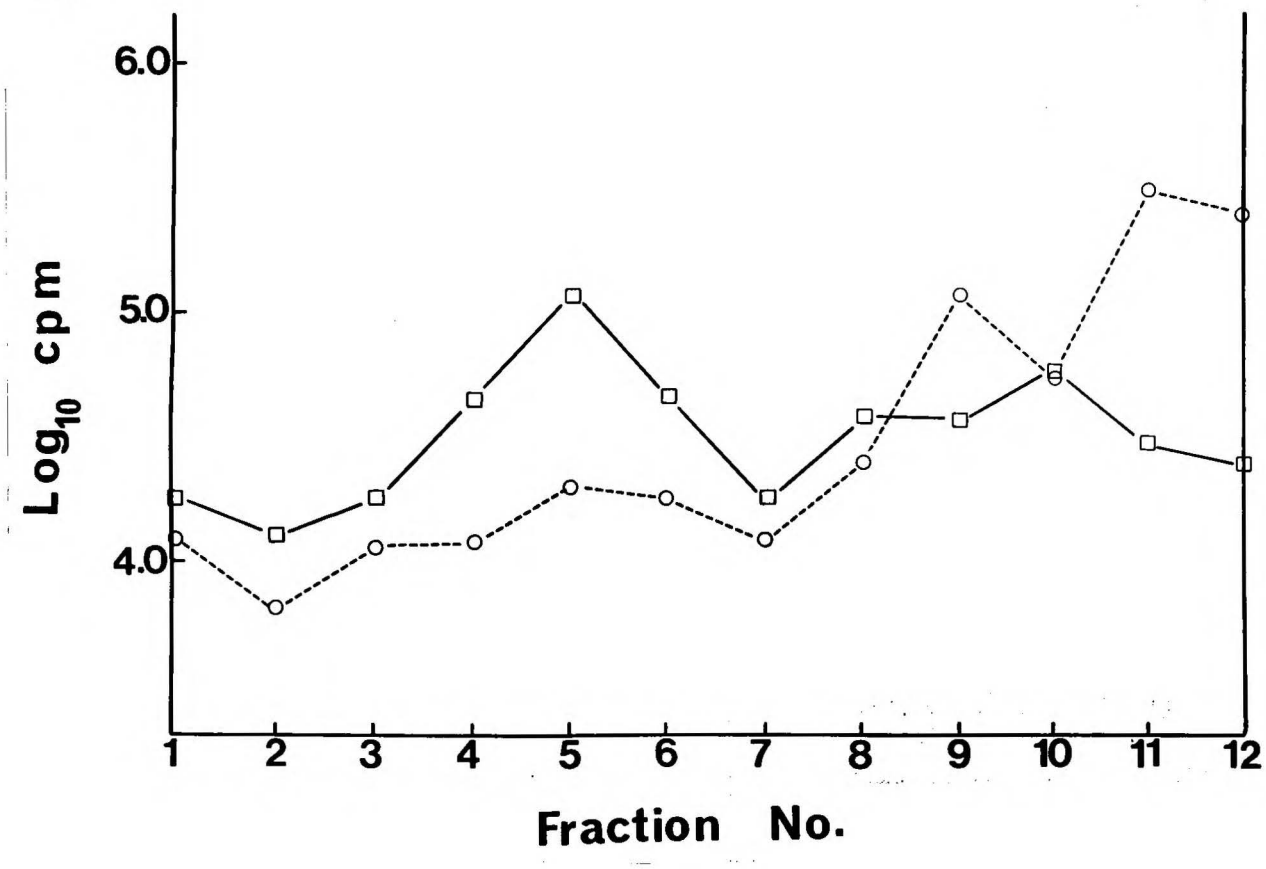


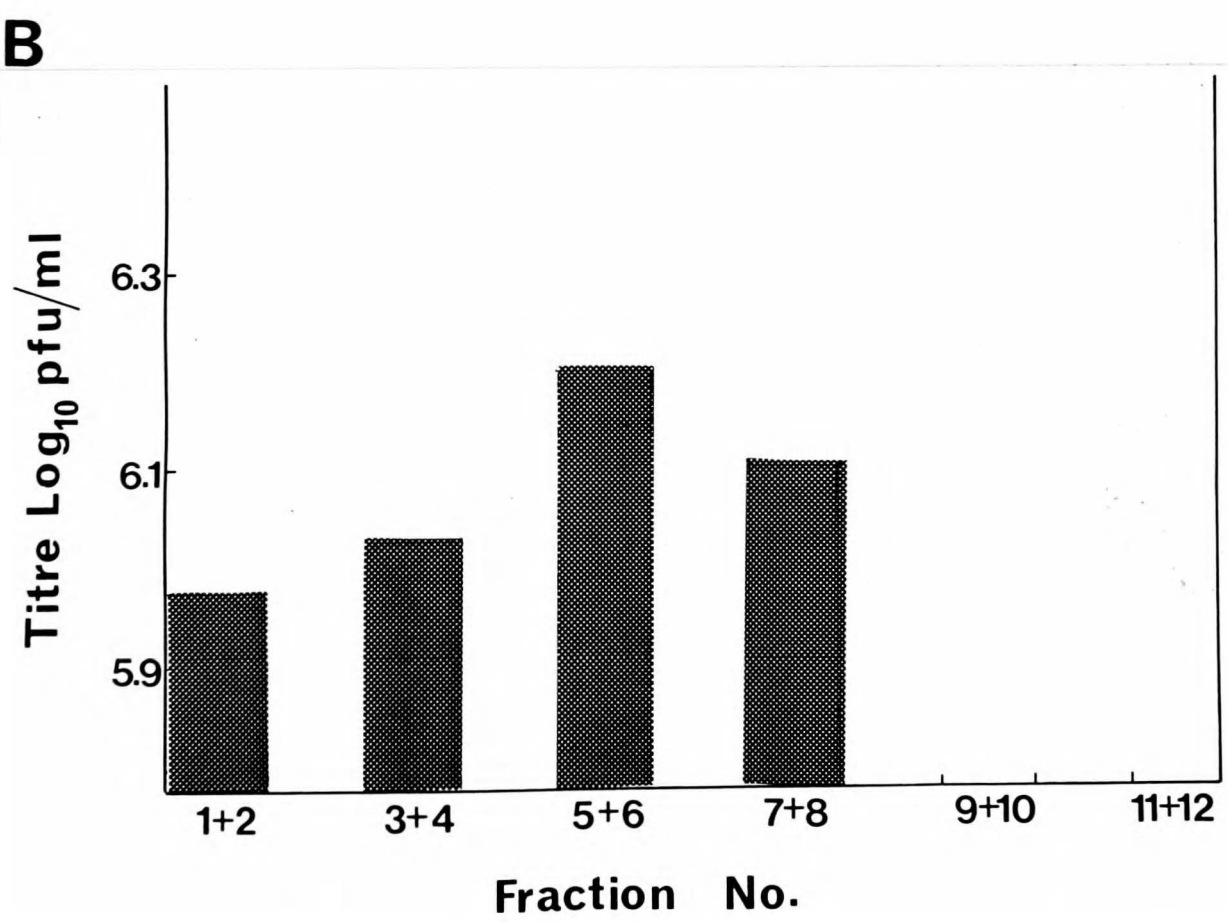
Figure 4.13. Immune-precipitation of Mill Door/79 virus induced proteins. An autoradiograph of a 12.5% PAG. The cells were labelled at 7-8 hours p.i. with [^{35}S] methionine (200μCi/ml). Track A, mock infected cell lysate; B, mock infected cell proteins incubated with pre-immune ascitic fluid; C, mock infected cells treated with immune ascitic fluid; D, infected cell lysate; E, infected cells treated with pre-immune ascitic fluid; F, infected cells treated with immune ascitic fluid.

Figure 4.14. Radioactivity and infectivity profiles from a 20-60% sucrose gradient (fraction No. 1 was the bottom of the gradient). Each fraction was 1ml and each sample for scintillation counting and titration was 10µl. In Figure 4.14A -○- represents the radioactivity of samples taken from the gradient fractions and —□— represents the radioactivity of fractions taken from the solubilized pellets after centrifugation of the gradient fractions. In Fig. 4.14B the shaded bars represent the titre of pooled fractions from the gradient. No infectivity was detected in fractions 9 and 10, and 11 and 12.

A


6.0
5.0
4.0
Log10 cpm
1
2
3
4
5
6
7
8
9
10
11
12
Fraction No.

B


6.3
6.1
5.9
Titre Log10 pfu/ml
1+2
3+4
5+6
7+8
9+10
11+12
Fraction No.

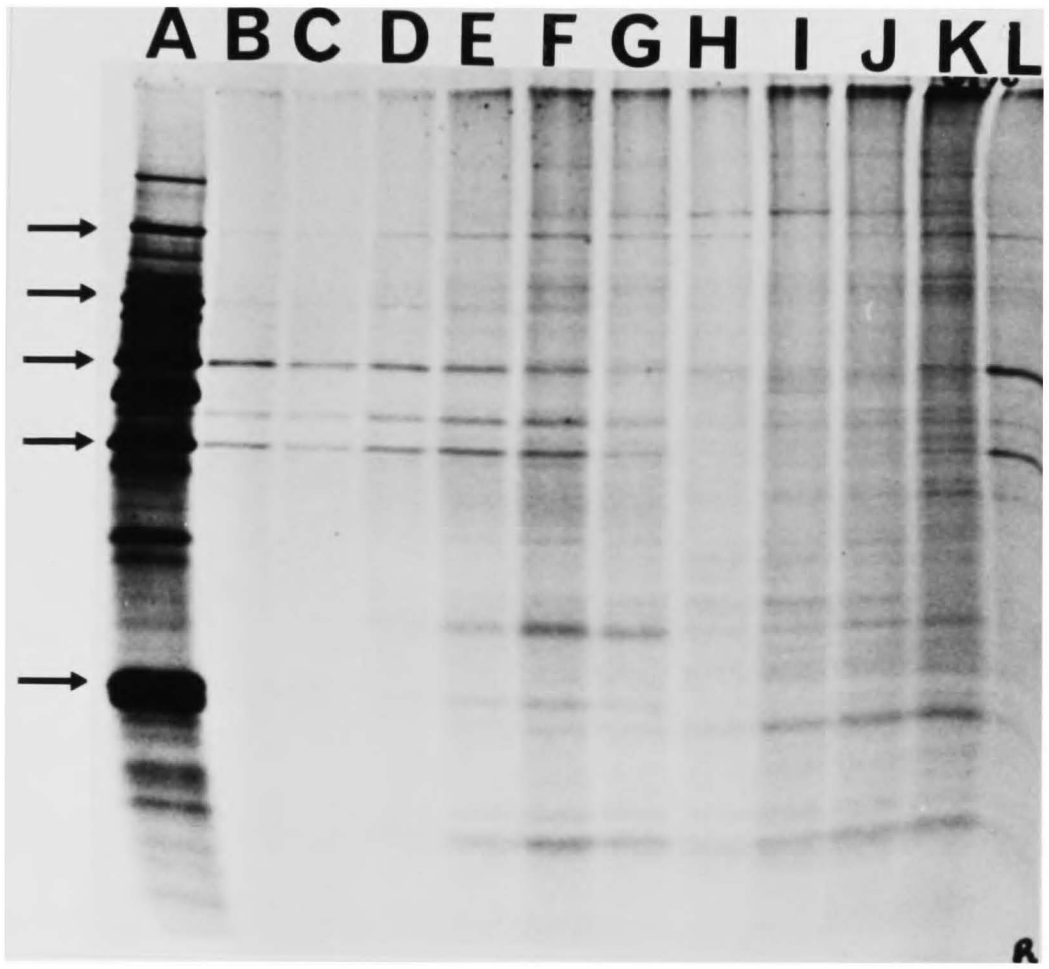
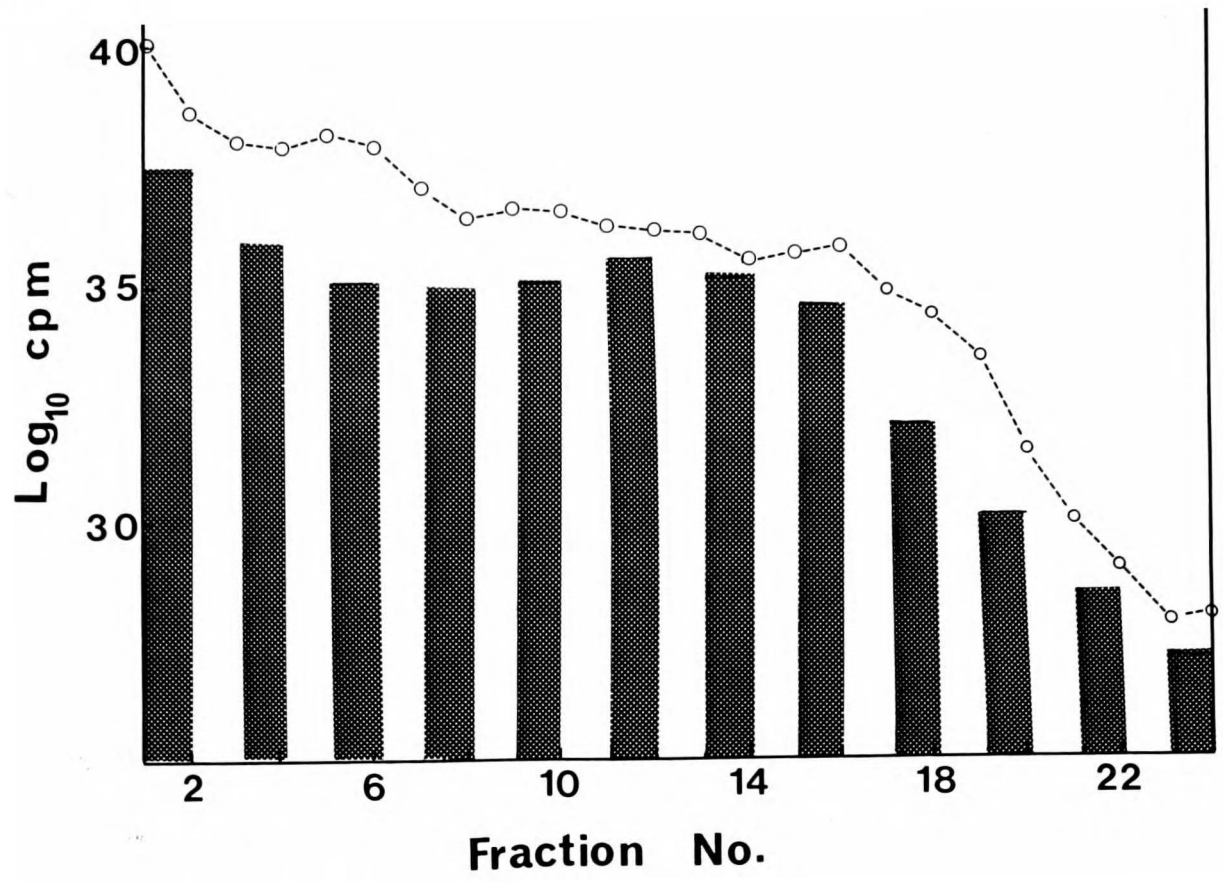


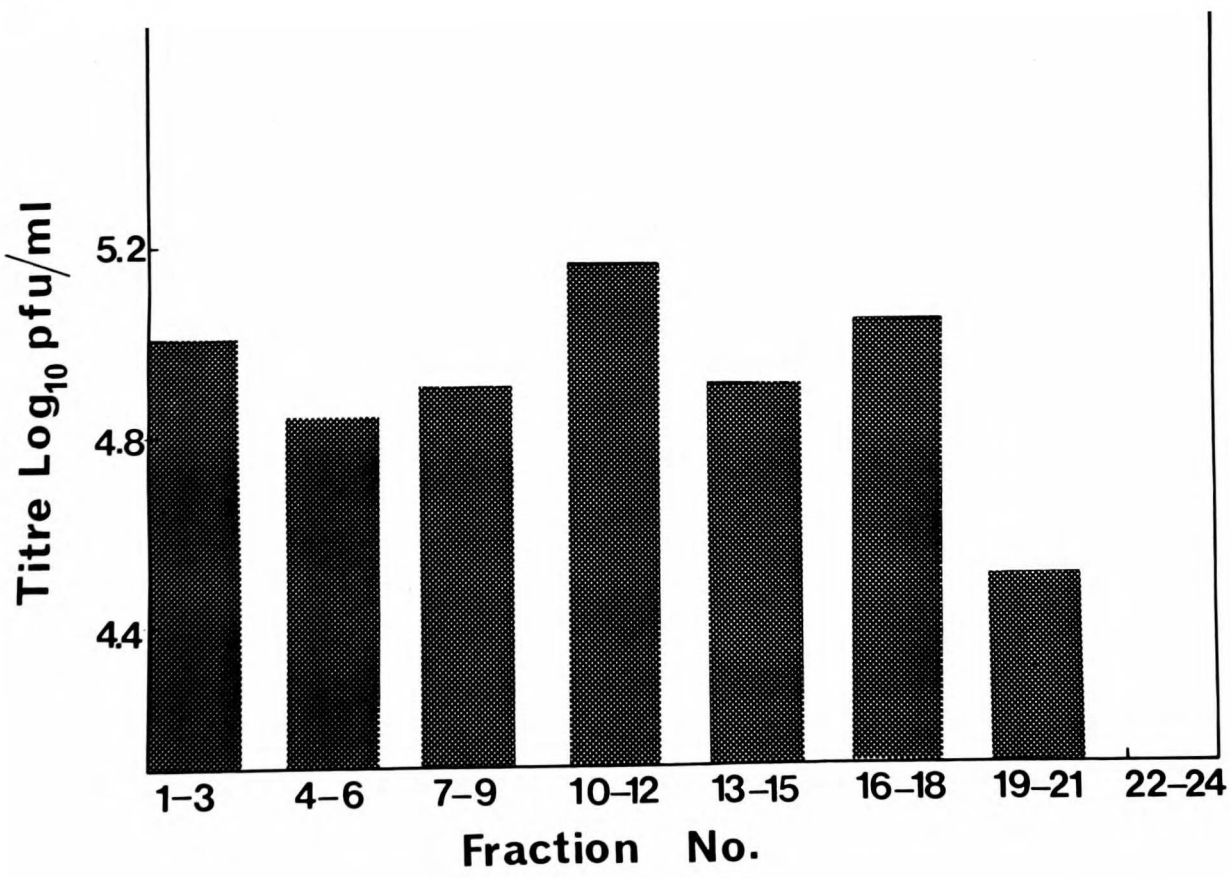
Figure 4.15. Mill Door/79 virus purification. Pelleted fractions after treatment with PEG-6000 followed by sucrose density centrifugation. An autoradiograph of a 12.5% PAG. Track A, infected cell lysate; B-L, pellets of fractions 1-11 respectively. Fractions described in Fig. 4.14. Arrows indicate virus induced polypeptides p93, p69, p53/51, p37, p21 and p20, from top to bottom respectively.

Figure 4.16. Radioactivity and infectivity profiles of a 10-50% sucrose gradient (fraction No. 1 was the bottom of the gradient). Each fraction was 0.5ml and each sample for scintillation counting and titration was 10µl. In Fig. 4.16A -O- represents the radioactivity of samples taken from the gradient fractions and the shaded bars represent the radioactivity of samples taken from the solubilized pellets after centrifugation of pooled gradient fractions 1 and 2, 3 and 4 etc. In Fig. 4.16B the shaded bars represent the titre of pooled fractions from the gradient. No infectivity was detected in pool 22-24.

A


40
35
30
Log10 cpm
2
6
10
14
18
22
Fraction No.

B


5.2
4.8
4.4
Titre Log10 pfu/ml
1–3
4–6
7–9
10–12
13–15
16–18
19–21
22–24
Fraction No.

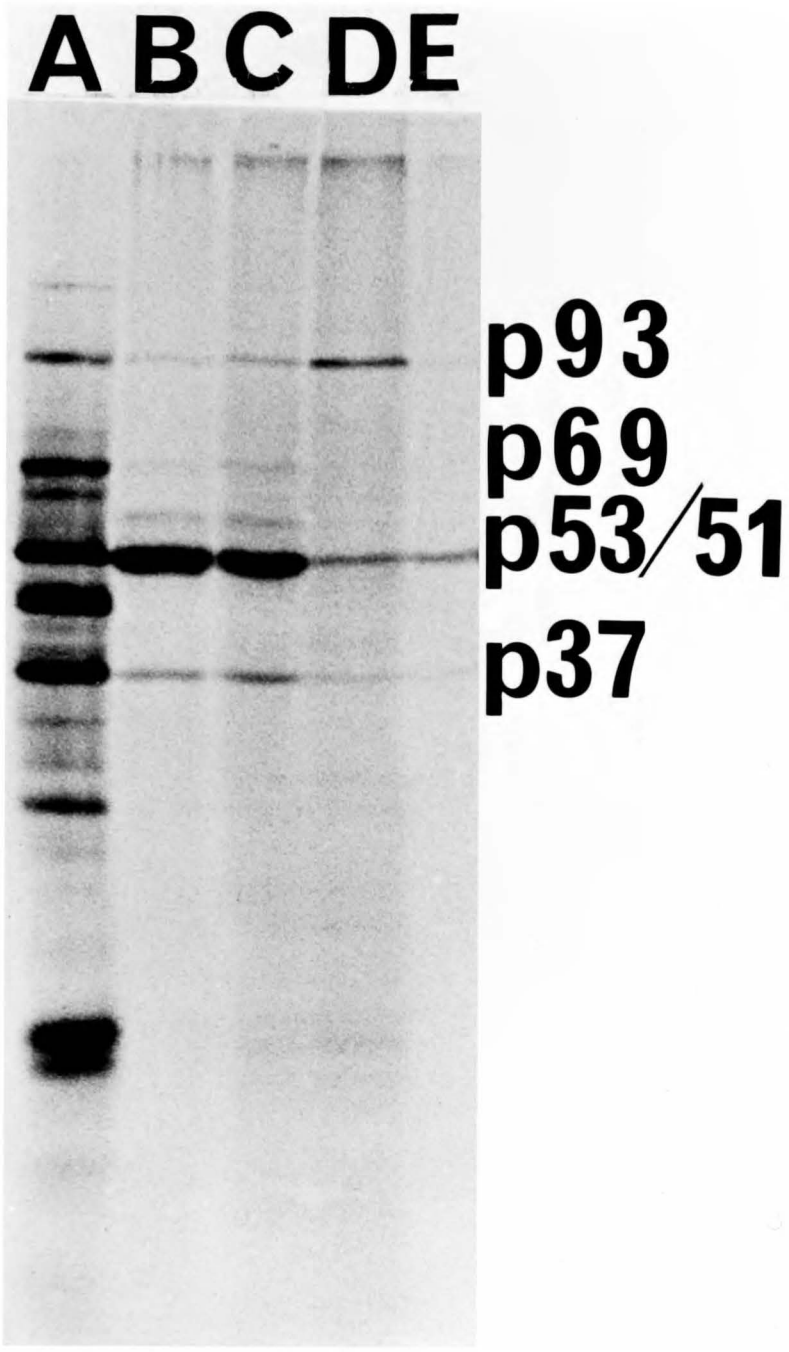


Figure 4.17. Mill Door/79 virus purification. Pelleted fractions after ether extraction and sucrose density centrifugation. The pellets were solubilized and electrophoresed on a 12.5% PAG before autoradiography. Track A, infected cell lysate; , pooled fractions 9-12; C, pooled fractions 17-20; D, pooled fractions 1-4; E, pooled fractions 5-8. Fractions described in Fig. 4.16.

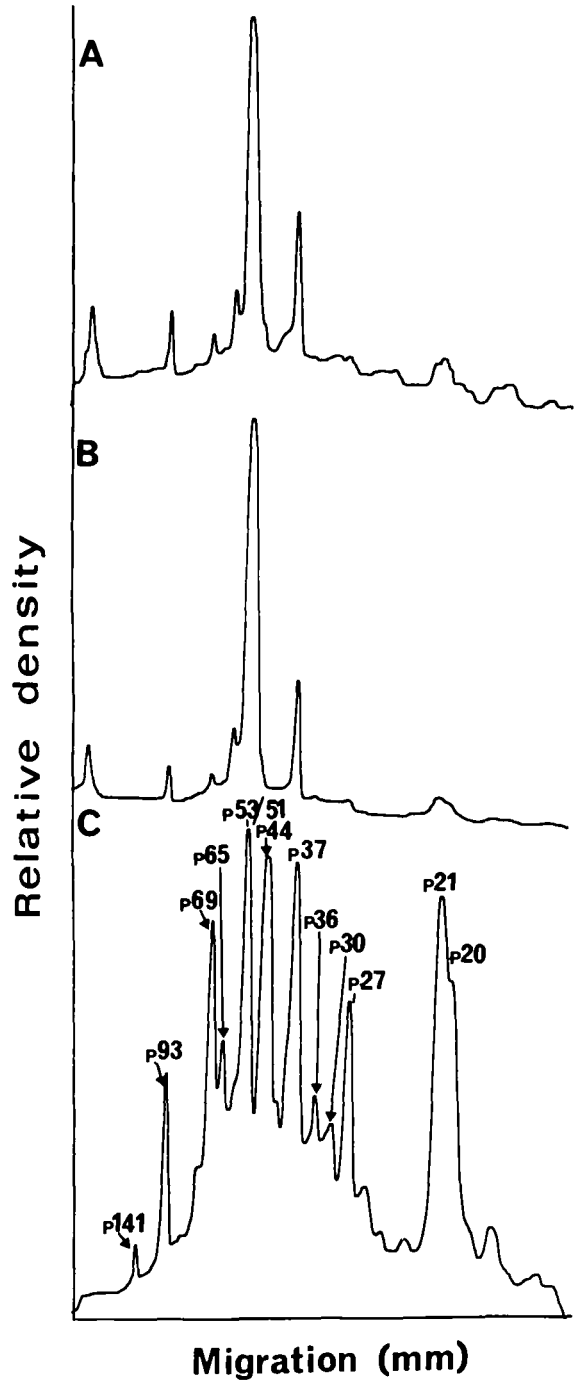


Figure 4.18. Electrophoretic profile of a 12.5% PAG of fractions from the pooled gradient (Fig. 4.16). A, top infectious fraction; B, bottom infectious fraction; C, infected cell lysate.

CHAPTER 5

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MODIFICATION OF THE POLYPEPTIDES PRODUCED BY MILL DOOR/79 VIRUS

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I. INTRODUCTION

The genome of Mill Door/79 virus was shown to comprise 10 segments of dsRNA (Chapter 6). However, in the preceding chapter it was shown that 13 virus specified polypeptides were produced in Mill Door/79 virus-infected cells. This apparent discrepancy may be due to the modification of virus-induced polypeptides. In this chapter post-translational modification, such as cleavage of primary gene products or the addition of some chemical component to the polypeptides, was investigated using the following methods: pulse-chase, temperature elevation and reduction, protease inhibitors, synchronous re-initiation of protein synthesis, detection of amino acid sequence homologies, glycosylation inhibitors and radiolabelled sugars. The rationale behind each of these approaches is dicussed below.

The basis of pulse-chase experiments is that the modification of polypeptides occurs after their translation. Therefore, unlike straightforward "pulse" experiments in which the cells are harvested directly after the labelling period, a "chase" period is introduced during which the radiolabel is removed and the cells re-incubated, after the addition of an excess of the appropriate unlabelled amino acids, for a variable period of time before harvesting. After separation on PAGs and autoradiography, the polypeptides detected are those translated during the pulse period since polypeptides translated during the chase period would not be radiolabelled. Protein modifications occurring during the chase period are detected by comparing this "pulse-chase" profile with a "pulse only" profile.

Raising the incubation temperature of infected cells can either stimulate or inhibit proteolytic cleavage, depending on

the system studied. Zweerink et al. (1971) reported the presence of virus products in reovirus infected cells incubated at 37°C that were not found when the cells were incubated at 31°C. Moore and Pullin (1983) demonstrated the inhibition of cleavage of Drosophila C virus polypeptides at 37°C whereas cleavage occurred normally at 28°C.

Protease inhibitors have been used extensively in the study of protein processing in the Picornaviridae (Korant, 1972; Korant, 1973; Butterworth & Korant, 1974; Korant & Butterworth, 1976; Moore et al., 1981; Reavy, 1982; Moore & Pullin, 1983), but their use has not been reported in studies of the Reoviridae. If proteolytic cleavage is inhibited cleavage products are not formed, whereas the precursor polypeptides accumulate in infected cells. By comparing the protein profiles of inhibitor-treated and untreated cells, polypeptides produced by proteolytic cleavages can be identified. If protein processing is required for the formation of infectious virus particles, inhibition of proteolytic cleavage will lead to a loss of virus infectivity. Several inhibitors were used during the following investigations, as it had been demonstrated previously that the different cleavage reactions of picornaviruses were blocked by different inhibitors (Korant, 1973). Iodoacetamide (IOA) is an alkylating agent which irreversibly inhibits enzyme activity by modifying cysteine and other side chains (Mahler & Cordes, 1971; Means & Feeny, 1971); it is water insoluble but dissolves readily in dimethyl sulphoxide (DMSO). Zinc ions inhibit protein cleavage in a reversible manner, the degree of inhibition increasing with increasing zinc ion concentration (Butterworth & Korant, 1974; Korant & Butterworth, 1976). Korant and Butterworth (1976) suggested that the zinc ions themselves participate directly in

cleavage inhibition, altering the precursor protein configuration so that it can no longer be processed normally. L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) and N-α-p tosyl-L-lysine chloromethyl ketone HCl (TLCK) inhibit chymotrypsin and trypsin respectively (Korant, 1972) whereas phenylmethyl sulphonyl fluoride (PMSF) is an enzyme inactivator which has been shown to inhibit mammalian acetyl cholinesterase (Moss & Fahrney, 1978).

Hypertonic concentrations of NaCl were shown to inhibit the initiation of protein synthesis in HeLa cells, while having little effect on elongation, and removal of the NaCl led to synchronous re-initiation of protein synthesis (Saborio et al., 1974). If infected cells are radiolabelled when protein synthesis is re-initiated, and incubated for a short period of time, the only polypeptides detected after PAGE and autoradiography are the primary gene products. However, as in the case of pulse-chase experiments, rapid cleavage and cleavage during translation may go undetected.

The proteolytic cleavage of a polypeptide will result in the formation of one or more products having an amino acid sequence identical to a region, or regions, of the original polypeptide. If both precursor and products are present in infected cells this homology can be demonstrated by partial proteolysis. Three enzymes were chosen for the study on Mill Door/79 virus. Staphylococcus aureus V8 protease cleaves polypeptides at the carboxyl side of aspartate and glutamate residues, whereas chymotrypsin (TLCK-treated to inhibit trypsin activity) cleaves at the carboxyl side of aromatic and other bulky, non-polar residues, and trypsin (TPCK-treated to inhibit chymotrypsin activity) cleaves at the carboxyl side of lysine and arginine residues. The products of each of the digested polypeptides,

which depend on their amino acid sequences, are unique for each enzyme.

Modifications can include the addition of polysaccharides to polypeptides, which can produce an apparent increase in their molecular weight during PAGE. The increase is greater than that expected solely from the addition of the polysaccharide. This is because less SDS is able to bind to the proteins in the presence of the polysaccharide, hence the net protein charge is reduced during electrophoresis. This leads to reduced mobility, yielding artifactually high molecular weight estimates (Hames, 1981). Inhibitors of glycosylation prevent the addition of polysaccharides to proteins thereby increasing their mobilities in PAGs. Hence, the comparison of inhibitor-treated and untreated polypeptide profiles would indicate the presence of glycosylated proteins. The glycosylation inhibitors chosen were tunicamycin (Tm) [which is known to block the formation of N-glycosidic type protein/carbohydrate linkages by inhibiting the transfer of N-acetyl glucosamine-1-phosphate to dolichol monophosphate (Mahoney & Duksin, 1979)] from Streptomyces spp., and 2-deoxy-D-glucose (2DG) [a mannose and glucose analogue which blocks lipid-linked oligosaccharide transfer to protein (Kaluza et al., 1973)]. Radioactive sugars can be employed to label glycoproteins and can be used in combination with glycosylation inhibitors. Glycosylation has been demonstrated in both reoviruses and rotaviruses and its inhibition led to a loss in infectivity in each case (Sabara et al., 1982; Lee, 1983). Both gel electrophoresis and infectivity studies were therefore performed on TM- and 2DG-treated Mill Door/79 virus.

II. RESULTS

A. Pulse-chase experiments

Vero cell monolayers were inoculated at a multiplicity of 20-30 pfu/cell and incubated for 5.5 hours at 37°C when they were washed with labelling medium, incubated for a further half hour, and labelled with 100μCi/ml [^{35}S] methionine for either 2 or 5 minutes. At this stage the cells were either harvested or the radioactive medium replaced with maintenance medium, containing extra (15mg/ml) unlabelled methionine to minimise further incorporation of [^{35}S] methionine into proteins, and incubated further. These "chased" monolayers were harvested after 5, 10, 30, 60 and 120 minutes and, to detect very slow processing 18 and 24 hours p.i. Once harvested, the cell lysates were subjected to PAGE followed by autoradiography.

Infected Vero cell monolayers labelled for 2 and 5 minutes, including those labelled for 5 minutes followed by a 5 minute chase, displayed reduced levels of p141 (Fig. 5.1, tracks B, C, D, G and H), whereas all the other virus-specified polypeptides [except p65 (see below) and p93 (tracks G and H)] were present in these samples. No change in the protein profiles was observed in the 2 hours after labelling, apart from the appearance of a polypeptide migrating above p44 after 5 minutes chase (tracks H to K, open arrows). However, after 18 hours chase this polypeptide was no longer present and the level of p27 was also reduced (tracks L and M). Differences between two experiments were observed involving both virus and host-cell products. p65 was not detected among the products of experiment 1 (tracks B-F) but it was produced in experiment 2 (tracks G-M). A host-cell polypeptide (arrowed) was detected in infected cells after a 5

minute pulse and in the pulse-chase samples in experiment 1. However, in experiment 2 an extra polypeptide migrating ahead of it was also detected until 24 hours chase when its level was reduced. p27 was present in all the samples from experiment 1 but appeared in much larger quantities in the 5 minute chase sample (track D).

B. Effect of temperature on the production of virus-specified polypeptides

Vero cells, inoculated with Mill Door/79 virus at a multiplicity of 20-30 pfu/cell, were incubated at either 31°C or 37°C for 6 hours when they were treated with 5µg/ml AMD. After incubation for a further 4 hours they were labelled with 50µCi/ml [^{35}S] methionine for 1 hour. Figure 5.2 shows the polypeptide profiles of both infected and uninfected cells. The reduction in temperature to 31°C had no effect on host cell polypeptide production with or without AMD. The level of [^{35}S] methionine incorporation in infected cells incubated in the presence of AMD (tracks D and H) was slightly higher than that of infected cells incubated without AMD (tracks C and G).

In a similar experiment, infected Vero cells incubated at 37°C and 42°C were treated with 5µg/ml AMD 2 hours p.i. and labelled with 50µCi/ml [^{35}S] methionine for 1 hour at 6 hours p.i. No differences between the polypeptide profiles of virus grown at these temperatures was observed (not shown).

C. Effect of treatment with protease inhibitors on the infectivity and production of virus specified polypeptides

For infectivity studies, Vero cells were inoculated at a multiplicity of 0.1 pfu/cell and incubated at 37°C for 24 hours

in the presence of one of the inhibitors, after which they were harvested for titration. For intracellular labelling, Vero cells were inoculated at a multiplicity of 20-30 pfu/cell, incubated at 37°C for 6.5 hours, washed with labelling medium and incubated for a further 15 minutes and then one of the inhibitors added. At 7 hours p.i. the cells were labelled with 50μCi/ml [^{35}S] methionine for 1 hour and then harvested.

(i) Iodoacetamide (IOA). Mill Door/79 virus infected cells were treated with a range of concentrations of IOA prepared in DMSO. A concentration of 1mM caused a 2.0 $\log_{10}$ pfu/ml reduction in titre compared to untreated infected cells (Fig. 5.3). At this concentration of IOA, DMSO was present in the medium at 5% at which concentration it alone reduced the titre by 1 $\log_{10}$ pfu/ml. Figure 5.5, tracks C and D show the polypeptide profiles of infected Vero cells treated with 0.1mM and 0.5mM IOA respectively. No changes were detected in comparison with untreated cells (track B).

(ii) Zinc acetate (ZnAc). Infected Vero cells were treated with a range of ZnAc concentrations. A concentration of 2mM caused a 2.5 $\log_{10}$ pfu/ml reduction in titre compared to untreated infected cells, whereas in the presence of 0.5mM ZnAc the reduction was only 0.5 $\log_{10}$ pfu/ml (Fig. 5.3). Cytopathic effects became evident after only 12 hours in cells treated with 1mM ZnAc compared to 24 hours in untreated cells. The profile of virus-specified polypeptides produced in infected cells treated with either 0.5mM or 1.5mM ZnAc is shown in Fig. 5.5, tracks E and F. Polypeptides p36 and p30 were not detected (position indicated by diamonds).

(iii) L-1-tosylamide-2-phenyl ethyl chloromethyl ketone (TPCK). Infected Vero cells were treated with various concentrations of

TPCK ranging from 0.01mM to 1.0mM. No reduction in the titre compared to untreated infected cells was observed (Fig. 5.4). Figure 5.5, tracks G and H show the polypeptide profiles of cells treated with 0.05mM and 0.1mM TPCK respectively. Polypeptides p36 and p30 (see diamonds, tracks E and F) could not be detected.

(iv) N-α-p tosyl-L-lysine chloromethyl ketone HCl (TLCK). Infected Vero cells were treated with a range of TLCK concentrations from 0.01 to 1.0mM. At 1.0mM, TLCK had no effect on infectivity (Fig. 5.4). The profile of virus specified polypeptides produced in infected cells after treatment with TLCK at 0.05mM or 0.1mM is shown in Fig. 5.5, tracks I and J. Polypeptides p36 and p30 were not detected (diamonds, tracks E and F).

(v) Phenylmethyl sulphonyl fluoride (PMSF). Infected Vero cells were treated with PMSF at a range of concentrations from 0.01mM to 1.0mM. Figure 5.4 shows that in the presence of 0.05mM PMSF the titre was reduced by 0.42 $\log_{10}$ pfu/ml, whereas 0.1mM PMSF reduced the titre by 0.64 pfu/ml compared with untreated infected cells. At these concentrations p36 was not detected in infected cells, whereas p30 was present at 0.05mM but was reduced at 0.10mM PMSF (Fig. 5.5, tracks K and L).

D. Inhibition and re-initiation of translation of virus-specified polypeptides using sodium chloride

Vero cells were inoculated with Mill Door/79 virus at a multiplicity of 20-30 pfu/cell, incubated at 37°C for 6.5 hours, and then washed in labelling medium. Fifteen minutes before labelling, two flasks were each treated with one of the following concentrations of NaCl: 175mM, 225mM, 275mM or 325mM. Immediately prior to labelling, one set of flasks containing each

of the NaCl concentrations were washed in labelling medium to remove the NaCl. The cells were labelled with 100μCi/ml [^{35}S] methionine at 7 hours p.i. for 5 minutes. Figure 5.6 shows the effect of NaCl on virus-specified polypeptide production. The level of radiolabel incorporated into proteins was reduced with increasing NaCl concentrations (tracks C-F). However, polypeptides were still detected after 15 minutes treatment with 325mM NaCl, but treatment either with higher NaCl concentrations, or for longer periods, caused the cells to round up and become detached from the flasks. Re-initiation of protein synthesis did not occur in these cells. When the NaCl concentrations were returned to normal from 325mM (track J) the level of protein synthesis was increased compared to cells still under treatment (track F). A polypeptide migrating between p96 and p65 with an approximate molecular weight of 75 x 10^3 was clearly visible in the cells labelled without NaCl (tracks G-J, arrowed) and was also present in reduced relative levels in cells labelled in the presence of 175mM NaCl (track C).

E. Partial proteolysis

Vero cells, inoculated with Mill Door/79 virus at a multiplicity of 20-30 pfu/cell, were incubated for 7 hours at 37°C and then labelled with 150μCi/ml [^{35}S] methionine, for 1 hour. After separation on 17.5% PAGs, the virus-specified polypeptides were digested with protease enzymes either individually or in strips. Figure 5.7 shows the products of the digestion of some individual virus-specified polypeptides using S.aureus V8 protease. The oligopeptide profiles of each of the polypeptides treated were unique. The virus polypeptides were not all digested individually because of the difficulty in

separating p69 from p65, and p37 from p36. In each case only the top quarter to third (slower migrating) of these bands was removed for digestion on the assumption that these would contain only the larger polypeptides, i.e. p69 and p37. p30 and p27 failed to produce oligopeptides that were detected on 17.5% gels when individually digested with S.aureus V8 protease. Figure 5.8 shows the proteolysis pattern produced by the digestion of a gel strip containing separated virus-specified polypeptides, using S.aureus V8 protease. p27 gave several products, four of which co-migrated with digestion products from p44 (open arrows). The oligopeptide profiles of the other virus polypeptides were each unique. Gel strips were also digested using α-chymotrypsin and trypsin. The oligopeptide profiles produced from each virus-specified polypeptide, after digestion with α-chymotrypsin (Fig. 5.9), were each distinct from the corresponding profiles after digestion with S.aureus V8 protease (Fig. 5.8). Three oligopeptides were produced by the digestion of p27 with α-chymotrypsin and these comigrated with products of p44 (arrows). The oligopeptide profiles produced by digestion of the virus-specified polypeptides with trypsin (Fig. 5.10) were distinct from the corresponding profiles observed after S.aureus V8 protease and α-chymotrypsin treatment (Figs. 5.8 and 5.9). A product from trypsin digestion of p44 comigrated with p27 (arrows), which was not cleaved.

F. Effect of inhibitors of glycosylation on the infectivity and production of virus-specified proteins

Vero cells were inoculated with Mill Door/79 virus at a multiplicity of 0.1 pfu/cell, incubated in the presence of either TM or 2DG for 24 hours, and harvested for titration. The

infectivity was unaffected by TM at concentrations up to 1.0μg/ml, but was reduced slightly (by 0.5 $\log_{10}$ pfu/ml) at concentrations of 10μg/ml (Table 5.1). The 2DG did not reduce infectivity significantly at concentrations up to 15mM (Table 5.1).

The effect of the glycosylation inhibitors on virus-specified protein synthesis was also investigated. Vero cells, inoculated at a multiplicity of 20-30 pfu/cell, were incubated at 37°C for 3 hours, after which time they were treated with either 10μg/ml TM or 6.0mM 2DG. At 7 hours p.i. the cells were labelled with 50μCi/ml [^{35}S] methionine for 1 hour then harvested for electrophoresis. Uninfected cells treated with TM displayed reduced levels of radiolabel incorporation, compared to untreated cells, and the loss of four host-cell polypeptides (Fig. 5.11, tracks A and C, open arrows). However, of these three polypeptides were present in 2DG treated cells. A polypeptide, present in untreated cells, was not detected in 2DG and TM treated cells, whereas they contained a polypeptide which was not visible in untreated cells (tracks A, B and C, arrows). Treatment of infected cells with TM reduced radiolabel incorporation compared to untreated infected cells and also prevented the appearance of p20 (track F). This polypeptide was also not seen when the experiment was repeated using 5μg/ml TM (not shown). Treatment with 2DG had no effect on the production of virus specified polypeptides (track E).

G. Incorporation of either [^{14}C] mannose or [^{14}C] glucosamine into virus specified polypeptides

Vero cells were inoculated with Mill Door/79 virus at a multiplicity of 20-30 pfu/cell and incubated at 37°C for 3

hours. The cells were then treated with either 10μg/ml TM or 6.0mM 2DG and, after a further hour incubation, were labelled with either 2μCi/ml [^{14}C] protein hydrolysate, 10μCi/ml [^{14}C] mannose or 10μCi/ml [^{14}C] glucosamine, and incubated for 4 hours before harvesting for electrophoresis. The incorporation of [^{14}C] mannose into host-cell polypeptides was not affected by 2DG (Fig. 5.12, tracks B and C), but was almost completely inhibited by TM (track D). The virus-specified polypeptides p93, p69, p53/51, p44, p37 and to a lesser extent p141, p65, p36, p27 and p21 were labelled with [^{14}C] mannose (track F). Both 2DG and TM failed to block this incorporation (tracks G and H). A host-cell specified polypeptide, present in both infected and uninfected cells, which was labelled by [^{14}C] mannose (tracks A and B, and F and G, indicated by CP) was not labelled in the presence of TM (tracks D and H). [^{14}C] glucosamine was incorporated onto the same polypeptides as [^{14}C] mannose (Fig. 5.13, tracks B and E). Treatment with 2DG inhibited [^{14}C] glucosamine incorporation completely (track F), whereas TM had no effect (track G).

III. DISCUSSION

Mill Door/79 virus expresses 10 segments of dsRNA (Chapter 6), but produces 13 virus-specified polypeptides in infected Vero cells (Chapter 4). This could occur in several ways: (1) post-translational modification, such as cleavage of primary gene products or the addition of some chemical component to the polypeptide chain, (2) incomplete translation of the genomic RNA possibly caused by either premature termination of translation, or initiation at more than one site or in more than one reading frame, (3) modifications at the level of transcription, i.e.

messenger RNA splicing. The experiments described here were carried out in order to determine whether post-translational modifications to the virus primary gene products could account for the apparent discrepancy between the number of genome segments and the number of polypeptides.

Pulse-chase experiments should identify polypeptides modified by either cleavage, or the addition of components such as sugar moieties, only if these alterations do not occur either during translation or rapidly (within minutes) afterwards. Consequently a failure to demonstrate processing by pulse-chase experiments should not imply that it does not occur. No evidence for protein modification was found. Pulse-chase experiments performed on bluetongue viruses failed to demonstrate processing (Huismans, 1979; Gorman et al., 1981), whereas processing was established among other members of the Reoviridae using this method (Zweerink et al., 1971; McCrae & Faulkner-Valle, 1981; Dyall-Smith & Holmes, 1981b).

To determine whether any rapid cleavage of virus polypeptides was occurring, two methods of inhibiting cleavage reactions were used. Changing the incubation temperature, successfully used to demonstrate protein cleavage with both reoviruses and insect picornaviruses (Zweerink et al., 1971; Moore & Pullin, 1983), did not produce evidence for cleavage with Mill Door/79 virus. However, the use of protease inhibitors indicated that two virus-specified polypeptides p36 and p30 were produced by cleavage of a precursor. p36 and p30 were not produced in the presence of ZnAc, TPCK and TLCK, and p36 was not produced in the presence of PMSF. The two polypeptides are probably produced by either rapid post-translational cleavage or cleavage during translation. The alkylating agent IOA failed to

inhibit the production of p36 and p30. Korant (1973) demonstrated that IOA did not block early cleavage reactions during the processing of poliovirus proteins whereas TPCK and TLCK did. This may indicate that a common mechanism is involved in the production of p36 and p30 and some picornavirus proteins. Virus infectivity was reduced by IOA, ZnAc and PMSF, although only ZnAc and PMSF affected the production of virus polypeptides. Since TPCK and TLCK, which blocked the production of p36 and p30 in cells, had no effect on infectivity, the significance of these polypeptides in virus replication was unclear. Treatment of cells with ZnAc caused a rapid appearance of cpe, which was probably unrelated to virus replication. The reduction of infectivity after treatment with ZnAc was probably due to a mechanism other than the inhibition of virus protein cleavage.

Re-initiation of translation in Mill Door/79 virus infected cells led to an increase in synthesis of 11 virus- specified polypeptides. This suggests that these polypeptides are either primary gene products, synthesized simultaneously, or that cleavage is occurring either rapidly after or during translation. The conditions of the experiment resulted in the appearance of a polypeptide of about 75 x 10^3 daltons, which may have been due to either inhibition of a cleavage reaction or inactivation of a control process preventing the translation of the mRNA. Since host-cell protein synthesis was shut-off by the virus, and host-cell proteins failed to appear after re-initiation of translation, the 75 x 10^3 dalton polypeptide was probably a virus product. Complete inhibition of protein synthesis using NaCl was not achieved as the salt concentration and time required to complete polypeptide chain elongation caused cpe and protein synthesis could not be re-initiated.

Cleavage of a virus polypeptide leads to the formation of products with amino acid sequences identical to those of the precursor. To determine the presence of homologous amino acid sequences, the virus-polypeptides were digested with 3 proteases having differing site specificities. Similar digestion profiles were produced for two polypeptides, p44 and p27, using each enzyme, indicating that they probably contained homologous sequences. This could be the result of protein processing by either rapid cleavage, or cleavage during translation. However, these polypeptides could also be produced by either the separate translation of homologous RNA sequences or the presence of multiple initiation sites.

The presence of glycosylated proteins can be detected by either an apparent increase in the weight of a polypeptide during pulse-chase experiments, the use of glycosylation inhibitors, or by labelling the glycoproteins with [^{14}C] sugars. Mill Door/79 virus polypeptides p141, p93, p69, p65, p53/51, p44, p37, p36, p27 and p21 were labelled by both [^{14}C] mannose and [^{14}C] glucosamine. The incorporation of [^{14}C] mannose into virus-specified proteins was unaffected by both TM and 2DG, which inhibit N-glycosidic and O-glycosidic bond formation, respectively. However, [^{14}C] glucosamine incorporation was blocked by 2DG, glucosamine, therefore, was probably involved in O-glycosidic bond formation. Since 2DG is a mannose analogue (Klenk & Schwarz, 1982), its failure of 2DG to block mannose incorporation is difficult to explain. Non-specific association of mannose with the virus polypeptides is unlikely because the small amount of virus protein present in infected cells would need to bind a prodigious quantity of sugar to label the proteins so strongly.

The infectivity of Mill Door/79 virus was unaffected by 2DG at 15mM, a concentration that reduced reovirus infectivity by about 30% (Lee, 1983). At 0.1µg/ml TM reduced the titre by only 0.1 $\log_{10}$ pfu/ml (20%), whereas this same concentration was sufficient to reduce bovine rotavirus infectivity by 99% (Sabara et al., 1982). Since at 1µg/ml TM increased the titre of Mill Door/79 virus by 0.1 $\log_{10}$ pfu/ml (20%) the effect of TM on infectivity at these concentrations was, therefore, insignificant.

The data presented in this chapter shows that the genome products of Mill Door/79 virus undergo modifications; polypeptides p36 and p30, and possibly p27, are formed by proteolytic cleavage, and 10 virus-specified polypeptides are glycosylated. The significance of these modifications on the replication of Mill Door/79 virus is unclear since the functions of these polypeptides are not known.

Table 5.1

Effect of tunicamycin (TM) and 2-deoxy-D-glucose (2DG) on the growth of Mill Door/79 virus

TM concentration µg/ml	Titre of virus
0	7.5* ± 0.2
0.1	7.4 ± 0.2
1.0	7.6 ± 0.1
5.0	7.2 ± 0.3
10.0	7.0 ± 0.2
2DG concentration mM	
0	7.5 ± 0.2
0.1	7.7 ± 0.1
1.0	7.5 ± 2.2
6.0	7.7 ± 0.1
15.0	7.4 ± 0.3

* Titres expressed as $\log_{10}$ pfu/ml in Vero cells, mean of three experiments

Figure 5.1. Pulse and pulse-chase experiments using Vero cells infected with Mill Door/79 virus. The cells were pulsed with 100μCi/ml [^{35}S] methionine for either 2 or 5 minutes at 6 hours p.i. Autoradiograph of a 12.5% PAG showing: track A, mock infected cells with a 5 minute pulse; B, infected cells and 2 minute pulse; C, infected cells and 5 minute pulse; D-F, infected cells following 5, 10 and 30 minute chases respectively after a 5 minute pulse; G-M, infected cells following 5, 10, 30, 60 and 120 minutes, then 18 and 24 hour chases respectively after a 5 minute pulse. The same polypeptides on different gels are indicated by connecting lines. Open arrows indicate polypeptides migrating slightly slower than p55. Closed arrows indicate a host-cell polypeptide (possibly actin).

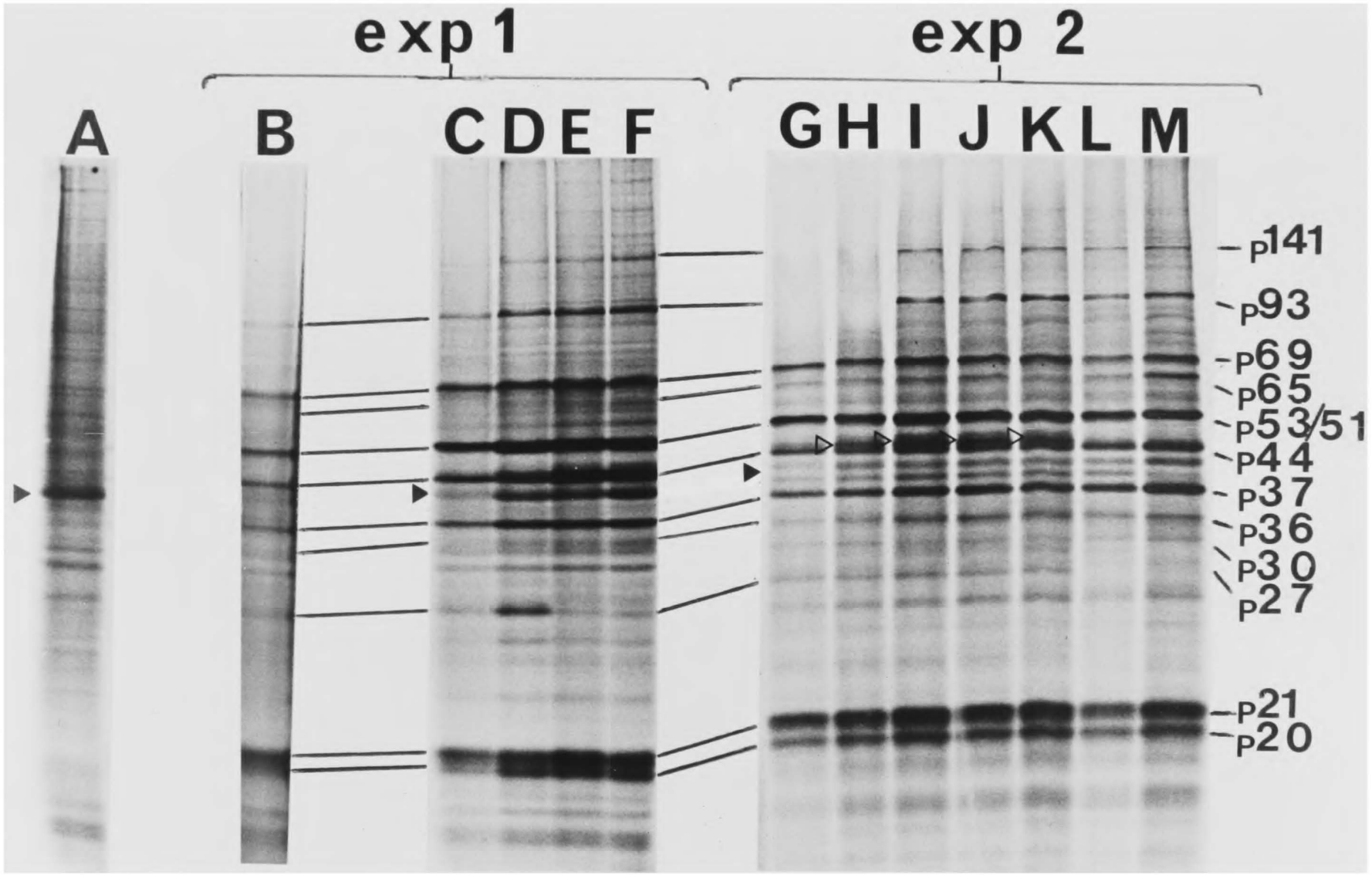
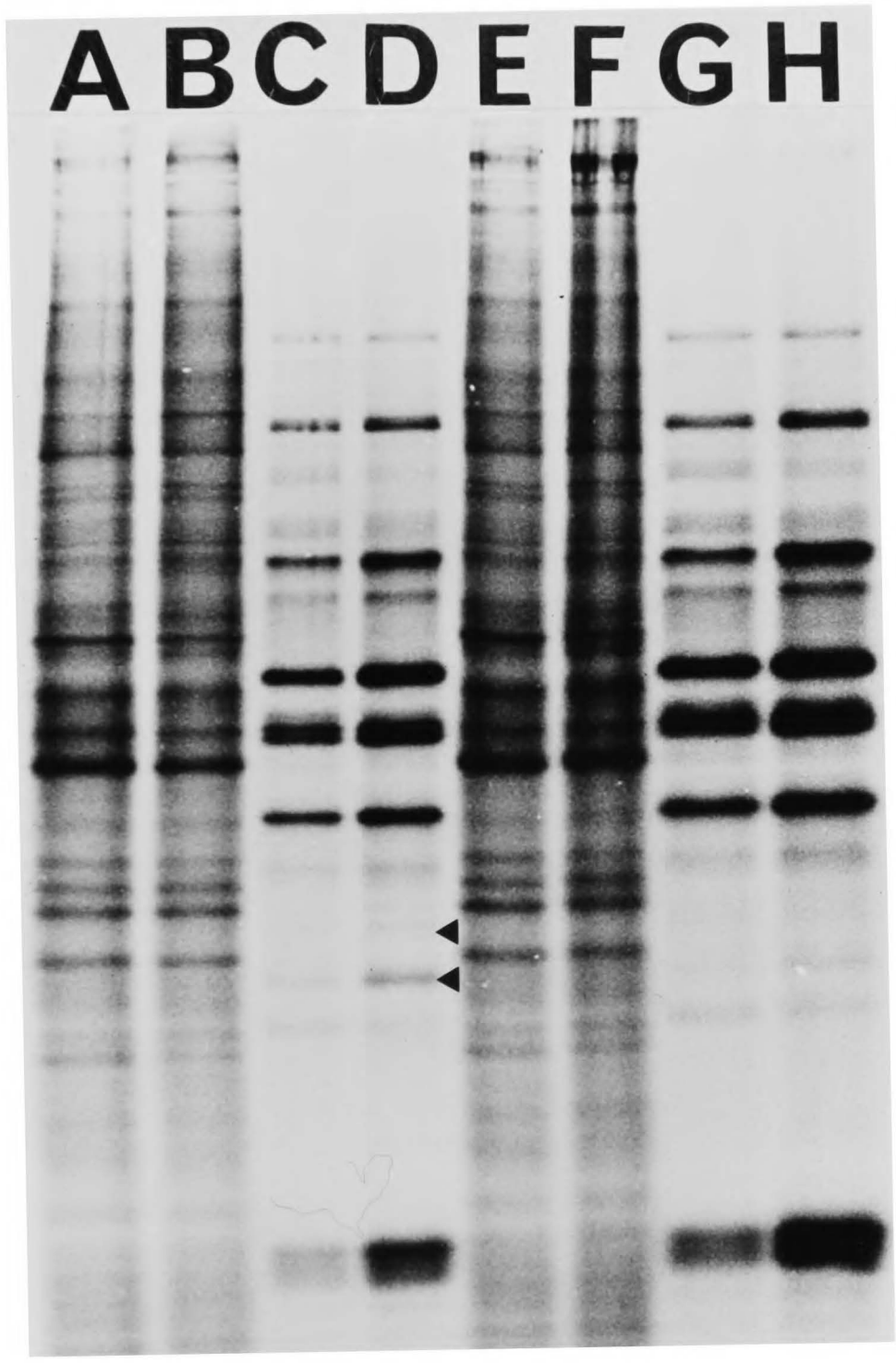

exp 1
exp 2
A
B
C D E F
G H I J K L M
P141
P93
P69
P65
P53/51
P44
P37
P36
P30
P27
P21
P20

Figure 5.2. Effect of incubation at 37°C and 31°C on Mill Door/79 virus polypeptides in Vero cells. Cells incubated at either 37°C or 31°C were pulse labelled with 50µCi/ml [^{35}S] methionine for 1 hour at 10 hours p.i. Autoradiograph of a 12.5% PAG showing: track A, uninfected cells incubated at 37°C; B, as A but treated with 5µg/ml actinomycin D (AMD) 4 hours before labelling; C, infected cells incubated at 37°C; D, as C but treated with 5µg/ml AMD 4 hours before labelling; E, uninfected cells incubated at 31°C; F, as E but treated with 5µg/ml AMD 4 hours before labelling; G, infected cells incubated at 31°C, H, as G but treated with 5µg/ml AMD 4 hours before labelling. Arrows indicate position of p30 (top) and p27 (bottom).


A B C D E F G H

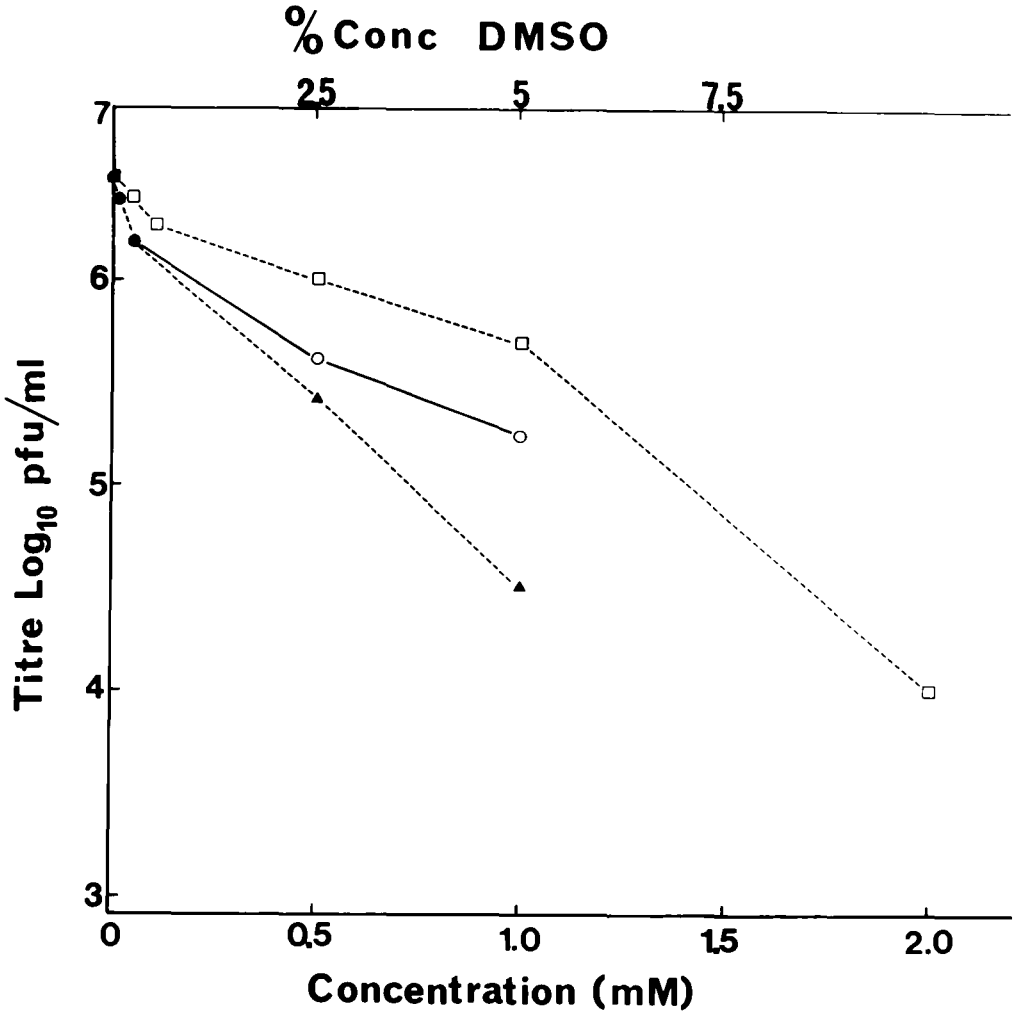


Figure 5.3. Effect of iodoacetamide (IOA) and zinc acetate (ZnAc) on the infectivity of Mill Door/79 virus. Both protease inhibitors were added immediately after inoculation. The cells were harvested 24 hours p.i. IOA was added as a stock solution in dimethyl sulphoxide (DMSO), and ZnAc was dissolved in maintenance medium. --▲-- represents titre with IOA in DMSO, —○— represents titre with DMSO and -□- represents titre with ZnAc.

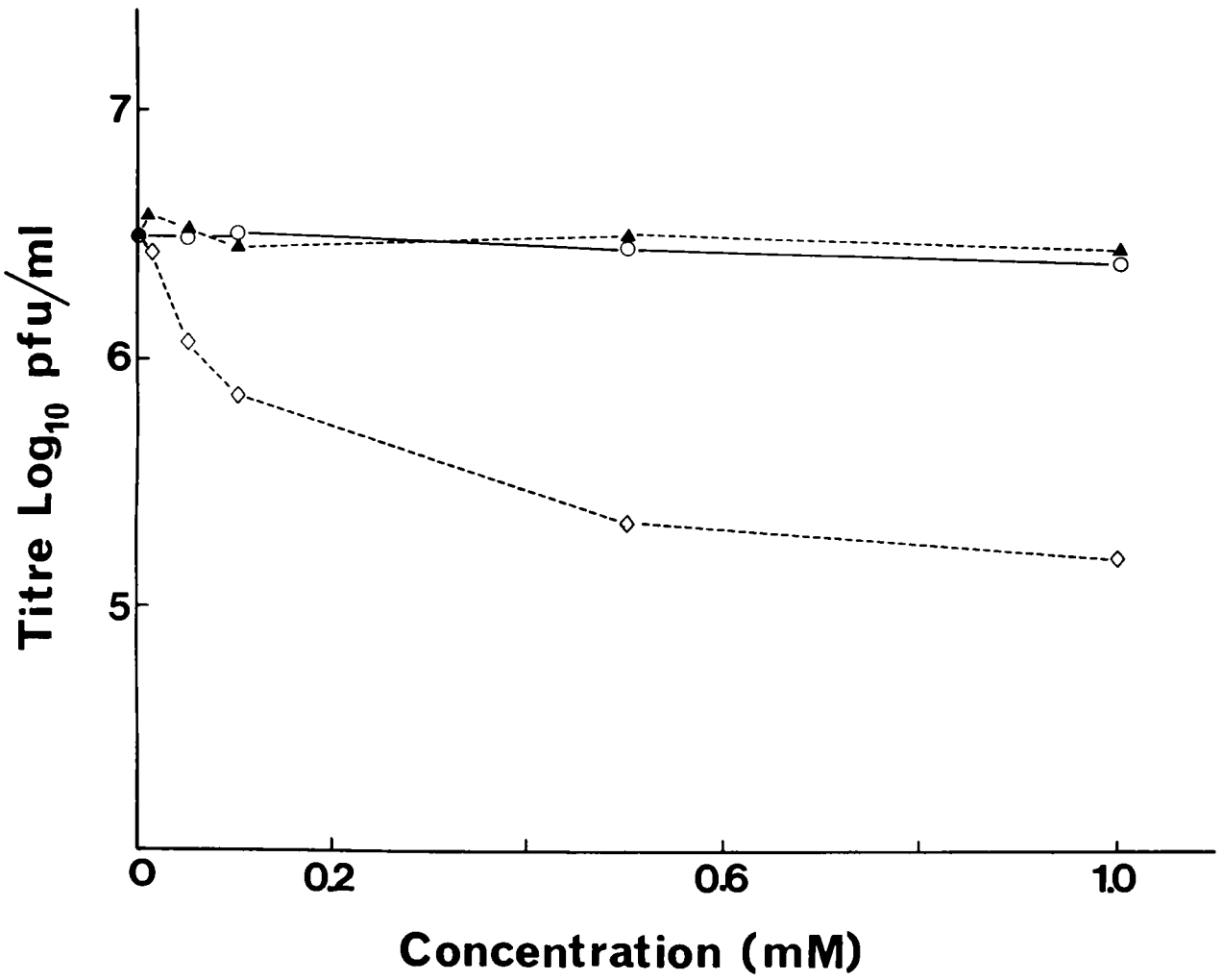


Figure 5.4. Effect of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), N-α-p tosyl-L-lycine chloromethyl ketone HCl (TLCK) and phenyl methyl sulphonyl fluoride (PMSF) on the infectivity of Mill Door/79 virus. Each inhibitor was added immediately after inoculation and the cells were harvested at 24 hours p.i. Virus titres after treatment with differnt concentrations of TPCK (--▲--), TLCK (—○—) and PMSF (-◇--).

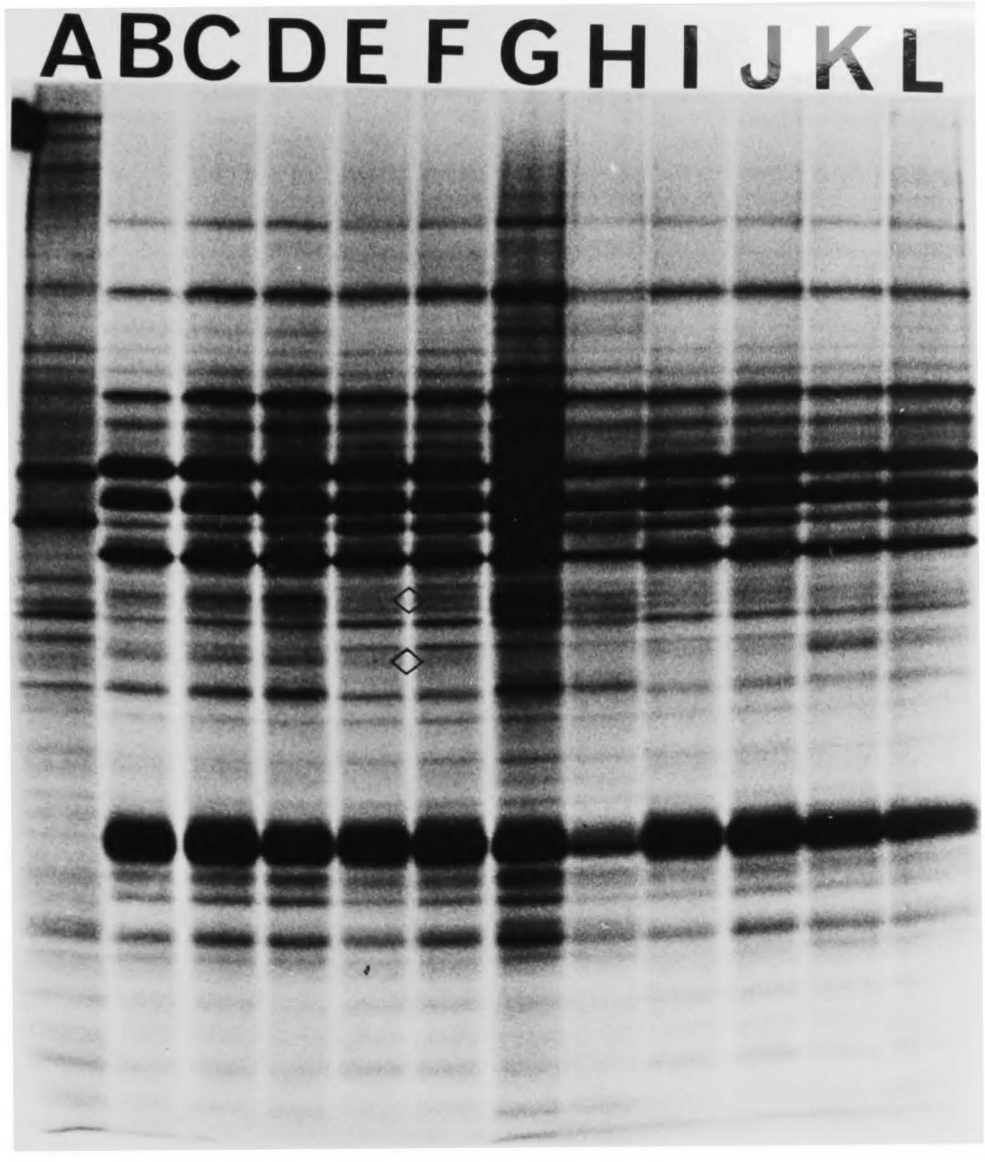


Figure 5.5. Effect of protease inhibitors on the polypeptides in Mill Door/79 virus infected Vero cells. Cells were treated with the inhibitors at 6.75 hours p.i. and labelled with 50μCi/ml [^{35}S] methionine at 7 hours p.i. for 1 hour. Autoradiograph of a 12.5% PAG showing: track A, uninfected cells and B-L, infected cells.. Track B, untreated; C, 0.1mM IOA; D, 0.5mM IOA; E, 0.5mM ZnAc; F, 1.5mM ZnAc; G, 0.05mM TPCK; H, 0.1mM TPCK; I, 0.05mM TLCK; J, 0.1mM TLCK; K; 0.05mM PMSF; L, 0.1mM PMSF. The diamonds indicate the positions of the polypeptides p36 (top) and p30 (bottom).

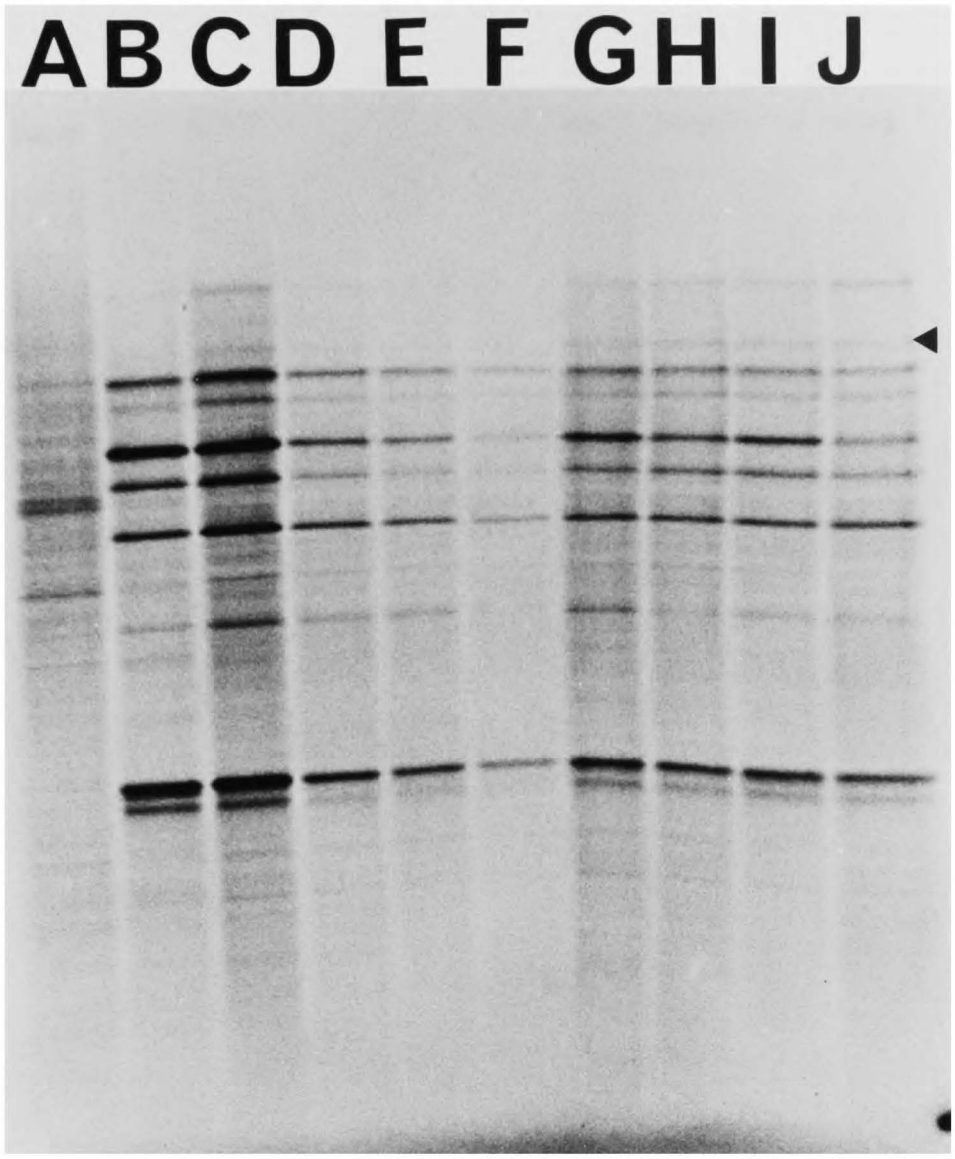


Figure 5.6. Inhibition and re-initiation of the translation of virus-specified polypeptides using NaCl. Mill Door/79 virus infected Vero cells were treated with various concentrations of NaCl 15 minutes before labelling for 5 minutes with 100µCi/ml [^{35}S] methionine, at 7 hours p.i. An autoradiograph of a 12.5% PAG showing: track A, uninfected cells; B, infected cells; tracks C–F, infected cells treated with 175mM, 225mM, 275mM and 325mM NaCl respectively and tracks G–J, infected cells treated as tracks C–F respectively, except that the cells were returned to isotonic medium immediately before labelling.

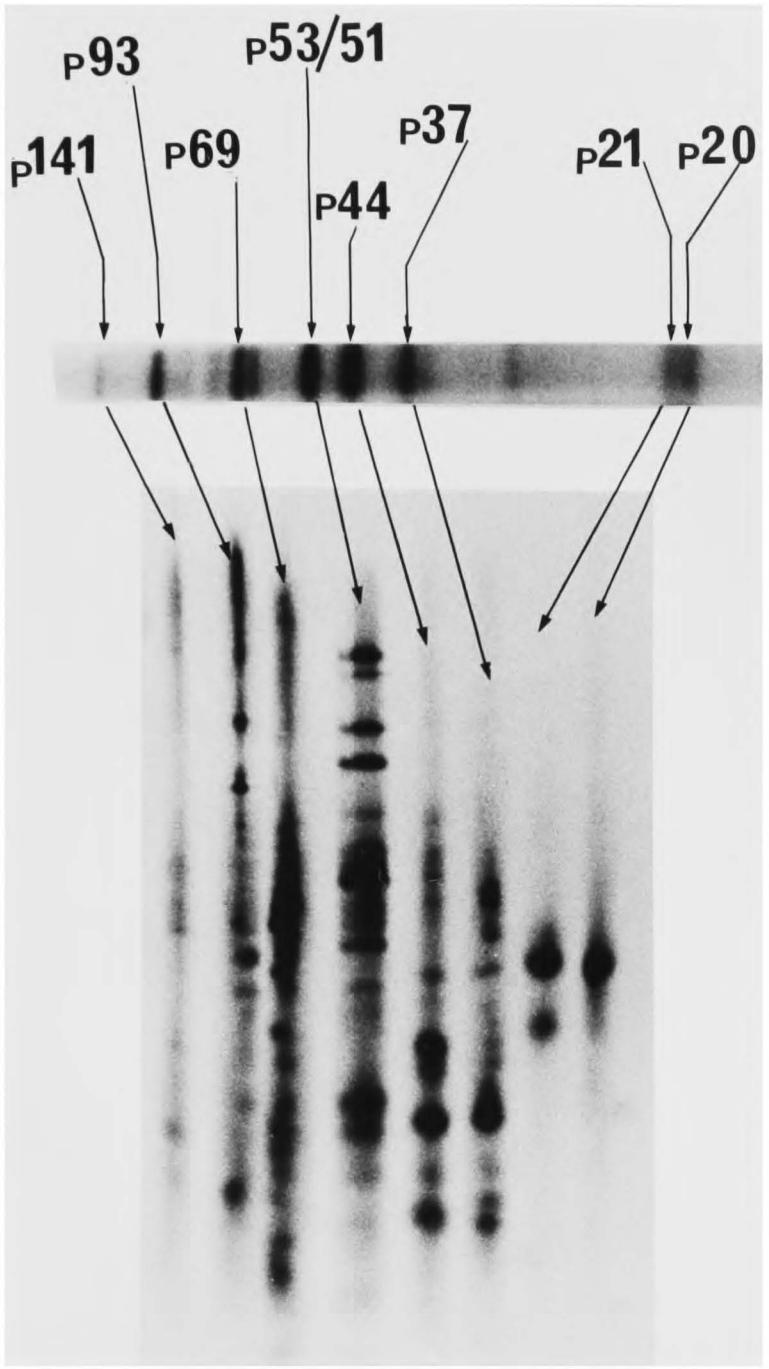


Figure 5.7. Partial proteolysis of Mill Door/79 virus-specified polypeptides. An autoradiograph of a 17.5% PAG of the digestion products of individual polypeptides each treated with 50µg/ml S.aureus V8 protease and separated on a 17.5% PAG.

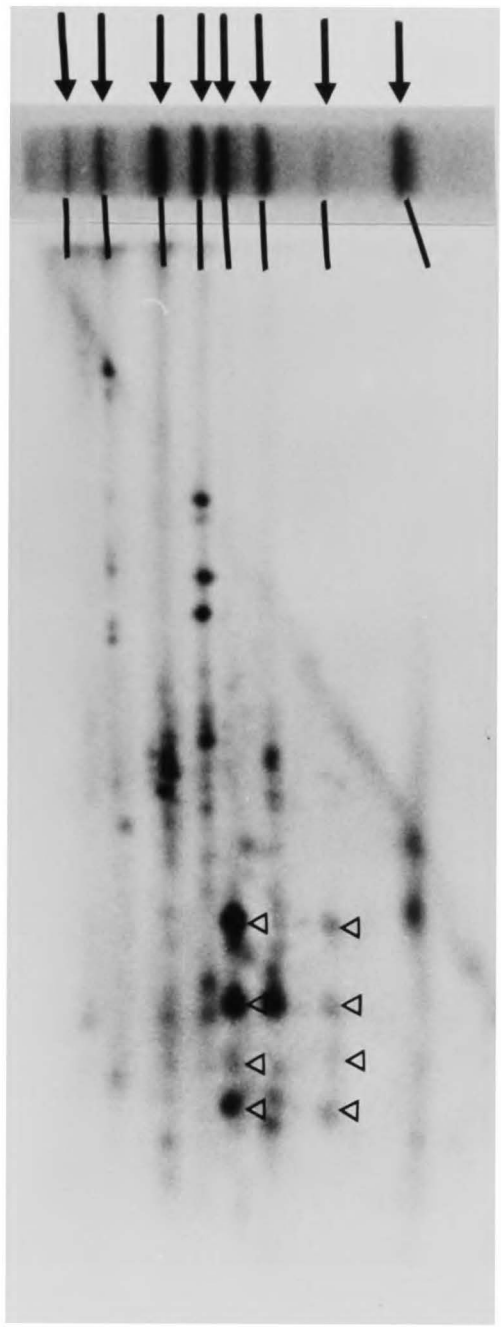


Figure 5.8. Partial proteolysis of Mill Door/79 virus-specified polypeptides. An autoradiograph of a 17.5% PAG showing the digestion products of a gel strip treated with 100µg/ml S.aureus V8 protease. Arrows indicate the virus polypeptides from left to right; p141, p93, p69, p53/51, p44, p37, p27 and p21 and p20. The triangles indicate co-migrating oligopeptides.

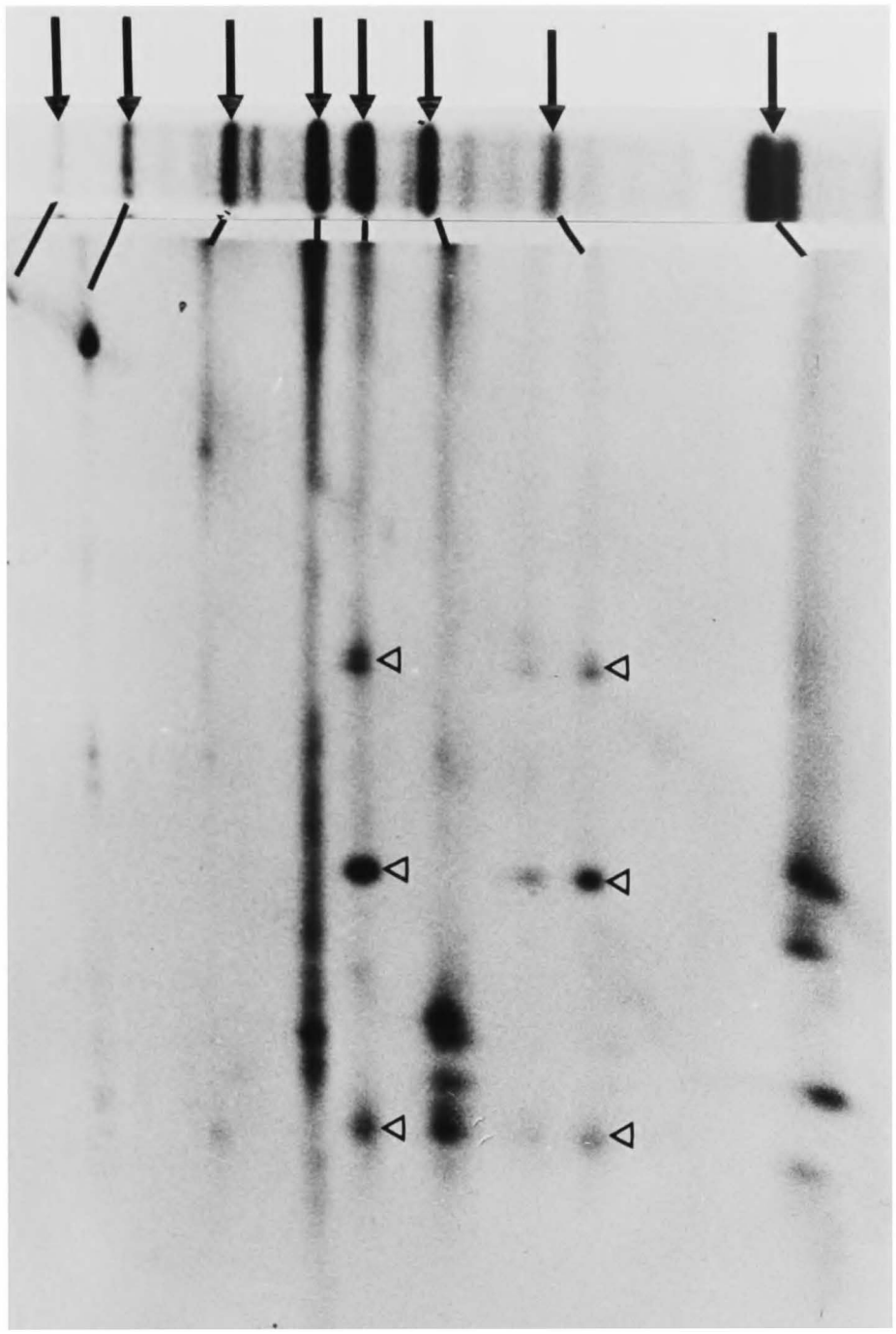


Figure 5.9. Partial proteolysis of Mill Door/79 virus-specified polypeptides. An autoradiograph of a 17.5% PAG showing the digestion products of a gel strip treated with 300µg/ml α-chymotrypsin. Arrows indicate the virus polypeptides from left to right; p141, p93, p69, p53/51, p44, p37, p27 and p21 and p20. The triangles indicate co-migrating oligopeptides.

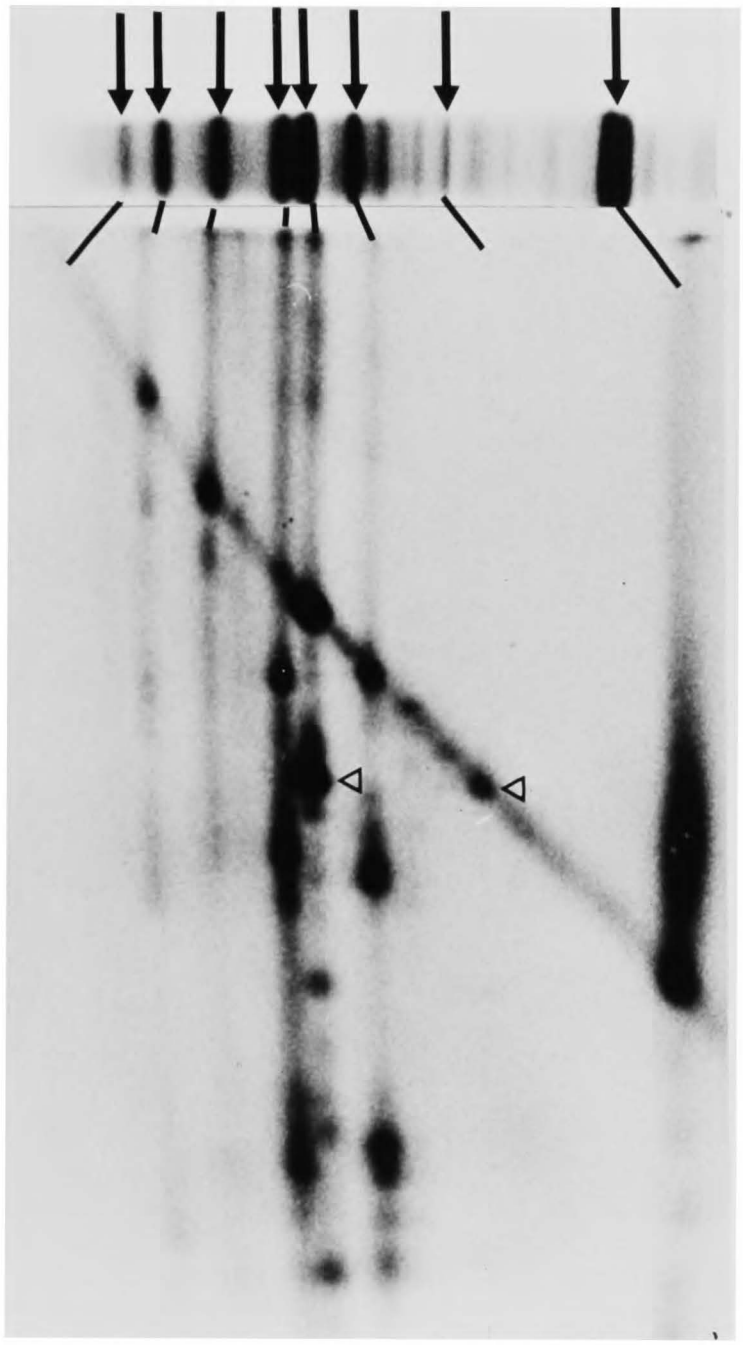


Figure 5.10. Partial proteolysis of Mill Door/79 virus-specified polypeptides. An autoradiograph of a 17.5% PAG showing the digestion products of a gel strip treated with 200µg/ml trypsin. Arrows indicate virus polypeptides from left to right; p141, p93, p69, p53/51, p44, p37, p27 and p21 and p20.

Figure 5.11. Effect of glycosylation inhibitors on Mill Door/79 virus-infected Vero cells. The cells were treated with either 6mM 2-deoxy-D-glucose (2DG) or 10µg/ml tunicamycin (Tm) at 3 hours p.i. and labelled with 50µCi/ml [^{35}S] methionine at 7 hours p.i. for 1 hour. Autoradiograph of a 12.5% PAG shows: track A, uninfected cells; B, uninfected cells treated with 2DG; C, uninfected cells treated with Tm; D, infected cells; E, infected cells treated with 2DG; F, infected cells treated with Tm. The closed arrows indicate variation between untreated and treated cells and the open arrows indicate the polypeptides not present in Tm treated cells.

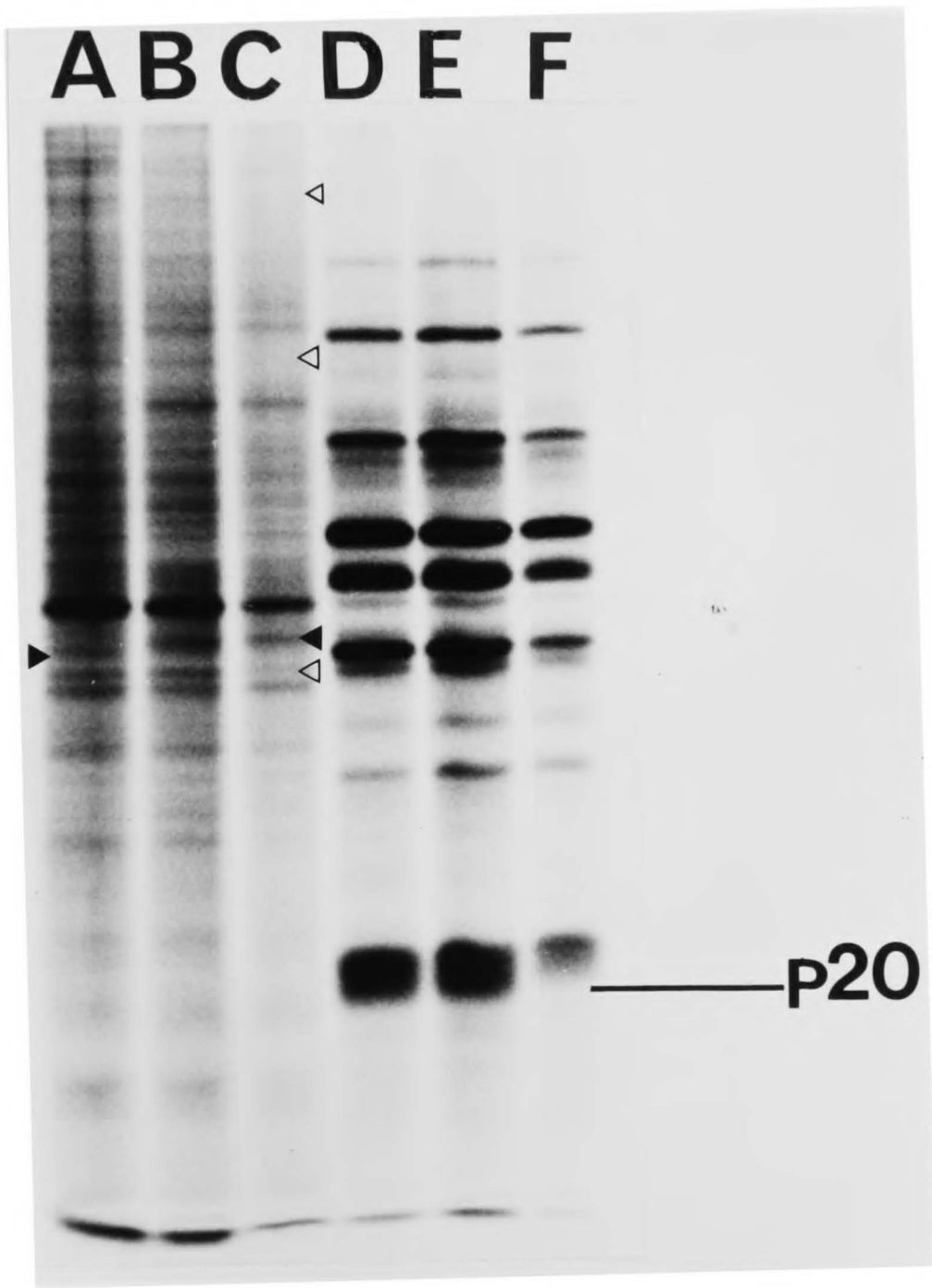

A B C D E F
P20

Figure 5.12. Effect of glycosylation inhibitors on Mill Door/79 virus infected Vero cells labelled with [^{14}C]-mannose. The cells were treated with either 6mM 2-deoxy-D-glucose (2DG) or 10µg/ml tunicamycin (Tm) at 3 hours p.i. and labelled with either 2µCi/ml [^{14}C]-protein hydrolysate or 10µCi/ml [^{14}C]-mannose at 4 hours p.i. for 4 hours. The autoradiograph of a 12.5% PAG shows: track A, uninfected cells labelled with [^{14}C]-protein hydrolysate; B, uninfected cells labelled with [^{14}C]-mannose; C, as B but treated with 2DG; D, as B but treated with Tm; E, infected cells labelled with [^{14}C]-protein hydrolysate; F, infected cells labelled with [^{14}C]-mannose; G, as F but treated with 2DG; H, as F but treated with Tm. Triangles indicate [^{14}C]-mannose labelled virus polypeptides, and cp indicates a host cell polypeptide.

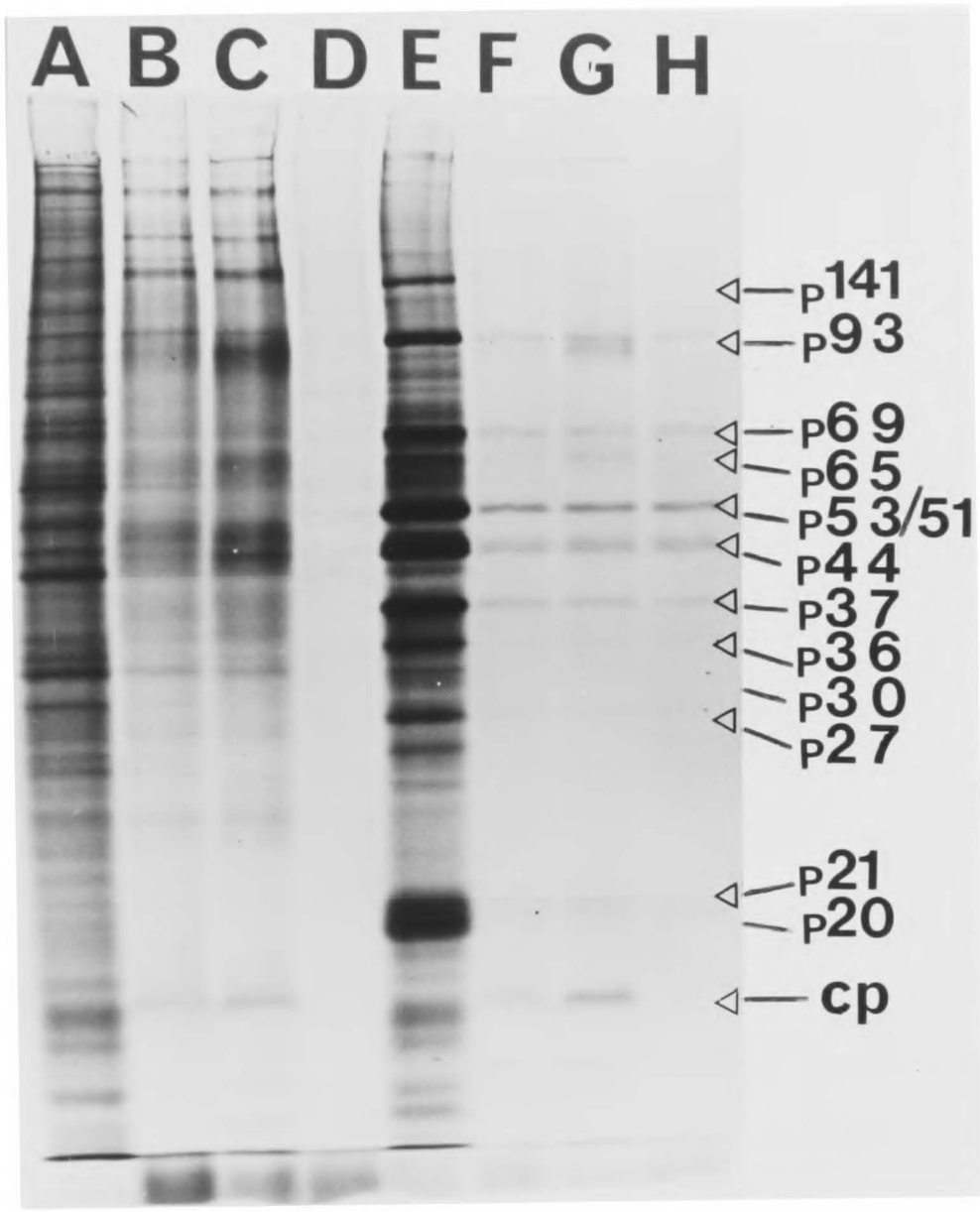
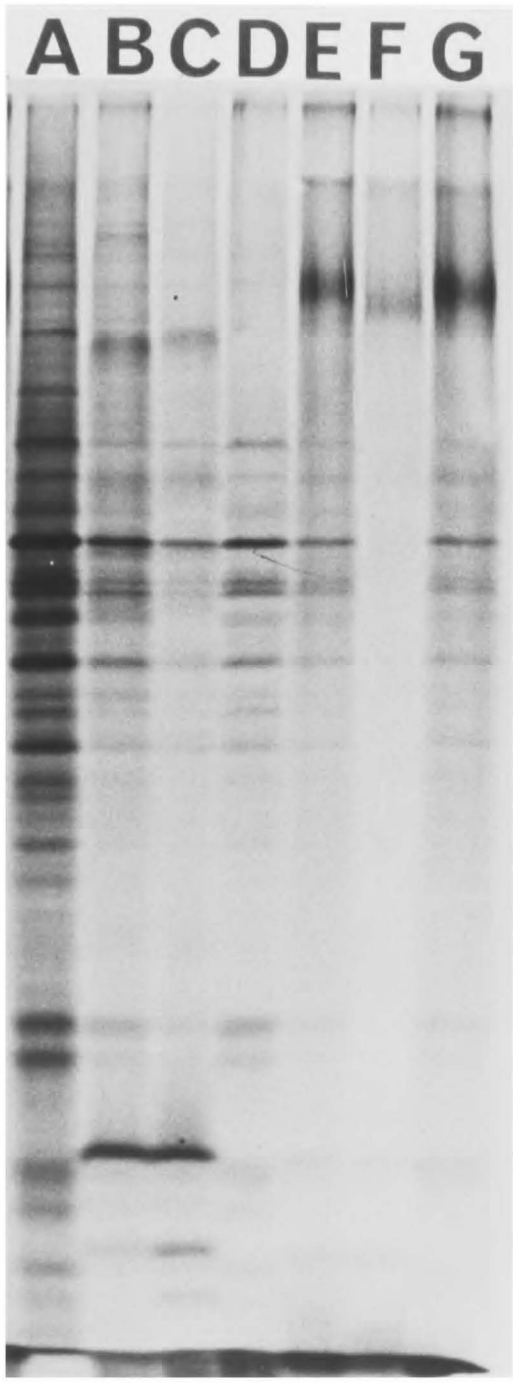

A B C D E F G H
P141
P93
P69
P65
P53/51
P44
P37
P36
P30
P27
P21
P20
cp

Figure 5.13. Effect of glycosylation inhibitors on Mill Door/79 virus infected Vero cells labelled with [^{14}C]-mannose or [^{14}C]-glucosamine. The cells were treated with either 6mM 2-deoxy-D-glucose (2DG) or 10µg/ml tunicamycin (Tm) at 3 hours p.i. and labelled with either 2µCi/ml [^{14}C]-protein hydrolysate, 10µCi/ml [^{14}C]-mannose or 10µCi/ml [^{14}C]-glucosamine at 4 hours p.i. for 4 hours. The autoradiograph of a 12.5% PAG shows: track A, infected cells labelled with [^{14}C]-protein hydrolysate; tracks B-D infected cells labelled with [^{14}C]-mannose; B, untreated; C, 2DG treated; D, Tm treated; tracks E-G, infected cells labelled with [^{14}C]-glucosamine; E, untreated; F, 2DG treated; G, Tm treated.


A B C D E F G

CHAPTER 6

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THE dsRNA EXPRESSED BY MILL DOOR/79 VIRUS AND ITS TRANSLATION PRODUCTS *IN VITRO*

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I. INTRODUCTION

The genomes of all orbiviruses studied so far, except Colorado tick fever virus, comprise 10 segments of dsRNA (Gorman et al., 1983). In Chapters 4 and 5 Mill Door/79 virus was shown to produce 13 virus-specific polypeptides in infected Vero cells, 3 of which may have been produced by the cleavage of primary gene products. Further investigations were carried out to determine the number of primary gene products, by first demonstrating the number of segments of dsRNA expressed by Mill Door/79 virus in cell culture, and then translating them in a cell-free translation system and analysing the products by PAGE. The dsRNA segments were also translated individually to identify their specific products, which were compared with with the virus-specified polypeptides synthesized in vivo.

II. RESULTS

A. Characterization of the Mill Door/79 virus dsRNA

The dsRNA was extracted directly from infected cell cultures and purified using each of several different procedures, which included the fractionation of nucleic acids using guanidium isothiocyanate (after which no dsRNA was obtained), CF 11 cellulose (low yields of dsRNA were obtained), or treatment with lithium chloride. This last procedure, which is described in Chapter 2, produced the highest yields of dsRNA and of sufficient purity for in vitro translation experiments (Section B). The yields of dsRNA calculated for each extraction using lithium chloride varied between 21-38μg/75cm^2 flask (7.0-12.7pg/cell). Figure 6.1 shows the dsRNA profile of Mill Door/79 virus, in

which 10 segments of dsRNA were identified. Segments 4 and 5 co-migrated under normal electrophoresis conditions (15mA, 160V for 16 hours) but were separated by further electrophoresis (30mA, 200V for 24 hours at 4°C) with the resultant loss of segment 10 (not shown). The molecular weight of each segment was determined by co-running the dsRNA of Mill Door/79 virus with that of type 2 CPV (Fig. 6.2). The molecular weights of type 2 CPV dsRNA segments had been calculated previously (Pullin & Moore, unpublished information). A calibration curve was drawn from which the molecular weights of Mill Door/79 virus dsRNA segments were derived (Fig. 6.3). These varied between 2.60×10^6 and 0.18×10^6 daltons (Table 6.1). The total molecular weight of the genome of Mill Door/79 virus was 11.64×10^6 daltons.

B. In vitro translation of the virus dsRNA

Mill Door/79 virus was translated in a cell-free protein synthesizing system prepared from rabbit reticulocyte lysate by the method of Pelham and Jackson (1976). Before translation the dsRNA was denatured with methyl-mercury hydroxide to separate the complementary strands. The denatured dsRNA was added to translation mixtures at final concentrations of 0, 25, 50, 100, 200 and 400µg/ml and incubated at 30°C. Samples were removed from these mixtures at 0, 5, 15, 30, 60 and 90 minute intervals and the amount of TCA-precipitated radioactivity determined. Figure 6.4 shows the effect of different RNA concentrations on the incorporation of [^{35}S] methionine into TCA-precipitated material. In the assays containing 100µg/ml and 200µg/ml RNA, stimulation was detected at 5 to 15 minutes after the start of incubation. The initial rate of incorporation was approximately

317 cpm/minute and 417 cpm/minute for the 100μg/ml and 200μg/ml RNA assays, respectively. These rates were reduced after 15 minutes, and after 60 minutes incorporation ceased. Incubation for a further 30 minutes led to a reduction of incorporation in the 100μg/ml RNA assay. [^{35}S] methionine was incorporated in the assays containing 25, 50 and 400μg/ml at a lower level than the 0μg/ml RNA assay (endogenous control). Since no radiolabel was incorporated into TCA-precipitated material between 60 and 90 minutes in any assay, incubations were stopped after 60 minutes in subsequent experiments. Figure 6.5 shows the effect of increasing RNA concentration on the incorporation of [^{35}S] methionine into TCA-precipitated material. Low levels of RNA (25 and 50μg/ml) appeared to reduce incorporation, compared with the endogenous control. However, at 100 and 200μg/ml the RNA stimulated incorporation by 2.3-3.1-fold, respectively, compared with the endogenous control, but there was a sharp reduction in stimulation when the RNA concentration was raised to 400μg/ml. The determinations of optimum Mg^{2+} and K^{+} concentrations were not made, since in vitro translations carried out using the cation concentrations already present in the lysate (1.7mM for Mg^{2+} and 107mM for K^{+}) gave adequate product yields, but dilution of the translation mixture to even a small degree, to alter these concentrations, reduced incorporation by a factor of 10 to 50.

The translation products of Mill Door/79 virus RNA, after 60 minute incubation at concentrations from 25-800μg/ml, were analysed on a PAG (Fig. 6.6). Under these conditions several proteins were synthesized in vitro (tracks B-E) which co-migrated with the virus-protein species synthesized 7-8 hours p.i. in Mill Door/79 virus-infected Vero cells (track A). At RNA concentrations of 400 and 800μg/ml (tracks F and G) the only visible

products co-migrated with the endogenous polypeptides (track H). Increasing the RNA concentration from 25 to 200µg/ml led to an increase in the amounts of products, reflecting the pattern of stimulation from the radiolabel incorporation studies (Fig. 6.5). Although the profiles of polypeptides produced *in vivo* and *in vitro* were basically similar, some differences were apparent (Fig. 6.6, tracks A and E and Fig. 6.7). *In vitro* a polypeptide was produced between p93 and p69 which was not detected *in vivo* (arrow). The *in vivo* polypeptides p30 and p27 were not detected *in vitro*, and the *in vitro* polypeptide co-migrating with p53/51 was observed as a single band at all dsRNA concentrations. The relative amounts of *in vivo* and *in vitro* products were similar, although proportionately less of p69, p53/51, p44, p37 and p36 were produced *in vitro* (Fig. 6.7). An endogenous product, synthesized *in vitro*, co-migrated with a host-cell polypeptide (Fig. 6.7, open arrows). The level of endogenous products observed *in vitro* varied in relation to the concentration of added RNA. From 25 to 200µg/ml endogenous polypeptides were present at low levels, but increased when either no RNA was added (Fig. 6.6, track H), or when 800µg/ml of RNA was present (track G).

C. Derivation of the dsRNA coding assignments by comparison of theoretical and measured polypeptide molecular weights

In order to determine the relationship between the size of the virus coded polypeptides and the size of the dsRNA segments, the molecular weight of the polypeptides were calculated based on the molecular weight of each segment. Assuming that each segment was fully transcribed and translated, a conversion factor of 18 was used, derived from the ratio of the average molecular weight

of dsRNA to that of a polypeptide of equivalent size (Verwoerd et al., 1972). Table 6.2 shows the molecular weights of 10 polypeptides calculated from the 10 genome segments, together with the observed molecular weights of the 13 polypeptides produced in vivo, and the 12 produced in vitro. The polypeptides are listed in order of decreasing molecular weight and no attempt is made to compare the various sizes. If the polypeptides regarded as possible cleavage products are ignored the following pairings between calculated and observed (in vivo) molecular weights can be made: 144×10^3 and 141×10^3, 65×10^3 and 69×10^3, 65×10^3 and 65×10^3 or 62×10^3 and 65×10^3, and 34×10^3 and 37×10^3. As well as making the matches listed above using the in vivo values the 75×10^3 dalton polypeptide produced in vitro could be matched with the calculalted product of 78×10^3 daltons.

Mismatching was clearly evident in two cases; the calculated size of the polypeptide produced from segment 10 was only 10×10^3 whereas the smallest product identified experimentally was twice this size, and the "theoretical polypeptide" of 127×10^3 daltons was 34×10^3 daltons larger than any product identified in vivo or in vitro (except the 141×10^3 dalton product). The total calculated size of the proteins produced from the Mill Door/79 virus genome was 647×10^3 ($11.64 \times 10^6/18$), whereas the total size of the polypeptides produced in vivo and in vitro (excluding the possible cleavage products) was 594×10^3 and 669×10^3 respectively. Because of the difficulty in matching the theoretical and experimentally determined values, complete coding assignments could not be determined using this approach.

D. Separation and individual translation of virus dsRNA segments

The dsRNA of Mill Door/79 virus was run on a 5% preparative PAG and the separated segments individually harvested. To ensure an adequate yield of each segment for translation, 1.5mg of dsRNA was loaded onto a single gel and electrophoresed to obtain maximum separation while retaining all 10 segments on the gel (Fig. 6.8). Segments 4, 5 and 6, which did not resolve into individual bands on the first preparative gel, were subjected to further electrophoresis for 24 hours at 30mA when segment 6 was individually resolved (not shown). However, segments 4 and 5 could not be separated and were extracted from the gel together. The separated segments were electroeluted from the gel, phenol-extracted to remove monomeric acrylamide, and ethanol precipitated. Samples (0.5µg) from each extraction were electrophoresed on a 10% PAG to check their purity (Fig. 6.9). All 10 dsRNA segments were successfully extracted. Segments 1, 2, 3, 9 and 10 were free of any cross contamination, as judged by the appearance of single bands after silver staining. However, segments 4 and 5 (extracted together) showed cross contamination with segment 6, and a small degree of cross contamination was detected between segments 7 and 8. The yields of dsRNA varied between 23 and 90µg per segment, i.e. 15-60% of that originally loaded onto the preparative gel.

Figures 6.10 and 6.11 show the products from 2 different translation experiments using the separated genome segments. It is clear from these figures that some genome segments translated more efficiently than others, although none of them failed to produce any products. More than one polypeptide was produced by the translation of segments 1 and 2. Segment 1 produced three distinct polypeptides (Fig. 6.11, arrowed), one of which

co-migrated with p141 (Fig. 6.10, track C and I), and two more diffuse products (open arrows). Segment 2 also produced 3 distinct polypeptides (arrowed) together with some diffuse ones (open arrows). The largest of these polypeptides co-migrated with p93, whereas the smaller one co-migrated with a product (migrating between p93 and p75) produced only in some *in vitro* translation experiments. Segment 3 produced a single product (arrowed) which co-migrated with p75, a polypeptide only produced *in vitro*. The combined segments 4 and 5 produced a major polypeptide (arrowed) and a minor one (open arrow). Polypeptides co-migrating with these products, together with an intermediate size polypeptide (arrowed), were also present when segment 6 was translated. These three products co-migrated with the *in vivo* products p69, p65 and p53/51 respectively. Segment 7 produced a polypeptide which co-migrated with p44 (Fig. 6.11, arrow) and a small amount of a polypeptide co-migrating with p37 (open arrow). In some translation experiments using segment 7 the major polypeptide was lost and the minor component could be seen clearly (Fig. 6.10, open arrow). Segment 8 produced a single major product which co-migrated with p37 (Fig. 6.10, arrowed). Segments 9 and 10 each produced only one polypeptide, co-migrating with p20 and p21 respectively.

E. Determination of coding assignments from the individual translation of dsRNA segments

Provisional coding assignments were made by assuming that co-migration of polypeptides during electrophoresis was an indication of identity (McCrae & Joklik, 1978; Mertens *et al.*, 1984). The largest *in vitro* translation products from segments 1 to 3 co-migrated with p141, p93 and p75 respectively whereas the

products from segments 4 and 5 co-migrated with p69 and p53/51. Segment 6 produced polypeptides co-migrating with p69, p65 and p53/51. The similarity between the products of segments 4 and 5 and segment 6 were probably due to cross contamination. Segment 6 gave an extra product (co-migrating with p65) which was not produced on translation of segments 4 and 5. This product was assigned to segment 6 while the other two products (co-migrating with p69 and p53/51) were assigned to segments 4 and 5. A slight degree of cross contamination was observed between segments 7 and 8 therefore the major product synthesized by each was regarded as the true product. A map of coding assignments was constructed from the above data in which 10 polypeptides were provisionally assigned to the 10 dsRNA segments (Fig. 6.12). Three polypeptides (p36, p30 and p27) were not assigned since they were not produced by the individual translation of any of the segments, although p36 did appear to be synthesized *in vitro* by the unfractionated dsRNA (Fig. 6.10,track T).

The assignments of p93, p65, p53/51, p21 and p20 were confirmed by partial proteolysis with the products of *in vitro* translation with which they co-migrated. No variations were detected among the digest products of co-migrating polypeptides (Fig. 6.13). The other *in vitro* products were produced in insufficient quantities to allow their detection after partial proteolysis.

III. DISCUSSION

Mill Door/79 virus, like the majority of orbiviruses studied to date, expressed 10 segments of dsRNA in cell culture. The dsRNA pattern on PAGs (2 + 4 + 3 + 1) was similar to Nugget virus

(a member of the Kemerovo serogroup) and Corriparta virus (Corriparta serogroup) (Gorman et al., 1983), but was distinct from bluetongue viruses and Changuinola virus, which displayed 3 + 3 + 3 + 1 and 3 + 3 + 1 + 1 + 2 patterns, respectively (Gorman et al., 1983; Knudson, 1981). The total size of the dsRNA segments of Mill Door/79 virus (11.46 x 10^6 daltons) was similar to that of Nugget virus (11.7 x 10^6 daltons) (Gorman et al., 1984), bluetongue virus (11.80 x 10^6) (Verwoerd et al., 1972) and Changuinola virus (11.40 x 10^6) (Knudson et al., 1981) and was significantly lower than the molecular weights of other members of the reoviridae (Joklik, 1974; Verwoerd et al., 1979). The largest segment of Mill Door/79 virus-expressed dsRNA (2.52 x 10^6 daltons) was similar in size to that of other orbiviruses whereas the smallest segment (0.18 x 10^6) was smaller than those of most other orbiviruses, e.g. bluetongue virus type 10 segment 10 was 0.30 x 10^6 daltons. However, the calculated size of the smallest segment may be inaccurate since, in estimates of molecular weight using CPV as the standard, segment 10 migrated far ahead of the smallest CPV segment (Fig. 6.2) and therefore the calibration curve had to be extrapolated.

When denatured using methyl mercury hydroxide, the dsRNA of Mill Door/79 virus was capable of stimulating the incorporation of [^{35}S] methionine into TCA-precipitated material. The optimum RNA concentration for incorporation was 200µg/ml, when stimulation was 3.1-fold greater than the endogenous levels. Lower levels produced reduced stimulation, whereas higher levels appeared inhibitory. Other workers, using different viruses and translation systems, have reported a range of optimum RNA concentrations. Using a wheat germ translation system and DMSO to denature the dsRNA McCrae and Joklik (1978) found the optimum

concentration for reovirus type 3 to be about 75μg/ml when stimulation was 20-fold greater than the control. Mertens (1979) using the same system, but without a denaturing agent, showed that optimum stimulation by CPV ssRNA occurred at concentrations of 160μg/ml (10 to 11-fold greater than the control), while higher RNA levels (240μg/ml) led to inhibition. The optimum levels of Mg^{2+} and K^{+} ions were not determined for Mill Door/79 virus RNA, since dilution of the lysate to alter the salt concentrations led to a reduction in stimulation which outweighed any advantages the new cation concentrations may be given.

The polypeptides synthesized in the translation system, using unfractionated dsRNA, were similar in size to those synthesized 7 to 8 hours p.i. in Mill Door/79 virus infected cells and their yields reflected the stimulation of the translation system discussed above. However, some differences were observed: a polypeptide, p75, was present *in vitro* but was not easily detected in infected cells. This polypeptide had previously been observed in cells treated with NaCl and its synthesis was re-initiated simultaneously with other virus polypeptides (Chapter 5, IID and Fig. 5.5). Therefore, p75 may represent a primary gene product that is either cleaved rapidly after or during synthesis, or the production of which is suppressed *in vivo*. Since only one "*in vitro*" polypeptide was observed that corresponded with p53/51 obtained *in vivo*, it was likely that the pronounced band, regarded as a doublet in *in vivo* studies, actually represented a single polypeptide. Polypeptides p30 and p27 were not produced *in vitro*. This was consistent with the observation that p30 was a possible cleavage product, and p27 may have been produced from the cleavage of p44 *in vivo* [since p27 and p44 share regions of homology (Chapter 5, Section IIE and

Figs. 5.6-8)].

The relative amounts of *in vivo* and *in vitro* products were similar, and the differences, although present, were not large enough to suggest a significant amount of translational control *in vivo*. If post-translational cleavage did not occur *in vitro*, and assuming that co-migration of polypeptides during electrophoresis was an indication of identity, the following polypeptides were probable primary gene products; p141, p93, p75, p69, p65, p53/51, p44, p37, p36, p21 and p20.

In order to individually translate the genome segments of Mill Door/79 virus they had first to be separated on a PAG. Separation was successfully achieved with 5 of the 10 genome segments. After extraction from the preparative gel, segments 7 and 8 showed a small degree of cross contamination, and the separation of segments 4 and 5 from segment 6 was only partially achieved. The yields of each segment varied, the major source of loss probably being the electroelution stage.

The separated genome segments were translated and produced polypeptides which co-migrated with those synthesized by the unfractionated genome and those obtained from infected cell cultures. The low protein yields produced on the translation of some of the individual segments was probably due to monomeric acrylamide contamination. One of the most striking features of these results was the production of more than one unique major translation product from a single RNA species, segments 1 and 2 producing at least 3 products each. Similar results have been reported for the translation of the genome segments of bluetongue virus and CPV (McCrae & Mertens, 1983; Sangar & Mertens, 1983). The smaller product species were probably produced either as a result of incomplete translation of the RNA (resulting from

either premature termination of translation or by initiation at more than one site) or by proteolytic cleavage. One of these smaller products, produced by segment 2, was not detected in all translation experiments. Since the dsRNA used throughout this work came from the same stocks, the polypeptide was probably produced as a result of differences between the batches of rabbit reticulocyte lysate used. The largest products from segments 1 and 2 co-migrated with polypeptides produced in infected cells, while the smaller products did not. These results contrasted with those of Sangar and Mertens (1983) who showed that the smaller products of the individually translated genome segments of bluetongue virus appeared to be produced in infected cells. Therefore, under the reaction conditions used, the translation of Mill Door/79 virus RNA in vitro, unlike that of bluetongue virus, does not appear to reflect all the processes involved in the production of Mill Door/79 virus proteins in vivo.

The production of more than one polypeptide in each case, following translation of segments 4 and 5, and segment 6 was almost certainly due to cross contamination. Segment 6 produced 3 polypeptides, two of which were also produced by segments 4 and 5. This suggested that segment 6 was contaminated with segments 4 and 5 while they in turn contained little or no RNA from segment 6. It was therefore concluded that segment 6 coded for the product not produced by the translation of segments 4 and 5. Segments 7 and 8 were cross contaminated to a small degree and this was reflected in the relative amounts of their translation products.

Individual translation of genome segments, and comparison of the genome and its product sizes, were used to determine the genome coding assignments of Mill Door/79 virus. Translation of

individual segments is specific and can be confirmed by protease digestion. Using this approach 10 polypeptides were assigned to the 10 genome segments and five were shown, by partial proteolysis, to be identical to the virus-specified *in vivo* products. The other *in vitro* polypeptides were not compared with the *in vivo* products of the same molecular weight, so their assignments must still be regarded as provisional. Polypeptides p69 and p53/51 were probably derived from segments 4 and 5 (each had the coding capacity for a 65 x 10^3 dalton protein). It was therefore possible that p53/51 may have been produced by the incomplete translation of one of these genome segments. Three polypeptides (two of which, p30 and p27, were not produced *in vitro*, whereas the third, p36 was possibly produced if the dsRNA was translated when unfractionated) could not be identified as products of any segment. It was stated in Chapter 5 that these polypeptides were possibly cleavage products. The data presented here supports this conclusion.

Attempts to derive coding assignments by comparing the calculated protein sizes produced by each dsRNA segment with the observed values of those present *in vivo* and *in vitro* were not successful. This was due both to ambiguities in some of the possible matches (since some calculated values were similar to more than one of the observed values) and matching failures (e.g. the calculated 127 x 10^3 and 10 x 10^3 dalton proteins were not found either *in vivo* or *in vitro*). In the latter case it was probable that an error in the calculated size of genome segment 10 was responsible. However, some matches were made; segment 1 coded for p141, segment 3 coded for p75, segments 4 and 5 coded for p69 and p65, or segment 6 coded for p65, and segment 7 coded for p37. These results agreed with those from individual *in*

in vitro translations only for segments 1 and 3, for one of the assignments of segments 4 and 5 and for segment 6. Problems with this method of gene assignment have been encountered by other workers. Verwoerd *et al.* (1972) produced unsatisfactory assignments when using this method for bluetongue virus type 10, although better results were obtained for reovirus type 3 (Zweerink *et al.*, 1971). This method relies on the assumptions that the dsRNA segments are transcribed and translated in their entirity, and that the molecular weights of the dsRNA and proteins are determined accurately. Since the molecular weight of a protein derived *in vivo* may also include non-protein components, e.g. polysaccharides, errors can easily be made with these determinations. Moreover, it cannot be assumed that each genome segment is entirely translated, since coding assignments already made for some members of the Reoviridae show smaller genome segments coding for larger polypeptides than larger genome segments, e.g. segments 1 and 2 of reovirus type 3 and segments 8 and 9 of simian rotavirus (Fig. 1.2). Therefore, a proportion of the larger segments must not be translated.

Analysis of the translation products of the dsRNA of Mill Door/79 virus *in vitro* and *in vivo* suggests that ten polypeptides are each derived from a unique dsRNA segment and that three polypeptides are cleavage products.

Table 6.1

Molecular weights of the dsRNA segments of Mill Door/79 virus compared with type 2 Cytoplasmic Polyhedrosis Virus

Segment	Type 2 CPV[1]	Mill Door/79 virus
1	2.52[2]	2.60 ± 0.05[3]
2	2.37	2.29 ± 0.03
3	2.17	1.41 ± 0.01
4	2.11	1.17 ± 0.02
5	1.16	1.17 ± 0.02
6	1.05	1.12 ± 0.02
7	1.00	0.62 ± 0.01
8	0.85	0.58 ± 0.01
9	0.76	0.50 ± 0.03
10	0.54	0.18 ± 0.05
Total M.W.	14.53	11.64 ± 0.25

[1] Pullin and Moore, unpublished information

[2] M.W. x 10^6 daltons

[3] mean and standard errors from 3 determinations

Table 6.2

Measured and calculated molecular weights of Mill Door/79 virus polypeptides

Equivalent genome segment[1]	Calculated[2] x 10^3	Observed x 10^3	
		In vivo[3]	In vitro[3]
1	144	141	141
2	127	93	93
3	78	69	75[4]
4	65	65	69
5	65	53	65
6	62	51	53
7	34	44	51
8	32	37	44
9	27	(36)[5]	37
10	10	(30)	(36)
		(27)	21
		21	20
		20	

[1] Equivalent to the calculated polypeptides

[2] Calculated using a conversion factor of 18 (Verwoerd, 1972) from values shown in Table 6.1

[3] In vivo = Vero cell grown virus, In vitro = products from rabbit reticulocyte lysate translation system

[4] Product of in vitro translation, not normally found in vivo

[5] Values indicated in brackets represent possible cleavage products

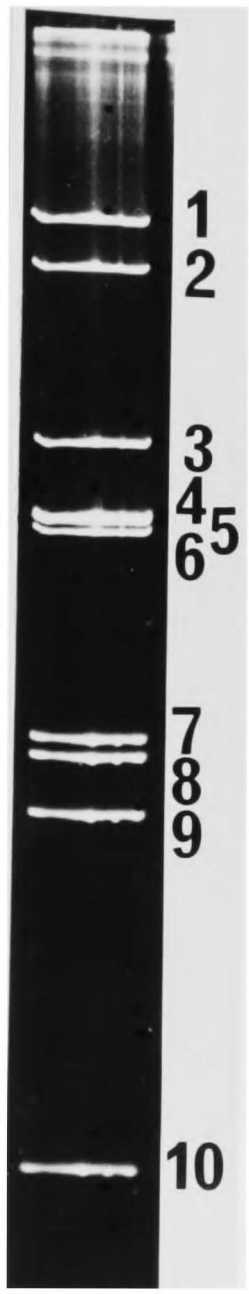


Figure 6.1. The dsRNA genome of Mill Door/79 virus. 2µg of dsRNA separated on a 10% PAG, stained with 0.5µg/ml ethidium bromide and photographed under UV light (approximately 302nm).

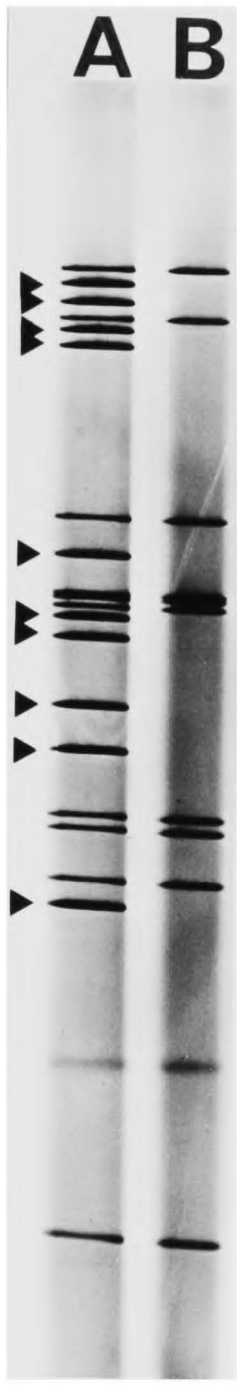


Figure 6.2. Comparison of dsRNAs of Mill Door/79 virus and type 2 Cytoplasmic Polyhedrosis Virus (CPV). 2µg of each virus dsRNA was separated on a 10% PAG and silver stained. Track A, separated dsRNA segments of both Mill Door/79 virus and type 2 CPV; B, dsRNA segments of Mill Door/79 virus. Arrows indicate the positions of the type 2 CPV genome segments.

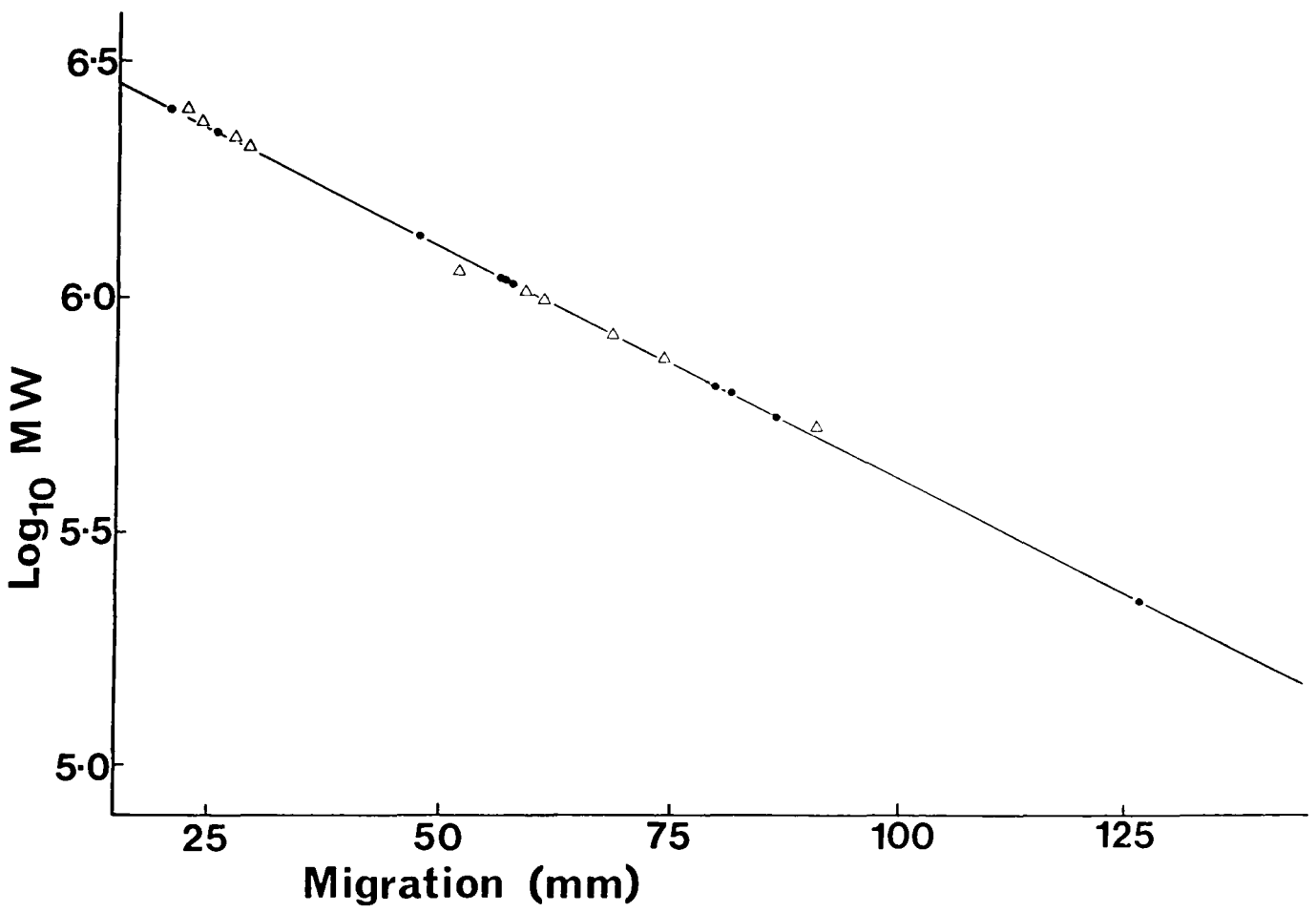


Figure 6.3. A calibration curve of type 2 Cytoplasmic Polyhedrosis Virus (CPV) dsRNA electrophoresed on a 10% PAG. Type 2 CPV genome segment sizes were calculated by Pullin and Moore (unpublished information) (Δ—Δ). The Mill Door/79 segments are indicated by ●—●

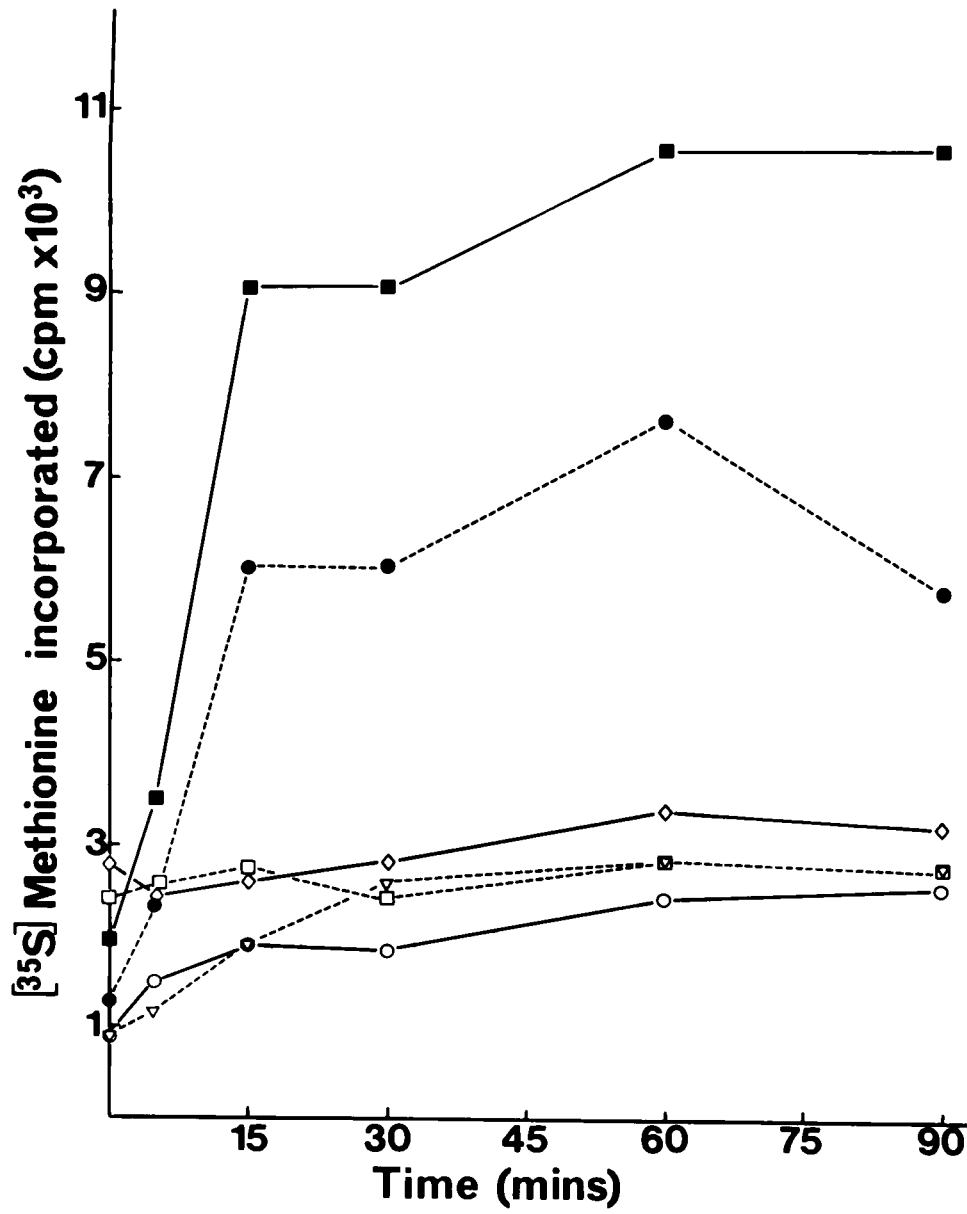


Figure 6.4. Stimulation of incorporation of [^{35}S]-methionine by denatured Mill Door/79 virus RNA. Translation assays were carried out as described in Materials and Methods. Samples of 1µL were removed at intervals from the 10µl reaction mixtures and processed to measure the incorporation of [^{35}S]-methionine into TCA-precipitated material. Endogenous —◇— ; 25µg/ml dsRNA -▽- ; 50µg/ml dsRNA —○— ; 100µg/ml dsRNA -●- ; 200µg/ml dsRNA —■— ; 400µg/ml dsRNA -□-.

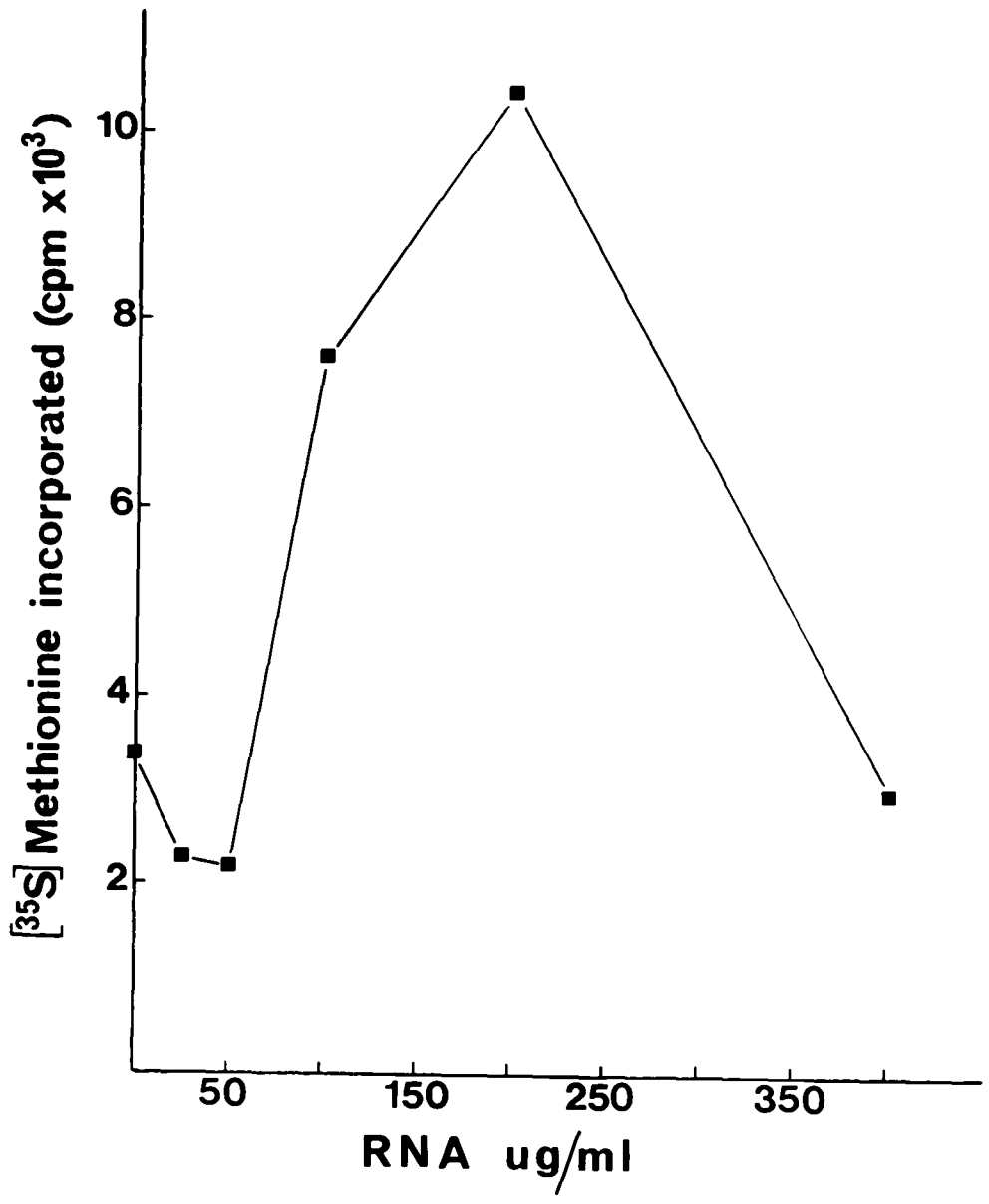


Figure 6.5. Stimulation of incorporation of [^{35}S]-methionine into TCA-precipitated products by increasing amounts of Mill Door/79 virus denatured dsRNA. Translation assays were carried out as described in Materials and Methods. The incorporation at 60 minutes is plotted against the concentration of RNA.

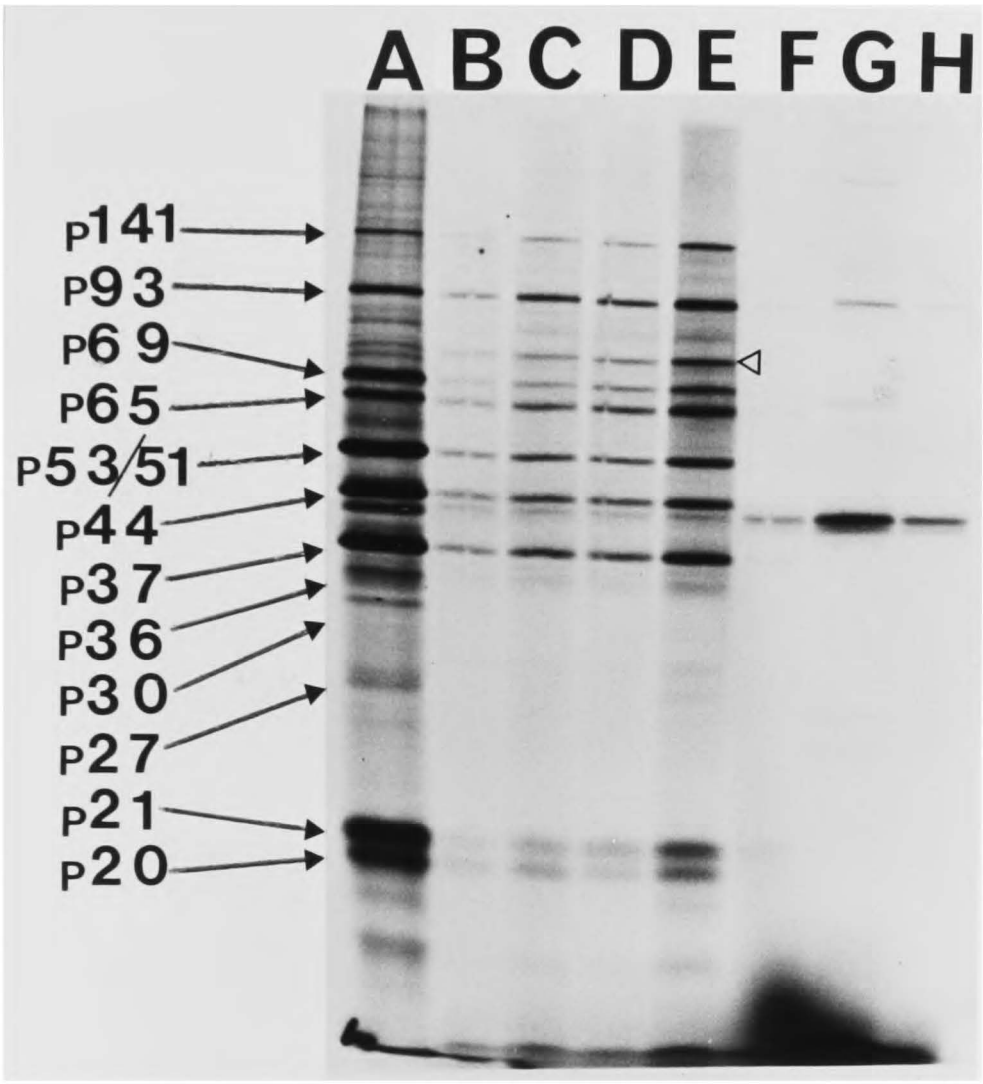
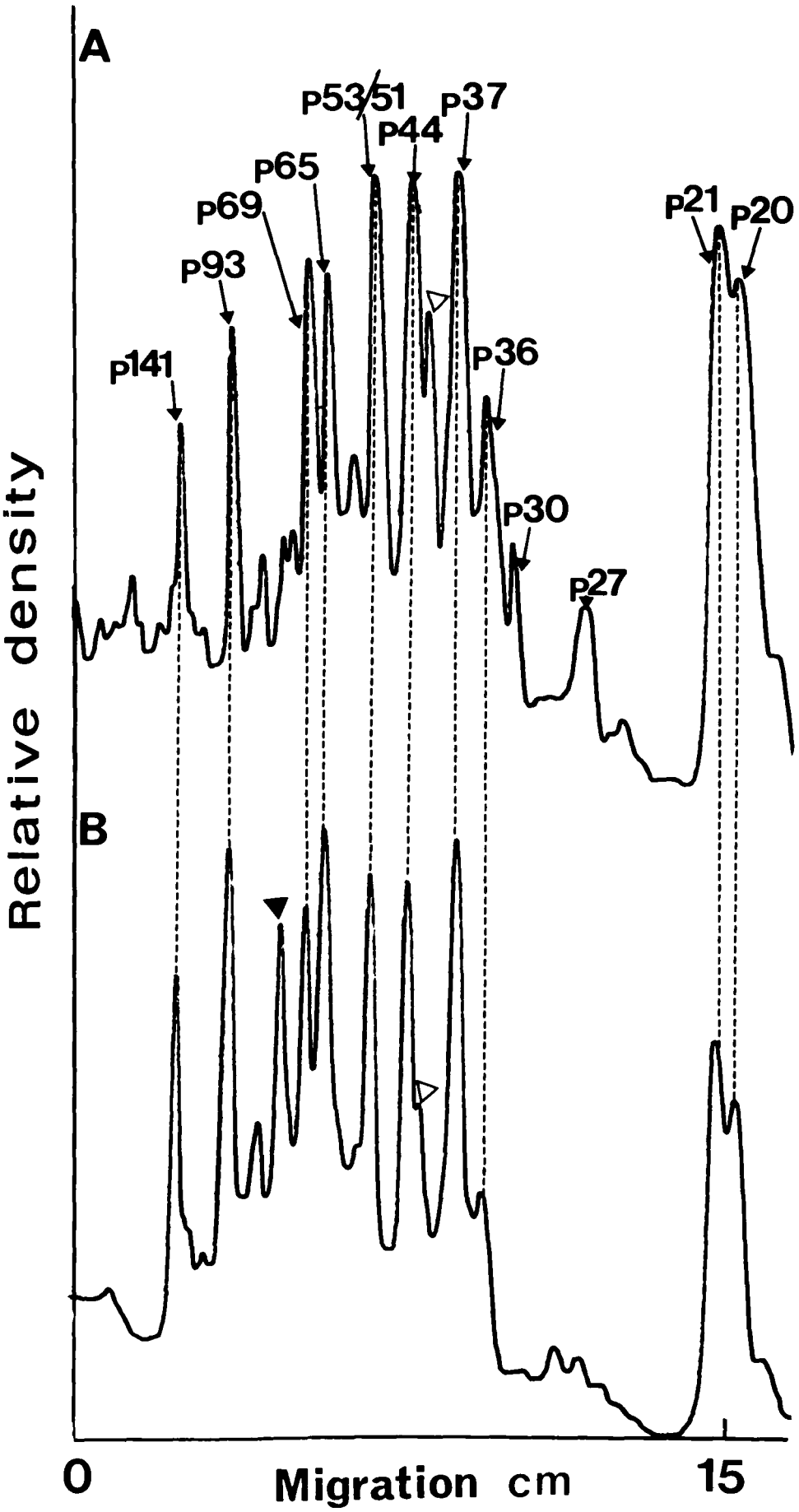


Figure 6.6. Comparison of the polypeptides synthesized in vitro with those from infected cells. Track A, infected Vero cells labelled with 50μCi/ml [^{35}S]-methionine between 7-8 hours p.i.; B-H, polypeptides synthesized in rabbit reticulocyte lysate after the addition of dsRNA at final concentrations of: B, 25μg/ml; C, 50μg/ml; D, 100μg/ml; E 200μg/ml; F, 400μg/ml; G, 800μg/ml; H, 0μg/ml. After solubilization the samples were electrophoresed on a 12.5% PAG and prepared for autoradiography. Open arrow indicates a product only synthesized in vitro.

Figure 6.7. Densitometer scan of the autoradiograph shown in Figure 6.6 (tracks A and E) indicating, A, the polypeptides produced in infected Vero cells and B, those produced by in vitro translation of denatured Mill Door/79 virus dsRNA. ▼ indicates a product of the in vitro system not found in infected cells, ▽ indicates a host specified polypeptide which co-migrates with an endogenous product of the translation system.


A
B
P53/51
P37
P44
P65
P69
P93
P141
P36
P30
P27
P21
P20
Relative density
0
Migration cm
15

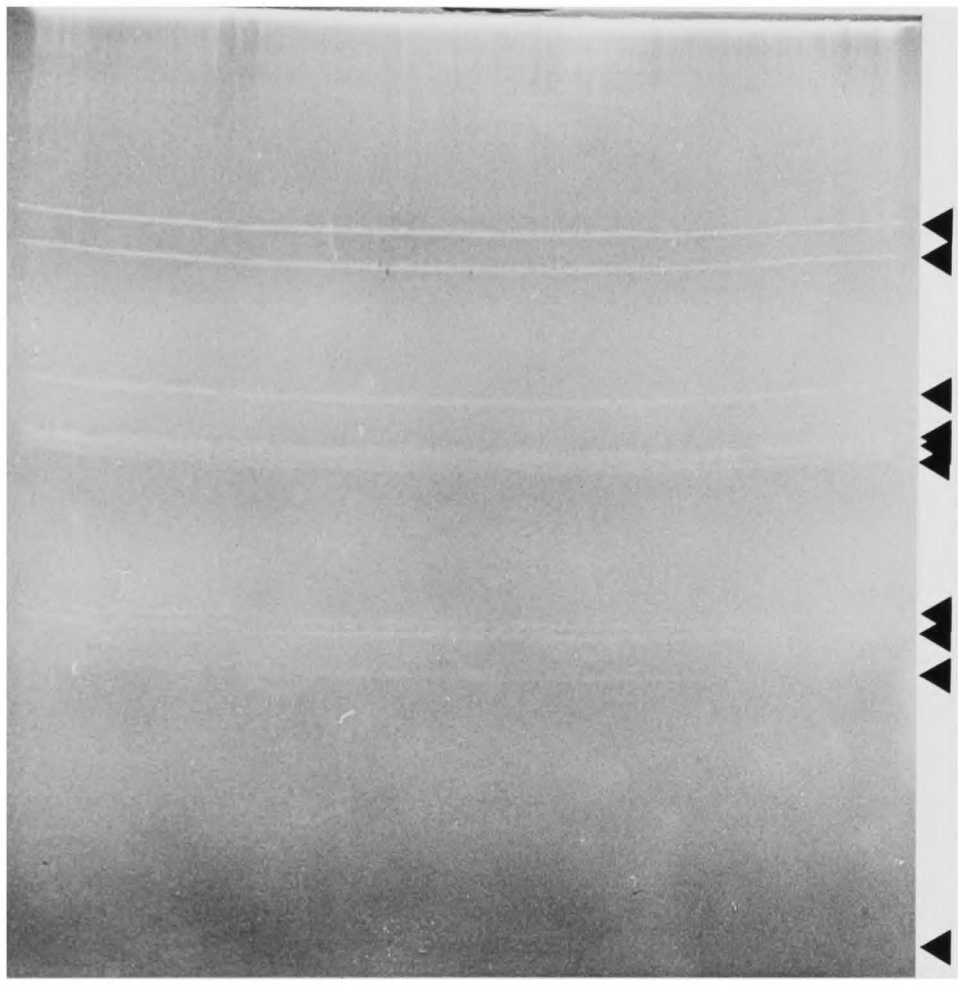


Figure 6.8. Preparative separation of Mill Door/79 virus dsRNA. A 5% PAG loaded with 1.5mg dsRNA and following separation the bands were visualized under UV light after staining with 0.5µg/ml ethidium bromide. Arrows indicate position of the segments, the smallest of which was not recorded on the film.

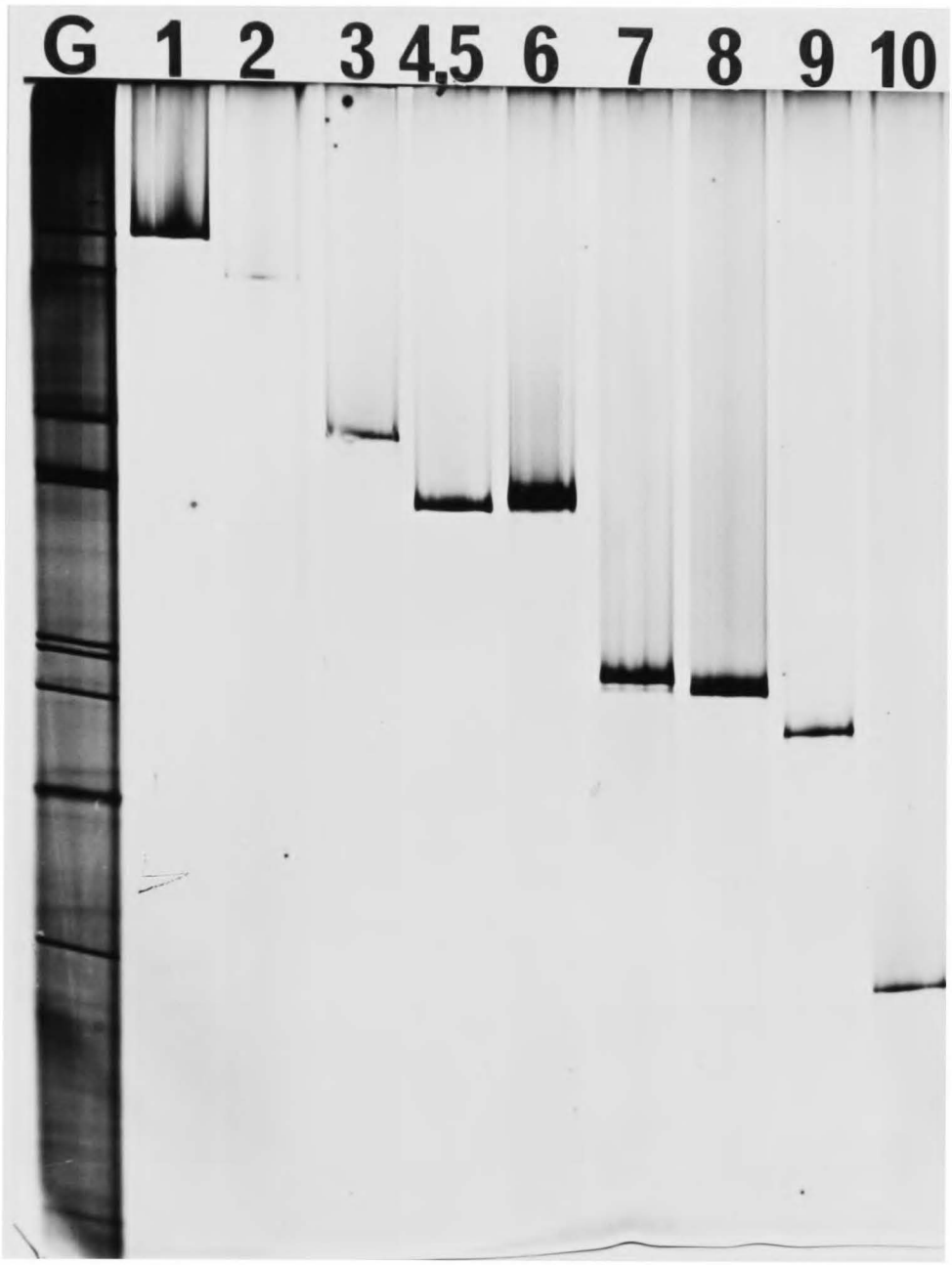


Figure 6.9. Separation of the 10 dsRNA segments of Mill Door/79 virus. The segments were electrophoresed on a 10% PAG and silver stained. Track G; Mill Door/79 virus whole genome; tracks 1-10, individual genome segments.

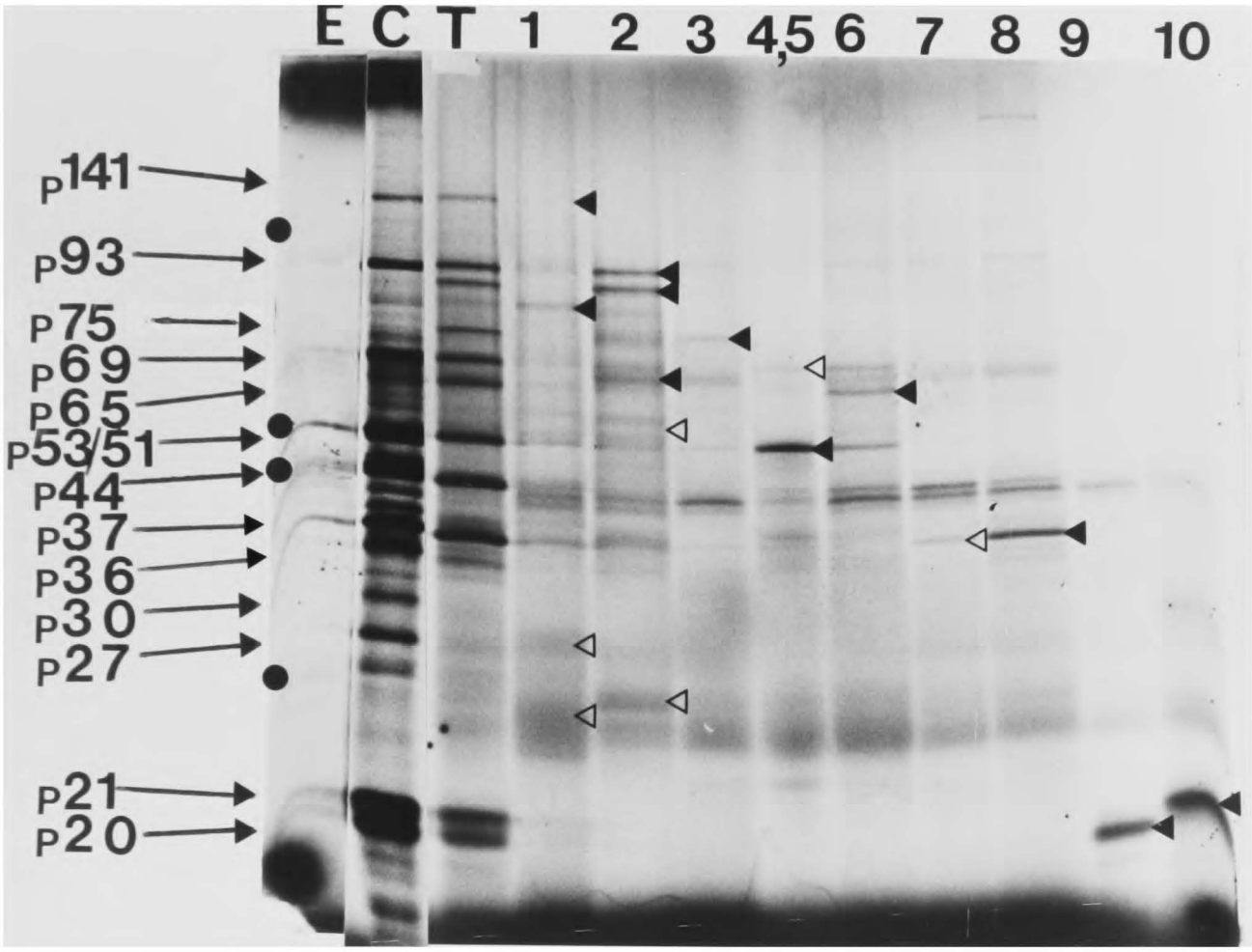


Figure 6.10. In vitro translation of the individual dsRNA segments of Mill Door/79 virus. The products from translation of the individual genome segments were separated on a 12.5% PAG which was then prepared for autoradiography. Track E, polypeptides synthesized in vitro with no added virus RNA; track C, polypeptides synthesized in infected Vero cells between 7 and 8 hours post infection; track T, polypeptides synthesized in vitro in response to the addition of unfractionated Mill Door/79 virus dsRNA; tracks 1-10, polypeptides synthesized in vitro in response to the individual RNA segments 1-10 respectively. Closed arrows indicate major products from the translation of the individual segments and open arrows indicate minor products. Endogenous products of the translation system are indicated by circles.

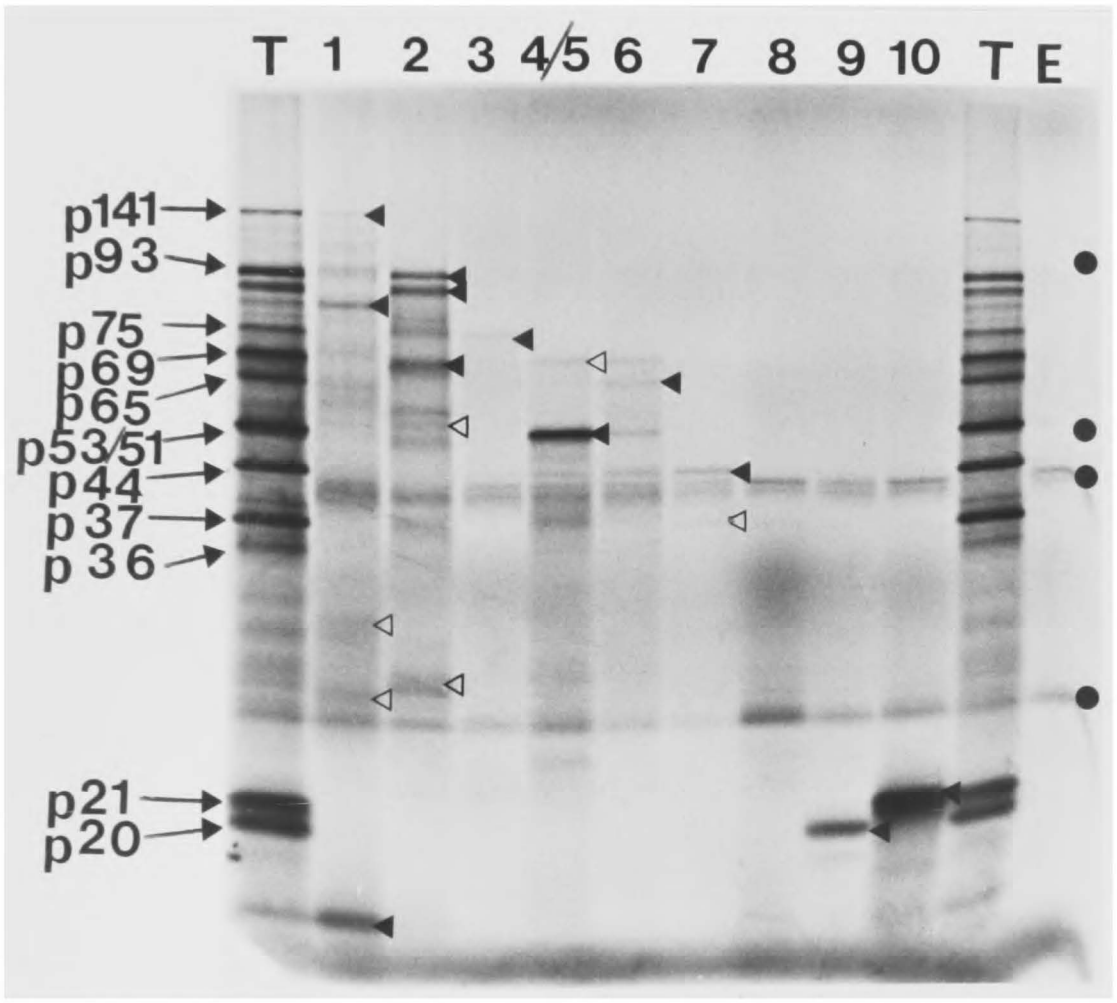


Figure 6.11. In vitro translation of the individual dsRNA segments of Mill Door/79 virus. The products from translation of the separate genome segments were separated on a 12.5% PAG which was then prepared for autoradiography. Track T, polypeptides synthesized in vitro in response to the addition of unfractionated Mill Door/79 dsRNA; track E, polypeptides synthesized in vitro with no added virus RNA; tracks 1-10, polypeptides synthesized in vitro in response to the individual RNA segments 1-10 respectively. Closed arrows indicate major products of the individual segments, and open arrows indicate minor products. Endogenous products of the translation system are indicated by circles.

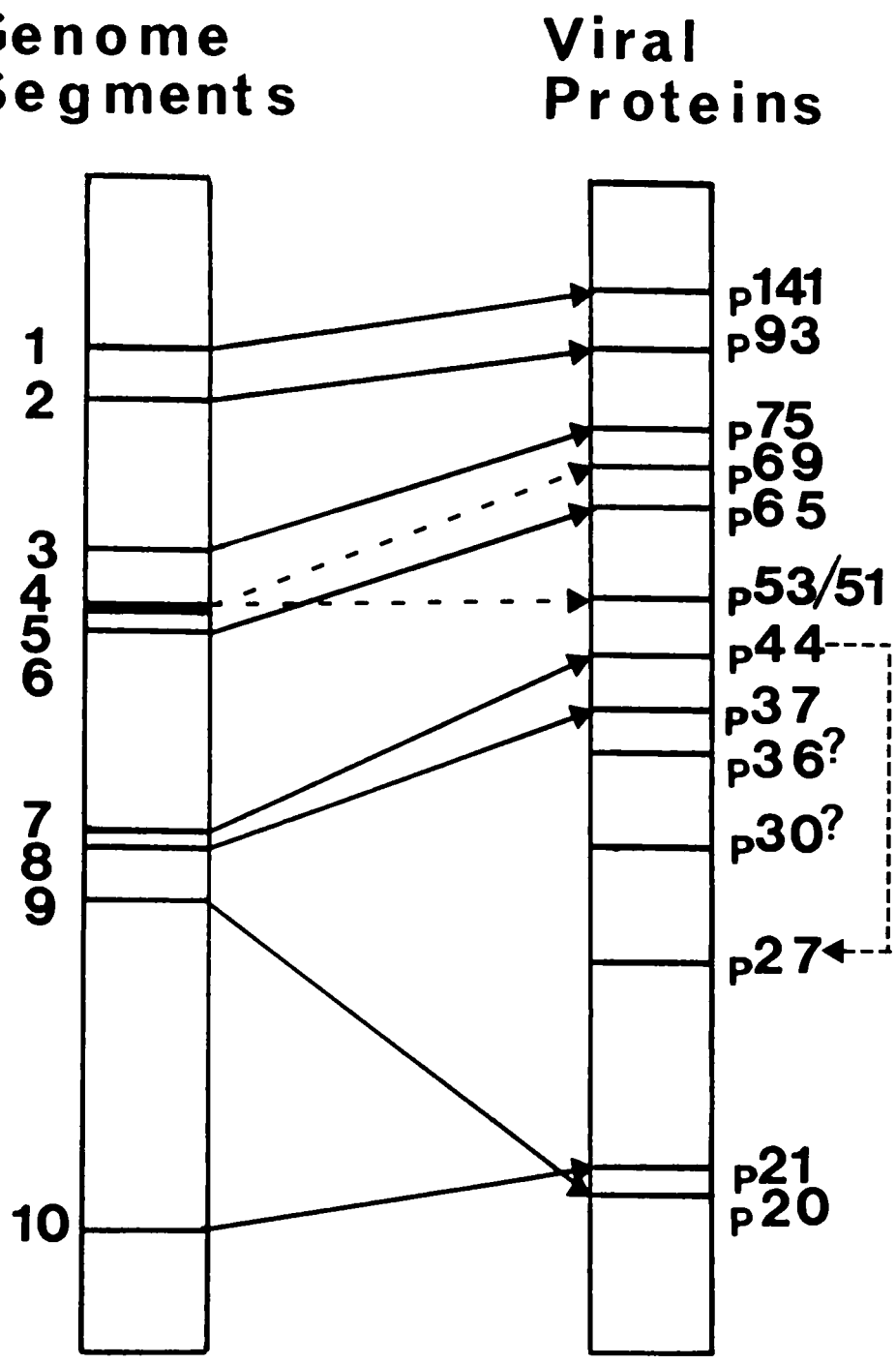


Figure 6.12. Coding assignments for the dsRNA segments of Mill Door/79 virus based on the data presented in Figs. 6.10 and 6.11. The viral proteins include those produced both in vitro and in infected cell cultures. The assignments of segments 4 and 5 are tentative as they were not translated separately. Polypeptides co-migrating with p36, p30 and p27 were not produced by any of the dsRNA segments (p27 may be a cleavage product of p44).

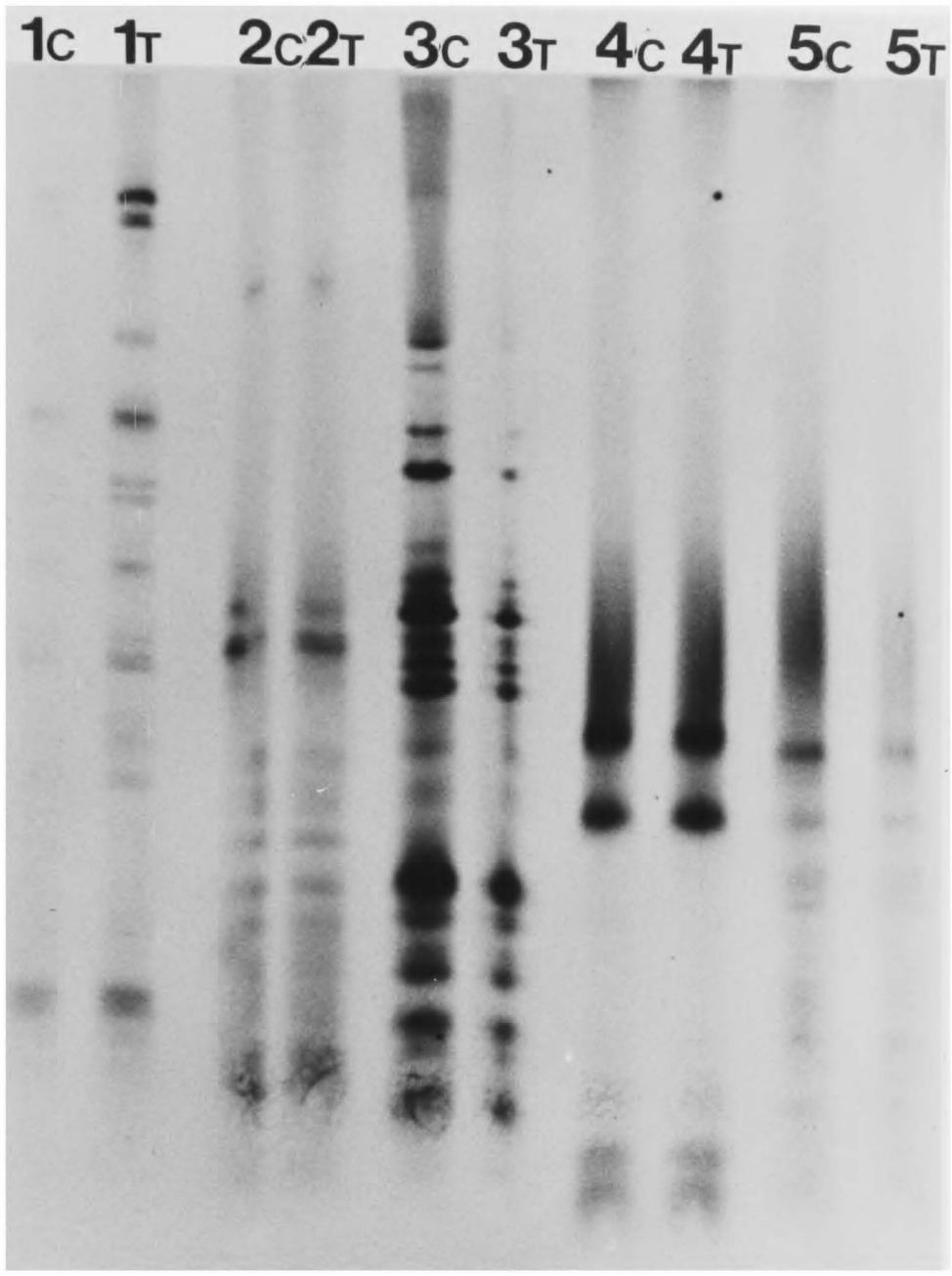


Figure 6.13. Products resolved after the partial proteolysis of p93, p65, p53/51, p21 and p20 produced in infected cell cultures (tracks 1c-5c respectively) compared to those of the co-migrating polypeptides produced in vitro (tracks 1T-5T). Digestion was carried out using 50µg/ml S.aureus V8 protease and electrophoresing for 20 hours at 5mA on a 17.5% PAG, which was subsequently prepared for autoradiography.

CHAPTER 7

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COMPARISON OF THE dsRNA PROFILES AND VIRUS-SPECIFIED POLYPEPTIDES OF THREE ORBIVIRUSES FROM THE ISLE OF MAY

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I. INTRODUCTION

In Chapter 3 it was shown that orbiviruses of the Kemerovo serogroup were isolated from three pools of ticks collected on the Isle of May. Each of the three isolates could be distinguished by neutralization tests. One isolate, Mill Door/79 virus, was further characterized to determine the nature of its dsRNA and the proteins it produced in cell culture. This chapter describes comparisons made between this isolate and the two others, Mill Door/81 and North Clett/81 viruses, at a molecular level. The dsRNA and polypeptide profiles of each of the isolates were compared on PAGs, followed by more detailed analysis of the proteins by partial proteolysis and immune precipitation.

II. RESULTS

A. Comparison of the dsRNA profiles

The dsRNA expressed by each isolate was extracted from infected Vero cells and the segments separated on a 10% PAG. To enable minor distinctions to be made between the migration rates of the corresponding segments of each isolate, samples were co-run in the same tracks (Fig. 7.1). All the isolates contained 10 dsRNA segments which gave similar separation patterns (2 + 4 + 3 + 1), although variations were observed on a segment to segment basis. (dsRNA profiles can only indicate the size of individual dsRNA segments and not their relationship to other segments of corresponding size from other viruses. However, for the purpose of the discussion below it was assumed that each segment migrated in the same relative position for each isolate). Mill Door/79

virus (tracks A and B) and North Clett/81 virus (tracks B and C) varied in the migration of 8 of the 10 segments, the exceptions being segments 3 and 6 (tracks A, B and C). The genome of Mill Door/79 virus (11.64 x 10^6 daltons) was 210 x 10^3 daltons smaller than the genome of North Clett/81 virus (11.85 x 10^6 daltons) (Table 7.1). The genome of Mill Door/79 virus was distinct from that of Mill Door/81 virus by the migration of 8 segments (E, F and G). The total molecular weight of the Mill Door/81 virus genome was 11.66 x 10^6, 20 x 10^3 daltons larger than that of Mill Door/79 virus. Mill Door/81 and North Clett/81 virus genomes were distinct in the migration of 9 segments (tracks C, D and E) and the North Clett/81 virus genome was larger by 190 x 10^3 daltons.

The largest variations in genome segment size were between the first segment of Mill Door/79 and Mill Door/81 viruses (250 x 10^3 daltons) and the fourth segment of Mill Door/79 and North Clett/81 viruses (180 x 10^3 daltons).

B. Comparison of the virus-specified proteins

(i) Polypeptide profiles. Each isolate was inoculated into Vero cells at a multiplicity of 20-30 pfu/cell and incubated at 37°C for 6.5 hours. After pre-treatment in labelling medium for 30 minutes the cells were pulse labelled for 15 minutes with 100μCi/ml [^{35}S] methionine and harvested for electrophoresis. Cells were also labelled between 7 and 8 hours p.i. with 10μCi/ml [^{14}C] protein hydrolysate before harvesting for electrophoresis.

Mill Door/79 virus (Fig. 7.2, track MD/79, Fig. 7.3, track A) produced 12 polypeptides (although p36 and p30 were not detected in Fig. 7.2) with molecular weights ranging from 141 x 10^3 to 20 x 10^3 (these were characterized in Chapters 4, 5

and 6). A polypeptide of about 46 x 10^3 daltons, migrating fractionally behind p44 (Fig. 7.2, open arrow), was also observed in Mill Door/79 virus infected cells.

North Clett/81 virus produced 12 polypeptides (Fig. 7.2, track NC/81; Fig. 7.3, track B), most of which co-migrated with those of Mill Door/79 virus. In the following discussion it has been assumed that the relative positions of the virus- specified polypeptides remained the same for each isolate. Therefore, although no North Clett/81 virus-specified polypeptide was detected co-migrating with p69 of Mill Door/79 virus, one was clearly visible migrating ahead of it, having a molecular weight of approximately 67-68 x 10^3 (Fig. 7.2, track NC/81). North Clett/81 virus-specified polypeptides were also detected migrating slightly behind p53/51 and slightly ahead of p37 and p21 of Mill Door/79 virus. Both the 46 x 10^3 dalton polypeptide (open arrow) and another one, migrating slightly behind it (small arrow) were detected in North Clett/81 virus infected cells.

Mill Door/81 virus produced 12 polypeptides (Fig. 7.2, tracks MD/81; Fig. 7.3, track C), all but one of which co-migrated with those of Mill Door/79 virus. No polypeptide co-migrating with p69 was detected, but one was present migrating ahead of it and co-migrating with the 67-68 x 10^3 dalton polypeptide produced by North Clett/81 virus. Both the polypeptides detected in North Clett/81 virus infected cells (Fig. 7.2, open arrows and small arrows) were also present in Mill Door/81 virus infected cells.

Figure 7.3 shows the polypeptide profile and densitometer scan of the three isolates after labelling with [^{14}C] protein hydrolysate. Indicated on the autoradiograph are the position of the polypeptide migrating behind the 46 x 10^3 dalton polypeptide

in North Clett/81 and Mill Door/81 virus infected cells (open arrow) and the position of p36 and p30 (closed arrows). p27 was not detected in cells labelled with [^{14}C] hydrolysate after infection with North Clett/81 virus. The relative amounts of the virus specified polypeptides were calculated, for each isolate, from the densitometer scan in Fig. 7.3 (for method see Chapter 4, Section F). The results indicate that, although the individual values varied, the pattern was similar for each virus (Table 7.2).

(ii) Partial proteolysis. Vero cells were inoculated with each of the three Isle of May isolates, incubated at 37°C for 7 hours and labelled with 100μCi/ml [^{35}S] methionine for 1 hour. Virus-specified polypeptides were separated in the first dimension on a 17.5% PAG, and strips containing the separated polypeptides were then digested with S.aureus V8 protease, and separated in the second dimension on a second 17.5% PAG. Figure 7.4 shows the partial digest patterns of Mill Door/79 and North Clett/81 virus polypeptides (separation on a single gel). No differences were detected between the products of the major polypeptides, however, a product (circle) of the polypeptide produced in North Clett/81 virus infected that co-migrated with p30 of Mill Door/79 virus, was not observed among the products of p30. Two additional oligopeptides (Fig. 7.5, circles) were detected among the products of the 67-68 x 10^3 dalton polypeptide produced in Mill Door/81 virus infected cells compared to the products of p69, although otherwise the polypeptide digestion patterns were similar. When the polypeptides expressed in North Clett/81 and Mill Door/81 virus infected cells were compared, differences could be detected among some of their products (Fig. 7.6, circles).

(iii) Immune precipitation. Vero cells, infected with each of the three isolates, were labelled for 1 hour at 7 hours p.i. with 100μCi/ml [^{35}S] methionine and solubilized in NTEP buffer. After pre-adsorption with S.aureus protein A the samples were mixed sequentially with hyperimmune mouse ascitic fluid raised against the isolates, rabbit anti-mouse IgG, and protein A, after which the precipitated proteins were subjected to electrophoresis. No proteins were precipitated from uninfected cells, whereas p93, p53/51 and p37, and the polypeptides produced in North Clett/81 and Mill Door/81 virus infected cells of identical or similar size, were precipitated from infected cells by both the homologus and heterologous ascitic fluids (Fig. 7.7). p37, and the corresponding polypeptides of North Clett/81 and Mill Door/81 viruses, appeared to be precipitated as a doublet (track F to P). p53/51, and corresponding polypeptides, were precipitated by preimmune ascitic fluid (track E). The densitometer scan of an infected cell profile and a Mill Door/79 virus homologous precipitation profile shows that p65 was also present in the precipitated material in small quantities (Fig. 7.8). The amount of p93 relative to one of the major virus products, p53/51, increased 6.3-fold in the precipitated material.

III. DISCUSSION

The three Isle of May isolates display distinctive dsRNA profiles. It was shown in Chapter 3 that they are probably strains of the same serotype, since they exhibit a degree of cross reactivity in neutralization tests. Travassos da Rosa et al. (1984) demonstrated that isolates belonging to the Changuinola serogroup, that cross neutralize, can possess

distinct dsRNA profiles, i.e. the Saraca and Caninde isolates. Squire et al. (1983) detected differences between the dsRNA genomes of different isolates of the same serotype of bluetongue virus types 10, 11 and 13 by their migration in PAGs; most of the variation appeared to occur between segments 3 and 9. Different strains of an orbivirus serotype can therefore possess distinct dsRNA profiles. However, a change in dsRNA profile does not indicate a major loss or gain of genetic material, as the total molecular weight of the dsRNA of each isolate can be similar e.g. Mill Door/79 virus (11.64×10^6) and Mill Door/81 virus (11.66×10^6).

The virus specified proteins of each isolate were examined to determine whether the variations in the dsRNA segments were reflected in their products. Differences were observed in the polypeptide profiles of each isolate, but these were less numerous than those seen in the dsRNA profiles. Assuming that the variations in the migration of the dsRNA segments of North Clett/81 and Mill Door/81 viruses relative to Mill Door/79 virus did not lead to altered gene coding assignments (an assumption probably only valid for the more distinct segments, e.g. 1, 2, 9 and 10), comparisons could be made between individual segments and their products. Segments 1 and 2 were distinct for each isolate but their products co-migrated. The differences between segments 9 and 10 for each of the isolates were probably too small to allow detection of differences in their products, although the polypeptide corresponding to p21 of North Clett/81 virus was slightly smaller than p21 of Mill Door/79 virus, reflecting the smaller size of North Clett/81 virus's dsRNA segment 10.

The origin of the 46×10^3 dalton polypeptide present in

infected cells, and the polypeptide migrating slightly behind it in North Clett/81 and Mill Door/81 virus infected cells, was not known. These polypeptides were only produced in infected cells and had previously been observed during pulse-chase experiments on Mill Door/79 virus infected cells (Chapter 5, Section IIA), but were not produced in vitro (Chapter 6, Section IIB).

After partial proteolysis most corresponding viral polypeptides were found to produce similar oligopeptide patterns. The differences that were detected were minor, comprising a few additional oligopeptides in basically similar digestion profiles. No completely unique digestion patterns were seen between co-migrating, or nearly co-migrating, polypeptides from each isolate. This suggests that, although differences occur between the virus dsRNA profiles, they may not be the result of major changes in the RNA sequences of the protein coding regions. The differences may be the result of addition, or removal, from genome segments, of non-translated dsRNA. However, partial proteolysis is only able to distinguish major differences between proteins and will not detect minor changes due to single base substitutions.

Immune precipitation of the viral polypeptides failed to distinguish between the three isolates. The hyperimmune ascitic fluids used in these experiments were the same as those used in neutralization tests, indicating that the latter approach was more sensitive in distinguishing between the isolates. Since the viruses were probably strains of the same serotype, precipitation of the polypeptide or polypeptides involved in virus neutralization may have been expected (Huismans & Erasmus, 1981). Huismans and Bremer (1981) demonstrated, using bluetongue virus isolates, that cross-immune precipitation of the serotype

specific virus polypeptide P2 was not enough to indicate cross neutralization. They suggested that cross neutralization between two isolates was only found between strains that had several antigenic determinants in common, whereas cross immune precipitation might require only one such determinant.

It appears therefore that, although the Isle of May isolates can be distinguished by neutralization tests, they cannot be so easily differentiated by their protein profiles, protease digest products or by immune precipitation. However, they can be clearly distinguished by their dsRNA profiles. Whether the differences between their dsRNA profiles has any significance is not clear, since it is the proteins (not the dsRNA) which comprise the structure and enzymatic composition of the virus.

Table 7.1

Molecular weights of the dsRNA segments of the three Isle of May isolates

Segment	Size (daltons x 10^{-6})		
	Mill Door/79[1]	North Clett/81[2]	Mill Door/81[2]
1	2.60	2.75	2.85
2	2.29	2.19	2.23
3	1.41	1.41	1.29
4	1.17	1.35	1.26
5	1.17	1.13	1.11
6	1.12	1.12	1.10
7	0.62	0.64	0.62
8	0.58	0.61	0.58
9	0.50	0.49	0.46
10	0.18	0.16	0.16
TOTAL	11.64	11.85	11.66

[1] Calculated by comparison with type 2 cytoplasmic polyhedrosis virus (Table 6.1)

[2] Calculated using the method described in Chapter 6, Section A with Mill Door/79 virus dsRNA as the standard

Table 7.2

Relative amounts of [^{14}C] labelled polypeptides of each Isle of May isolate

Polypeptide[1]	Relative amount in cells[2]		
	Mill Door/79	North Clett/81	Mill Door/81
pl41	3.3	4.8	5.3
p93	7.4	7.7	7.4
p69	14.2	7.2	9.9
p65	7.6	5.1	5.4
p53/51	14.7	18.1	13.8
p44	17.9	17.9	12.9
p37	8.4	9.9	11.7
p36	6.1	5.4	7.2
p30	1.6	4.3	2.6
p27	2.3	0	4.9
p21	8.6	9.8	8.8
p20	7.9	9.8	10.1

[1] Molecular weights calculated for Mill Door/79 virus only (some proteins from North Clett/81 and Mill Door/81 viruses do not co-migrate)

[2] Calculated from Figure 7.3

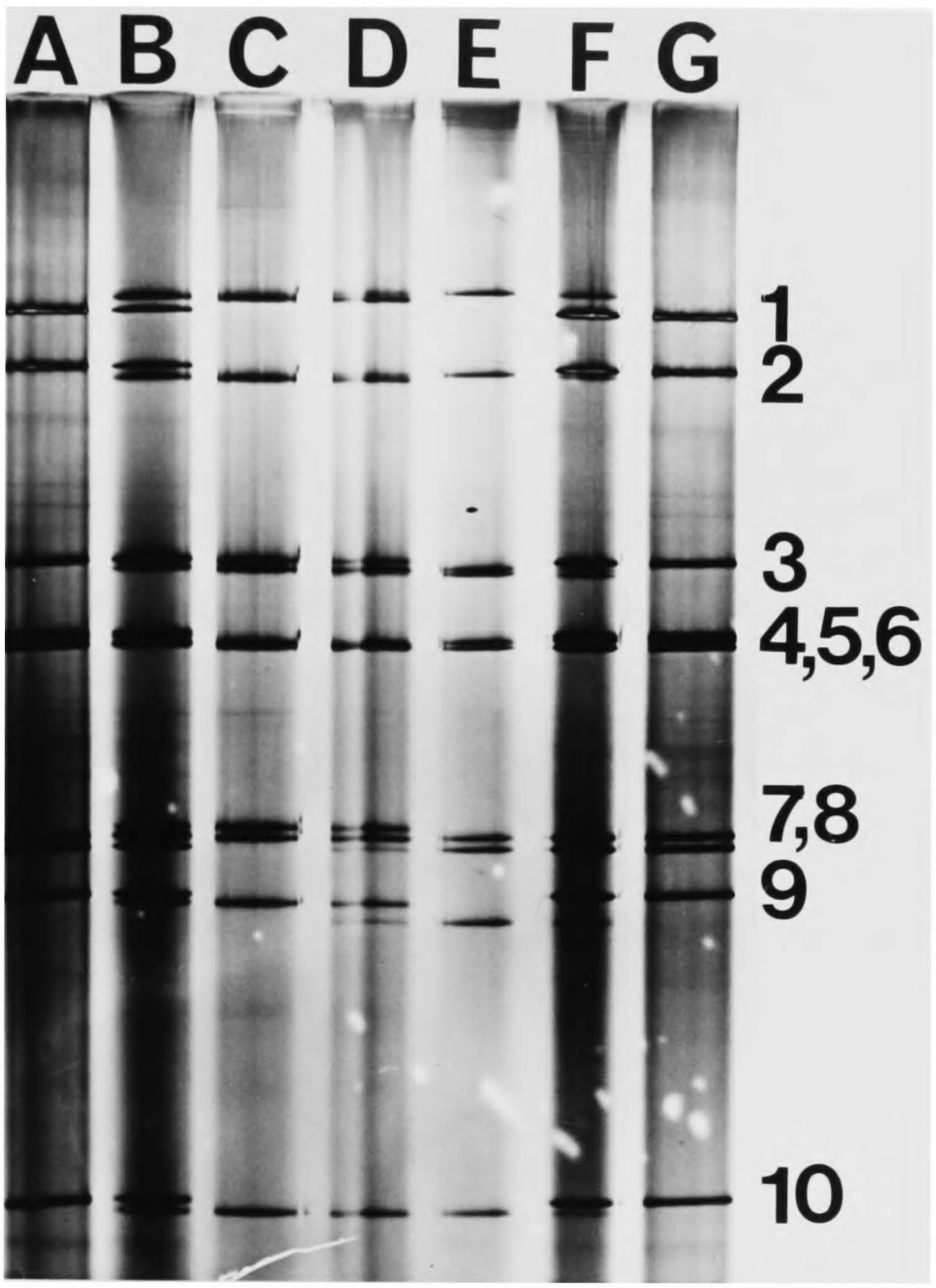


Figure 7.1. Genome profiles of Mill Door/79, North Clett/81 and Mill Door/81 viruses. 1µg of dsRNA from each virus was separated on a 10% PAG and silver stained. Track A, Mill Door/79 virus; B, Mill Door/79 and North Clett/81 viruses; C, North Clett/81 virus; D, North Clett/81 and Mill Door/81 viruses; E, Mill Door/81 virus, F; Mill Door/81 and Mill Door/79 viruses; G, Mill Door/79 virus. The genome segments are numbered for Mill Door/79 virus (track G).

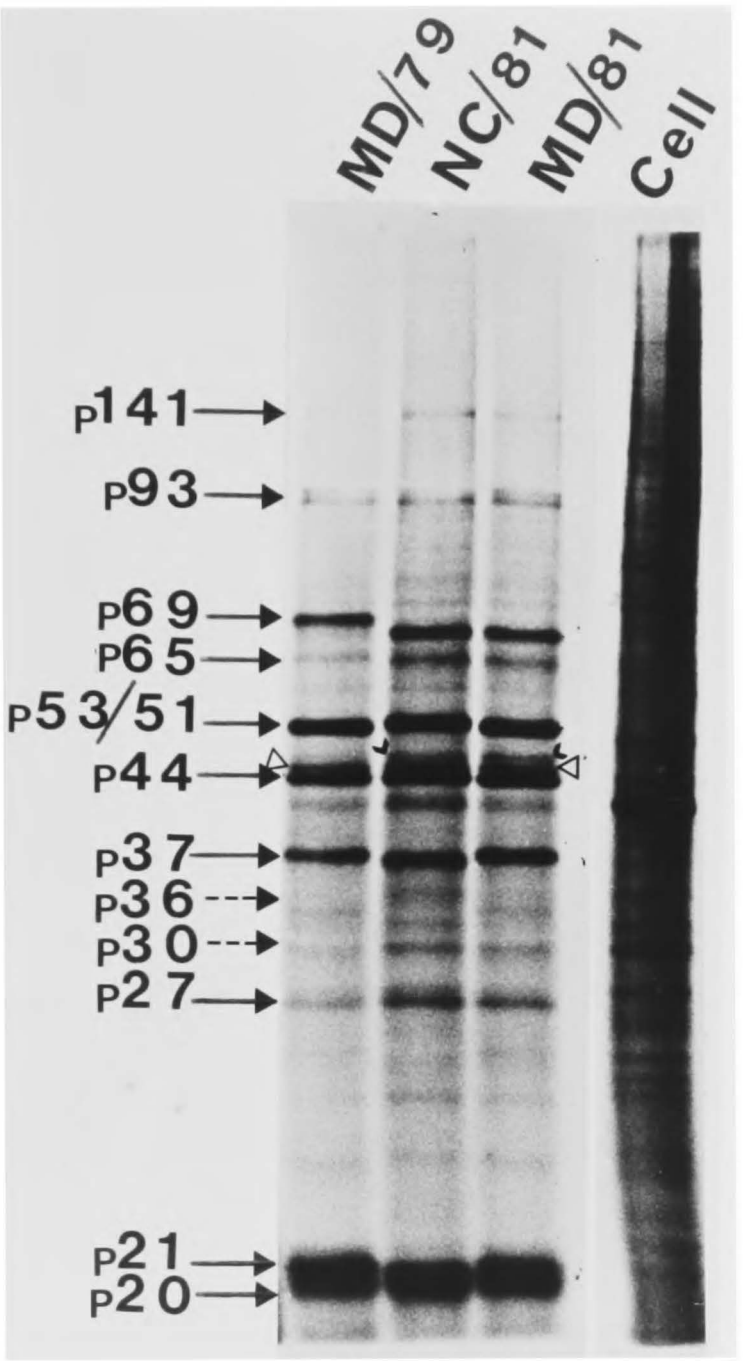
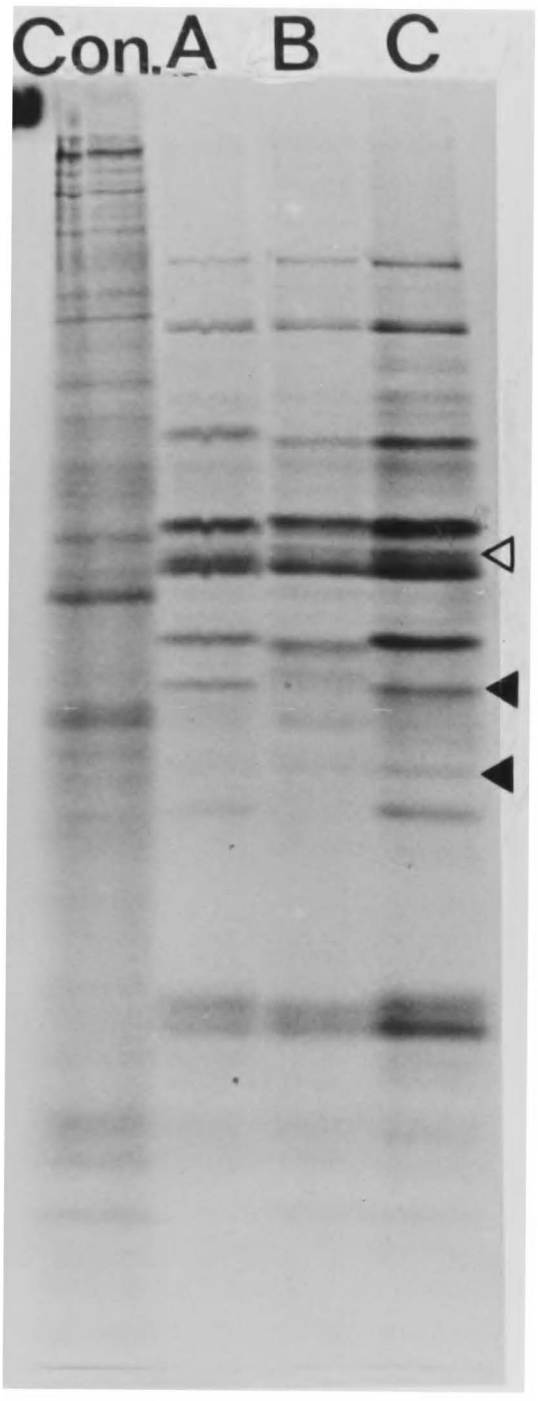


Figure 7.2. Intracellular induced polypeptides of Mill Door/79, North Clett/81 and Mill Door/81 viruses. Autoradiograph of a 12.5% PAG showing Mill Door/79 virus (MD/79), North Clett/81 virus (NC/81), Mill Door/81 (MD/81) infected Vero cells and uninfected Vero cells (cell). The triangles indicate the position of a polypeptide migrating above p44 and the small arrows indicate a polypeptide unique to North Clett/81 and Mill Door/81 infected cells. The broken arrows indicate the approximate position of two virus-specified polypeptides not visible on this autoradiograph.

Figure 7.3. Intracellular induced polypeptides of Mill Door/79, North Clett/81 and Mill Door/81 viruses. A densitometer scan and autoradiograph of a 12.5% PAG of virus-specified polypeptides. Infected cells were labelled with 10μCi/ml [^{14}C] protein hydrolysate between 7 and 8 hours p.i. The PAG shows uninfected cells (con) and cells infected with Mill Door/79 virus (A) North Clett/81 virus (B) and Mill Door/81 virus (C). The closed triangles indicate the positions of p36 (top) and p30 (bottom) and the open triangle the position of a polypeptide present in only North Clett/81 and Mill Door/81 virus infected cells. The densitometer scan represents tracks A, B and C from the PAG. The polypeptides of Mill Door/79 virus (A) are labelled and are connected by a dotted line to co-migrating polypeptides from the other isolates.


Con.
A
B
C

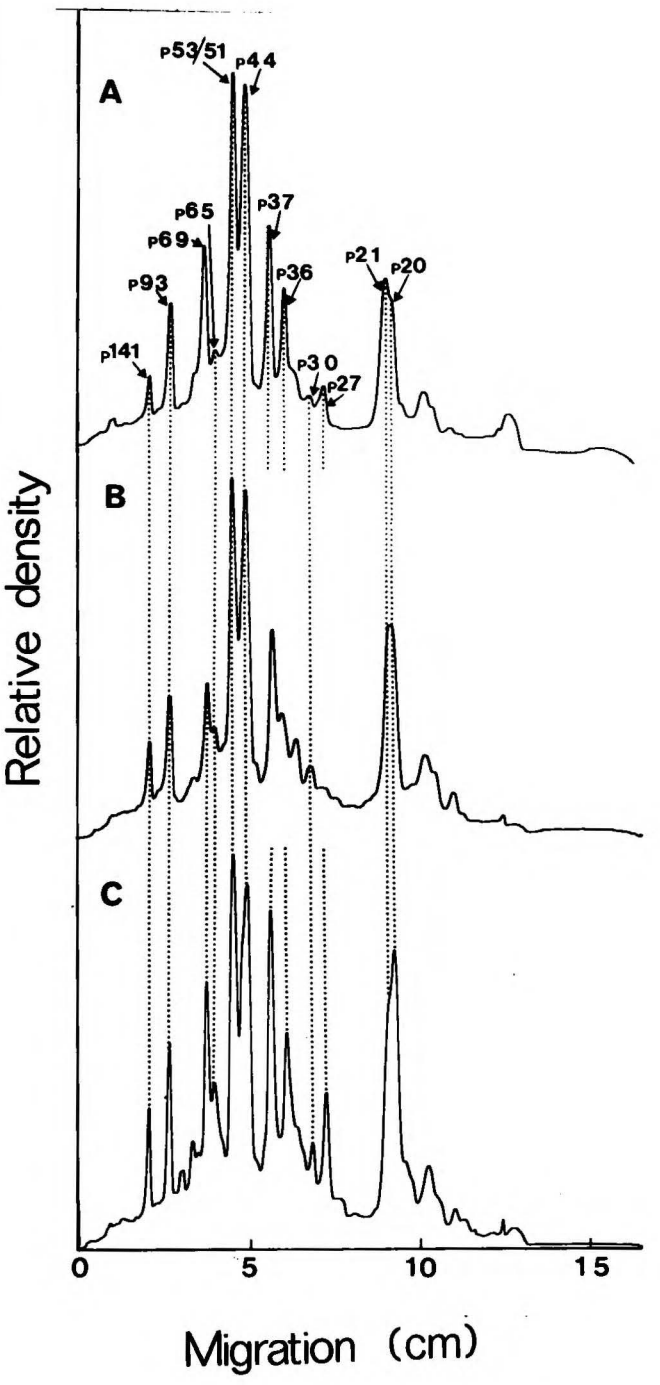

A
B
C
P53/51
P44
P65
P37
P69
P36
P21
P20
P93
P141
P30
P27
Relative density
0
5
10
15
Migration (cm)

Figure 7.4. Partial proteolysis of Mill Door/79 virus and North Clett/81 virus-specified polypeptides. Autoradiograph of a 17.5% PAG of the digestion products of gel strips, containing Mill Door/79 (MD/79) and North Clett/81 (NC/81) virus polypeptides, treated with 175µg/ml S.aureus V8 protease. The arrows indicate the polypeptides corresponding to, from left to right, p141, p93, p69, p65, p53/51, p44, p37, p36, p30, p27, p21 and p20 of each isolate as determined for Mill Door/79 virus. The circles indicate the position of oligopeptides identified only in the products of one isolate and the triangles indicate the polypeptides, produced by each isolate, which did not comigrate.

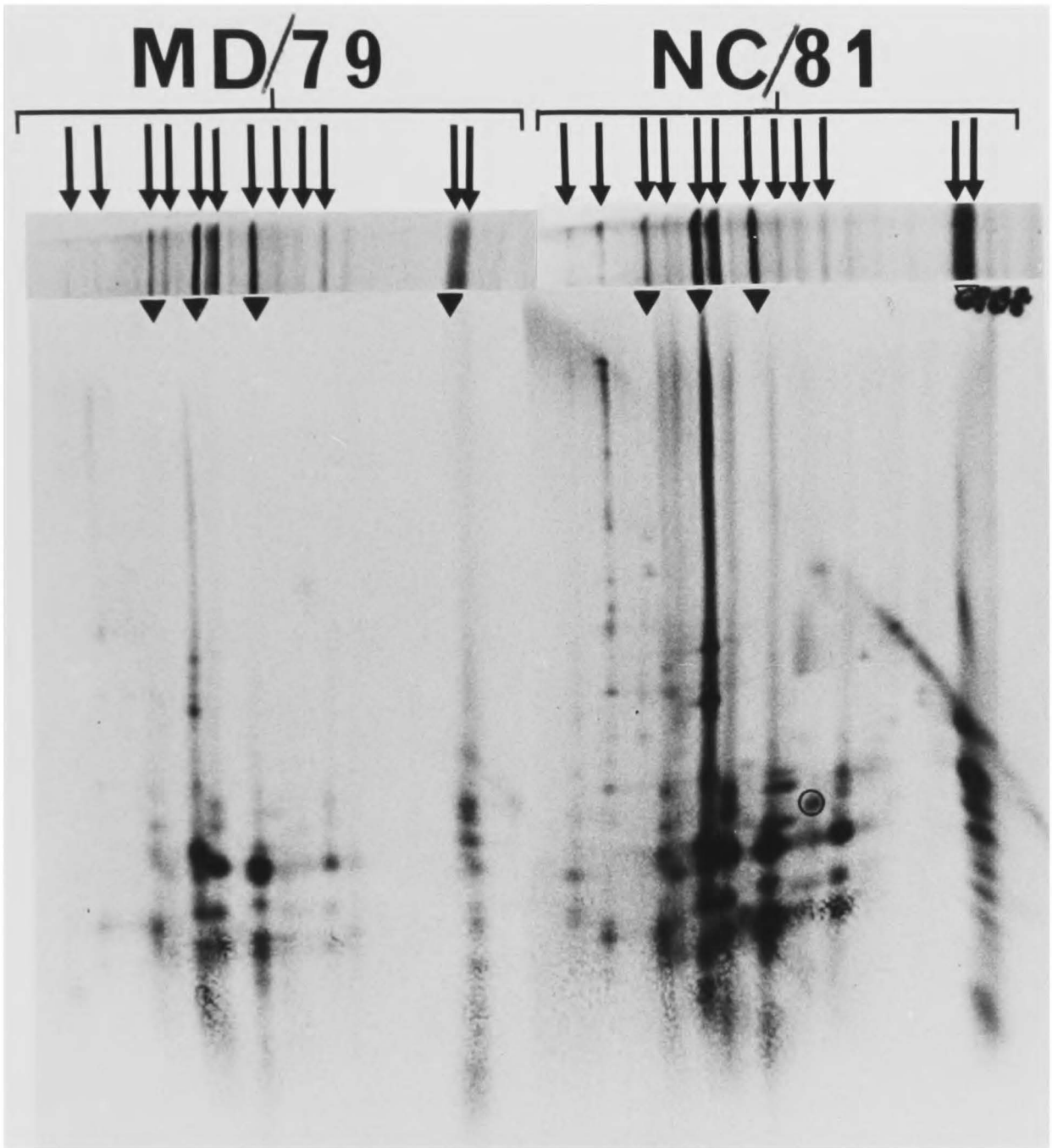

MD/79
NC/81

Figure 7.5. Partial proteolysis of Mill Door/79 and Mill Door/81 virus-specified polypeptides. Autogradiograph of a 17.5% PAG of the digestion products of gel strips, containing Mill Door/79 (MD/79) and Mill Door/81 (MD/81) virus polypeptides, treated with 175µg/ml S.aureus V8 protease. The arrows indicate the polypeptides corresponding to, from left to right, p151, p93, p69, p65, p53/51, p44, p37, p36, p30, p27, p21 and p20 of each isolate as determined for Mill Door/79 virus. The circles indicate the position of oligopeptides identified only in the products of one isolate and the triangles indicate the polypeptides, produced by each isolate which did not co-migrate.

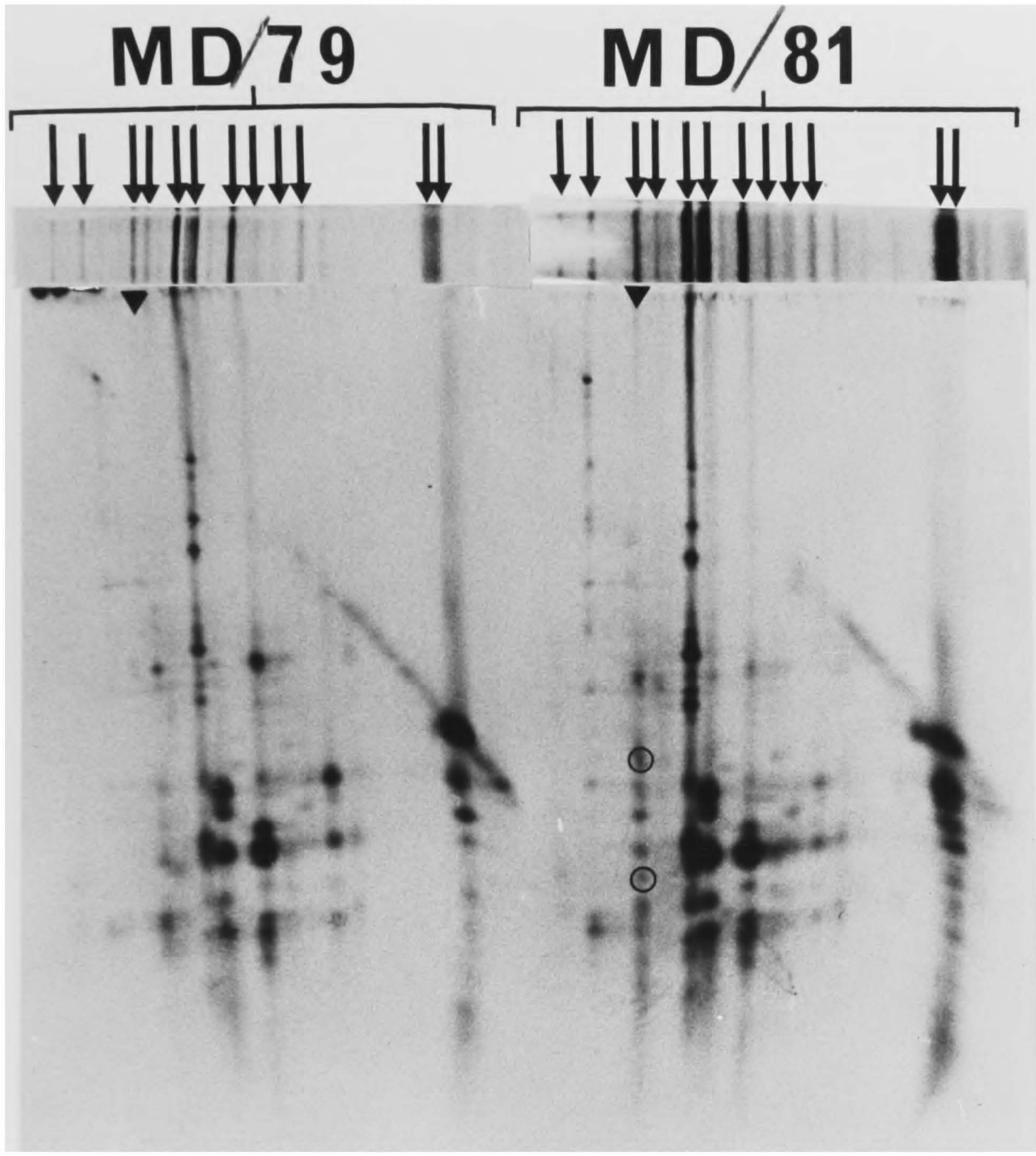

MD/79
MD/81

Figure 7.6. Partial proteolysis of North Clett/81 and Mill Door/81 virus-specified polypeptides. Autoradiograph of a 17.5% PAG of the digestion products of gel strips, containing North Clett/81 (NC/81) and Mill Door/81 (MD/81) virus polypeptides, treated with 175µg/ml S.aureus V8 protease. The arrows indicate polypeptides corresponding to, from left to right, p151, p93, p69, p65, p53/51, p44, p37, p36, p30, p27, p21 and p20 of each isolate, as determined for Mill Door/79 virus. The circles indicate the position of oligopeptides identified only in the products of one isolate and the triangles indicate the polypeptides, produced by each isolate which did not co-migrate.

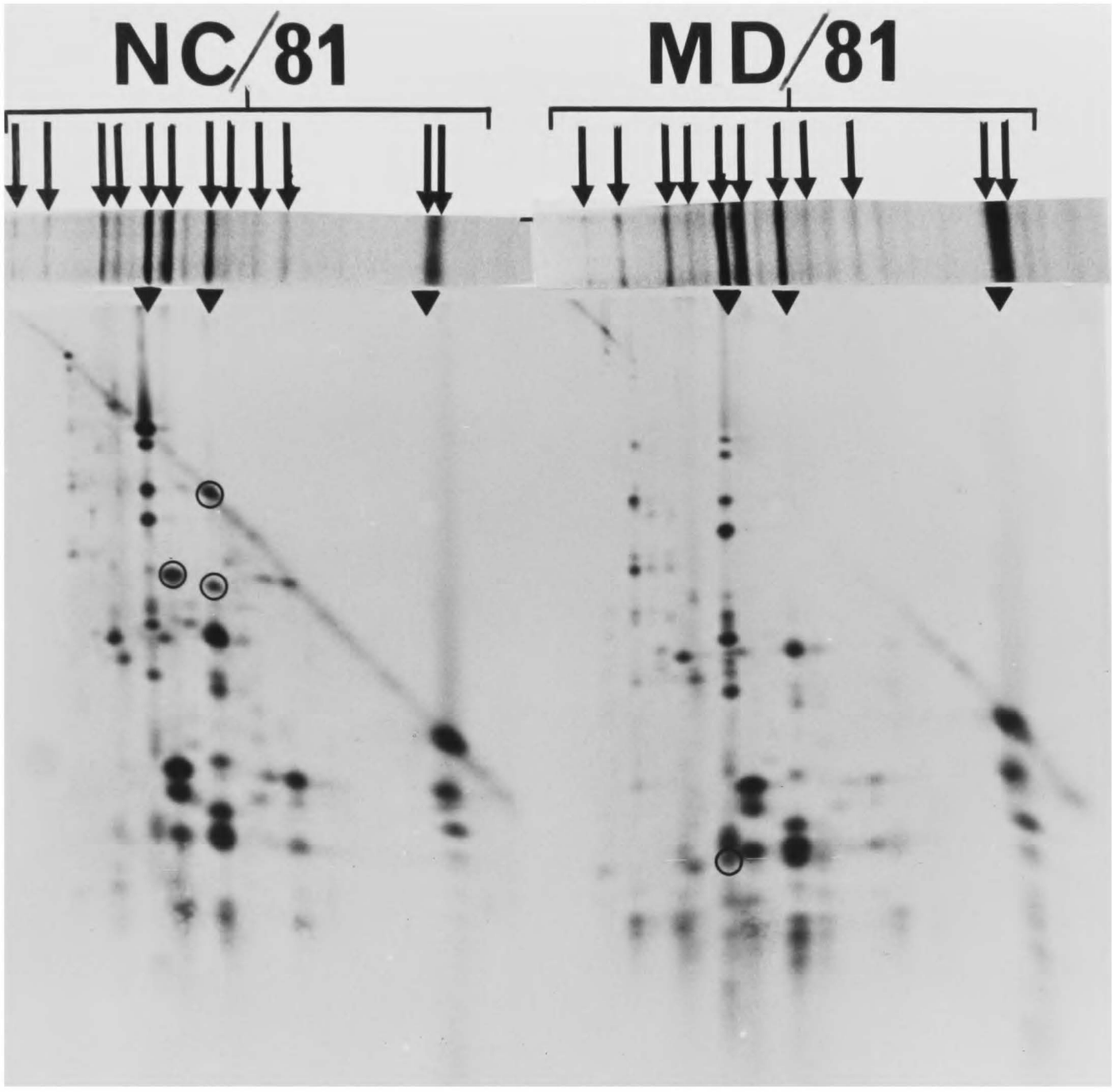
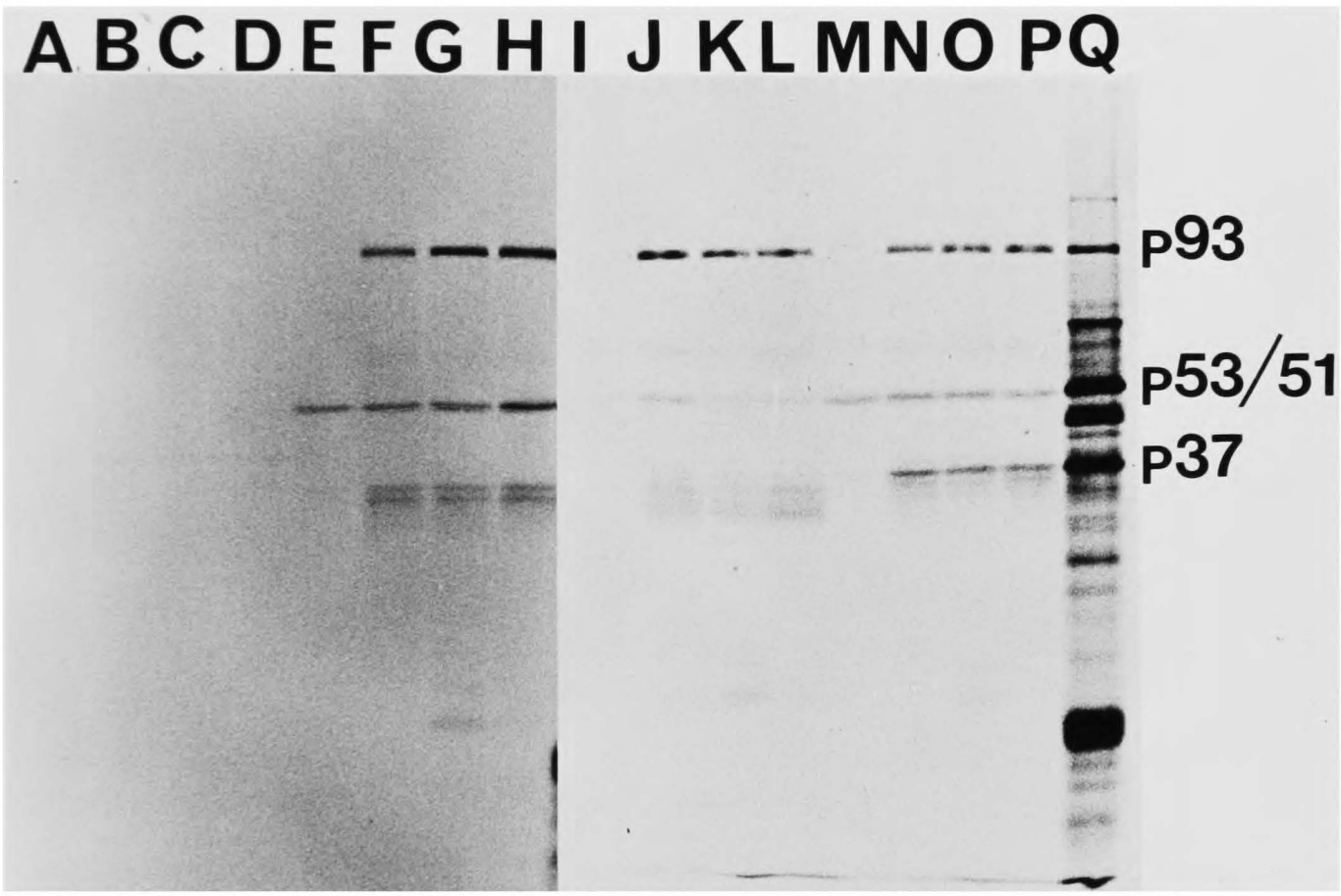

NC/81
MD/81

Figure 7.7. Cross-immune precipitation of the intracellular polypeptides of Mill Door/79, North Clett/81 and Mill Door/81 viruses. Autoradiograph of a 12.5% PAG of immune precipitates of [^{35}S]-labelled virus-specified proteins. Tracks A-D, uninfected cells treated with either pre-immune ascitic fluid (A) or hyper-immune ascitic fluid against Mill Door/79 (B), North Clett/81 (C) and Mill Door/81 (D) viruses. Tracks E-H; Mill Door/79 virus-infected cells treated with either pre-immune ascitic fluid (E) or hyperimmune ascitic fluid against Mill Door/79 (F), North Clett/81 (G) and Mill Door/81 (H) viruses. Tracks I-L; North Clett/81 virus infected cells treated with either pre-immune ascitic fluid (I) or hyperimmune ascitic fluid against Mill Door/79 (J), North Clett (K) and Mill Door/81 (L) viruses. Tracks M-P; Mill Door/81 infected cells treated with either pre-immune ascitic fluid (M) or hyperimmune ascitic fluid against Mill Door/79 (N), North Clett/81 (O) and Mill Door/81 (P) viruses. Track Q; intracellular polypeptides induced by Mill Door/79 virus.


A B C D E F G H I J K L M N O P Q
P93
P53/51
P37

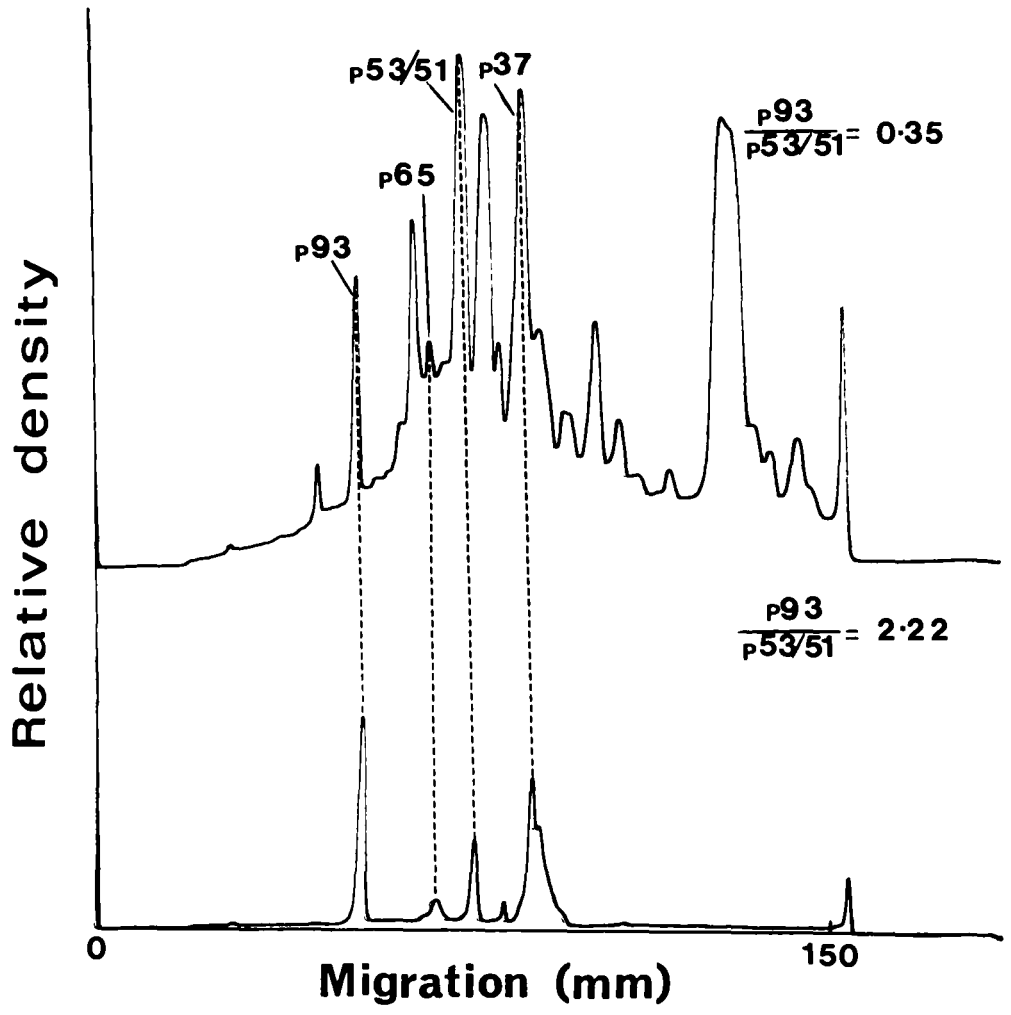


Figure 7.8. Densitometer scans of Mill Door/79 virus-specified intracellular polypeptides and the products of their homologous precipitation. An autoradiograph of a 12.5% PAG showing the intracellular polypeptides induced by Mill Door/79 virus (top) and those precipitated by homologous hyperimmune ascitic fluid (bottom). The values recorded by each profile represent the ratio of the amount of p93 compared to p53/51.

CHAPTER 8

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I. INTRODUCTION

The aim of this thesis was to isolate viruses from Ixodes uriae ticks collected on the Isle of May and to characterize them both serological and biochemically. Viruses were isolated from three tick pools, one from ticks collected during 1979 and two from ticks collected during 1981. They were identified by their physicochemical properties, electron microscopy and CFT as members of the Kemerovo serogroup, Great Island Complex, of the orbivirus genus. One isolate, Mill Door/79 virus, was further investigated to determine the nature of its dsRNA and products in cell culture. It produced 10 segments of dsRNA and 12 polypeptides, nine of which were probably primary gene products. In vitro translation identified a tenth primary gene product and led to provisional gene coding assignments. The three isolates were compared at a molecular level and the dsRNA profiles were shown to be distinct, whereas the virus-specified polypeptides were similar.

Orbiviruses are members of the family Reoviridae and display several structural and replicative features in common. Their genomes are composed of 10-12 segments of dsRNA of a similar total molecular weight to other members of the Reoviridae (Shatkin et al., 1968; Fujii-Kawata et al., 1970; Verwoerd et al., 1970; Reddy et al., 1974; Newman et al., 1975), and their capsids are about 70nm in diameter and comprise a double shell (Streissle & Granados, 1968; Els & Verwoerd, 1969; Luftig et al., 1972; Holmes et al., 1975). [CPVs possess a single shell (Lewandowski & Traynor, 1972)]. They replicate in the cell cytoplasm, and it is likely that transcription occurs after the removal of the outer capsid (Shatkin & Sipe, 1968b; Martin &

Zweerink, 1972; Galster & Lengyel, 1976). In the following sections some properties of the Isle of May orbivirus isolates, determined during the course of this thesis, are discussed in relation to the properties of other members of the family Reoviridae.

II. ANTIGENIC AND GENETIC DIVERSITY AMONG MEMBERS OF THE REOVIRIDAE

A. Antigenic Diversity

Members of the genus orthoreovirus are all serologically related, as judged by a variety of tests. Mammalian reoviruses comprise three serotypes, whereas avian reoviruses comprise possibly five (Joklik, 1983). A common group antigen is also shared by rotaviruses isolated from several mammalian and avian species (Holmes, 1983). Orbiviruses, on the other hand, do not possess a group antigen, but viruses within serogroups share antigens detected by CF, immunodiffusion and fluorescent-antibody tests (Gorman et al., 1983). Thus, the degree of antigenic diversity among orbiviruses appears to be greater than that found in other genera of the Reoviridae. This may be a function of their ecology, because unlike reoviruses and rotaviruses, which are both enteric viruses (and respiratory in the case of reoviruses) infecting vertebrates, the orbiviruses are arthropod-borne and therefore possess both vertebrate hosts and invertebrate vectors. Some orbiviruses, such as those of the Kemerovo serogroup, are tick-borne, with birds as the predominant vertebrate host, whereas others, such as bluetongue viruses, are transmitted by Culicoides, with sheep as the main vertebrate hosts. The different host specificities and habitats of viruses

belonging to these two serogroups therefore prevent their genetic interaction under normal circumstances, allowing them to evolve independently. The more restricted natural environment of reoviruses and rotaviruses would not lead to such a clear-cut isolation of virus populations, especially since reoviruses and rotaviruses may not be completely species specific (Rosen, 1960; Woode et al., 1976).

The Isle of May isolates are members of the Kemerovo serogroup, Great Island complex. They reacted in CFTs with an ascitic fluid raised against Cape Wrath virus, which was known to belong to this complex (Main et al., 1976), but ascitic fluids raised against other Kemerovo serogroup viruses failed to react with the isolates. Therefore, the Isle of May viruses were placed in the Kemerovo serogroup, although they showed no evidence of shared CF antigens with some of its members. This suggests an even greater degree of antigenic diversity among Kemerovo viruses than among other orbivirus serogroups. Some orbivirus serogroups cross-react by CFT; bluetongue and epizootic haemorrhagic disease of deer viruses (EHDV) from the USA have been shown to be related, as EHDV strains isolated in Nigeria share CF antigens with bluetongue virus strains from South Africa and EHDV strains from the USA (Moore & Lee, 1972). Therefore some orbivirus serogroups appear to share CF antigens. Thus, it may be possible to demonstrate serological relationships between other orbivirus serogroups using more sensitive immunological assays to identify "intermediate" virus strains. Gorman (1979) found that when complement-antibody-antigen mixtures were incubated at 4°C overnight, rather than at 37°C for 30 minutes evidence for cross-reactions between Eubenangee, Tilligerry and bluetongue viruses were found. The quality of the ascitic fluids

and antigens used is also important, as poor reagents may not reveal relationships between viruses that would otherwise be recognised.

Viruses within each serogroup are commonly distinguished by neutralization tests, those that cross-react belonging to the same serotype. Mill Door/79 and North Clett/81 viruses did not cross-react, so on this basis belonged to separate serotypes although the quality of reagents may not have been good enough to identify low level cross-neutralization reactions, e.g. greater than four-fold differences. However, Mill Door/81 virus cross-reacted with North Clett/81 virus and immune ascitic fluid raised against Mill Door/81 virus neutralized Mill Door/79 virus. Therefore all three isolates were related directly or indirectly by neutralization tests. Travassos da Rosa et al (1984) demonstrated a similar range of relationships among Changuinola serogroup viruses.

How did antigenic diversity among the Isle of May isolates come about? Two mechanisms can lead to changes within the genomes of members of the Reoviridae, and these, in turn, can lead to antigenic changes. Antigenic "shift" can occur due to a slow, progressive accumulation of changes in nucleic acid sequence, whereas antigenic "drift" is due to the interchange of genome segments. Antigenic drift can only come about when more than one virus "type" replicates in a single cell at the same time, and is therefore dependent on the presence in a virus population of adequate numbers of more than one virus "type". Antigenic "drift" can occur through geographical isolation. Viruses were isolated from pools of ticks from two distinct colonies on the Isle of May. Only three isolates were studied, consequently the conclusions drawn here are necessarily limited.

Contact between the two Isle of May colonies is restricted: seabirds tend to use the same site for breeding each year, and ticks move relatively short distances (a few metres). This may explain differences between the isolate from one seabird colony (North Clett/81 virus) and those from another colony (Mill Door/79 and Mill Door/81 viruses). Although the two Mill Door isolates were from the same colony they were collected two years apart. This suggests that genetic/antigenic changes occur among the viruses within a colony and/or that several antigenic variants can be present within a colony.

It was concluded that the three Isle of May isolates were related by NT and were, therefore, members of the same serotype. For the purpose of classification, virus families are arranged into genera which are further divided into species. The International Committee on Taxonomy of Viruses defines a virus species thus: A virus species is a concept that will normally be represented by a cluster of strains from a variety of sources, or a population of strains from a particular source which have a common set or pattern of correlating stable properties that separates the cluster from other clusters of strains (Matthews, 1982). Given this definition, the Isle of May isolates would be members of the same species. However, biological species are groups of actual or potential inter-breeding natural populations that are reproductively isolated from other such groups (Gorman, 1983). D.W. Kingsbury has suggested that a virus species represents a virus population or gene pool, normally separated from other gene pools. Unfortunately not enough is known about the Isle of May Kemerovo serogroup virus interactions between, or within seabird colonies, and whether reassortment takes place between these viruses, therefore the three Isle of May isolates

may either belong to the same species, or according to the definitions above, represent separate species. The International Committee on Taxonomy of Viruses has stated that "virus taxonomy at its present stage has no evolutionary or phylogenetic implications" (Matthews, 1982). However, antigenic "drift" and "shift", which occur within the Reoviridae, are evolutionary phenomena, therefore it would, under these circumstances, be helpful to define a species as a population or gene pool separated from other gene pools. This definition could be extended, since two populations could belong to the same species if they are actually or potentially able to exchange genes (Gorman, 1983). Thus, the serotype relates to properties of surface proteins with a common gene pool, but it is that common gene pool, and not the serotype, which defines the evolutionary unit. It is quite possible, although it has not been proven, that Kemerovo serogroup viruses within a seabird colony can interact through gene reassortment, although the extent to which this may occur can only be guessed at. Therefore, defining serotypes within a colony may be meaningless, since they only indicate a common/unique sequence of perhaps a part of one gene in an interacting population of thousands.

B. Genetic Diversity

The dsRNA profiles of different members of the Reoviridae are distinct, both between genera and among members of the same genus. Figure 1.1 shows the profiles of representative members of each genus. Intrageneric variation has been demonstrated for reoviruses (Hrdy et al., 1979), rotaviruses (Espejo et al., 1981), CPVs (Payne & Rivers, 1976), phytoreoviruses (Reddy & Black, 1974) and orbiviruses (bluetongue) (Gorman et al., 1981).

Variation between serologically closely related viruses has also been observed (Gorman & Taylor, 1978; Hrdy et al., 1979). The three Isle of May isolates, although related by both CFT and NT, possess quite distinct dsRNA profiles. Mill Door/81 and North Clett/81 viruses cross neutralize, but their dsRNA profiles differ by the migration of nine segments, and Mill Door/79 and North Clett/81 viruses, which do not cross neutralize, differ by eight segments. This suggests that serological data and dsRNA profiles cannot be easily compared, although this does not appear to be the case with CPVs (Payne et al., 1983). However, if the dsRNA profiles of each of the isolates is studied closely it becomes evident that the 1981 isolates possess a similar separation pattern for dsRNA segments 3 to 6, whereas the Mill Door/79 virus dsRNA separation pattern for these segments is quite distinct. It may be of value, therefore, to compare the general separation patterns of dsRNA from virus isolates, rather than the migration of individual segments, when attempting to correlate genome profiles with serological data. Payne et al. (1983) suggested that low resolution (low concentration) gel systems may prove useful in grouping viruses which appear related by antigenic properties.

The size of a genome segment does not in itself give any precise indication as to the size or amino acid composition of the protein for which it codes. Only complete sequence data would indicate this. However, less involved procedures can be used to compare genome segments and their products in detail, and these can produce valuable information concerning the relationships between viruses. Three techniques, namely oligonucleotide fingerprinting, hybridization and northern blot studies, compare viral RNAs, while partial proteolysis can be used to compare

viral products. Analysis of oligonucleotide fingerprints of each of the RNA genome segments of serotypes of Wallal virus has shown substantial sequence heterology, even among genome segments which co-migrate (Walker et al., 1980). Huismans and Howell (1973) attempted to correlate antigenic differences with molecular structure by hybridization studies between the RNA of bluetongue virus strains. Cross-hybridization of the RNAs showed considerable mismatching in each of the genome segments. Since no antigenic differences could be detected amongst the strains used it appears that the altered base sequences need not necessarily be reflected in alterations to the antigenic sites on the virus proteins. Gorman (1979) suggested that the apparent stability of the antigenic determinants was due to the failure of conventional serological tests to detect minor variation.

Schroeder et al (1983) and Street et al. (1982) demonstrated sequence diversity amongst human and animal rotaviruses by northern blot. Their results indicated that both antigenic "drift", owing to slow but progessive accumulation of changes in nucleic acid sequence, and "shift", due to interchange of genome segments, was occurring. Fewer differences were detected between the partial proteolysis products of the Isle of May isolates than would have been expected from the differences in their dsRNA profiles, although the former method was relatively insensitive. Gentsch and Fields (1981) compared tryptic peptides of the outer capsid polypeptides of reovirus serotypes 1, 2 and 3. The μ1c polypeptide showed an extraordinary degree of conservation of its tryptic peptides, whereas the type specific polypeptide σ1 contained both conserved and unique tryptic peptides. Gaillard and Joklik (1980) showed that the antigenic determinants of most of the reovirus proteins, of all three serotypes, were

conserved. However, only the RNA sequences of serotypes 1 and 3 showed substantial homology, whereas that of serotype 2 displayed little (Martinson & Lewandowski, 1974). The proteins of different reovirus serotypes and different orbivirus isolates appear to vary comparatively little, while the genomes appear to be quite distinct.

One would expect the capsid structure and mode of replication among serotypes within different genera of the Reoviridae to vary little, so the conformations of their proteins should also be similar. Too large a change in the amino acid composition of any protein would almost certainly impair its function. Consequently, changes in amino acid composition of a protein are restricted. Partial proteolysis, tryptic cleavage, and serological methods do not compare proteins by their amino acid sequences; serological methods detect conformational similarities between proteins (functional proteins have restricted conformations i.e. enzymes and capsid proteins), whereas enzyme digestion methods are not absolutely specific. Trypsin does not differentiate between lysine and arginine residues, both basic amino acids between pH 6 and 7, therefore it is quite possible that these two amino acids could be interchanged without impairing protein function. However, if one of them were replaced with a different amino acid, such as proline, the conformation would change and the protein function would probably be impaired. With isolates as closely related as those from the Isle of May it would probably be of value to select a more sensitive method of comparison, such as oligonucleotide fingerprinting or RNA-RNA hybridization, otherwise variation may not be detected.

III. PROTEIN PRODUCTION OF MILL DOOR/79 VIRUS AND OTHER REOVIRUSES

A. Protein Cleavage

Much of the work in this thesis was concerned with the production of polypeptides by Mill Door/79 virus (Chapters 4, 5 and 6). Since little information has been published on the production and possible processing of orbivirus proteins the following section deals mainly with a comparison of Mill Door/79 virus polypeptides with those of rotaviruses and reoviruses.

Twelve Mill Door/79 virus-specified polypeptides were identified in infected cells. Three of these were probably cleavage products. Pulse-chase experiments failed to demonstrate cleavage but the appearance of two polypeptides in infected cells was prevented by serine protease inhibitors (TPCK and TLCK) and other protease inhibitors (ZnAc and PMSF). Intracellular labelling experiments sometimes failed to detect these cleavage products, suggesting they were not always produced, or could not always be detected, although the reason for this was unclear. Protein cleavage was demonstrated in reovirus and rotavirus infected cells by pulse-chase experiments (Zweerink & Joklik, 1970; McCrae & Faulkner-Valle, 1981) and in reovirus infected cells using monoclonal antibodies (Lee et al., 1981a). In contrast to the Mill Door/79 virus cleavage products, which appeared to be produced immediately after or during translation, the cleavage products of reoviruses and rotaviruses were produced during the 90 to 120 minute chase periods. Pulse-chase experiments give little indication of the mechanism of cleavage, apart from when cleavage occurs. The effect of TPCK and TLCK on the production of Mill Door/79 virus cleavage products suggests

they were formed through the action of a serine protease enzyme (i.e. chymotrypsin or trypsin). None of the cleavage products of either rotaviruses (McCrae & McCorquodale, 1982), or Mill Door/79 virus were produced *in vitro*.

Some of the cleavage products of both rotaviruses and reoviruses were present in the virus capsids (Tables 1.2 and 1.3), whereas no Mill Door/79 virus cleavage products were detected amongst the possible capsid polypeptides after purification (Figures 4.15 and 4.17). The functions of the Mill Door/79 virus cleavage products are, therefore, obscure. It is possible that they represent the products of host-cell directed cleavage of virus polypeptides and are artifactual. Only when a more detailed understanding of the functions of each of the virus-specified polypeptides has been achieved can these problems be addressed.

Only 9 to 10 virus-specified polypeptides have been detected in cells infected with bluetongue virus isolates, and no processing was demonstrated using pulse-chase experiments (Huismans, 1979; Gorman *et al.*, 1981). However, Gorman *et al.* (1984) identified 11 virus-specified polypeptides in cells infected with Nugget virus, a member of the Kemerovo serogroup, and therefore more closely related to Mill Door/79 virus. These differences in the number of intracellular virus polypeptides reported indicates that either Kemerovo serogroup viruses and bluetongue viruses are distinct in their modes of replication and/or structure, or that Huismans (1979) and Gorman *et al.* (1981) failed to detect some of the polypeptides associated with Mill Door/79 virus and Nugget virus infected cells.

B. Protein glycosylation

Both rotavirus and reovirus proteins are glycosylated (see Sections IID and IIID). However, they vary in the type of sugar-peptide bond involved and the number of proteins glycosylated. The two rotavirus glycoproteins are not formed in the presence of tunicamycin (Arias et al., 1982) which indicates that the sugar-peptide bond is an N-glycosidic linkage, common in both cellular and viral glycoproteins (Klenk & Schwarz, 1982). The oligo-saccharides were also cleaved by treatment with endo H (Arias et al., 1982) suggesting that they contained residues of the "high mannose" type (Robbins et al., 1977). Reovirus structural proteins, with the exception of σ2 (a core protein), were glycosylated, but only glucosamine and galactose were shown to be incorporated (Lee, 1983). The antibiotic TM did not exhibit any inhibition of sugar incorporation into reovirus proteins (Lee, 1983). All Mill Door/79 virus polypeptides, with the exception of p30 and p20, were glycosylated, although the amount of sugar incorporation into some of the polypeptides was very low. None of the polypeptides were labelled by [^{14}C] glucosamine in the presence of 2DG, whereas labelling by [^{14}C] mannose was unaffected. Incorporation of [^{14}C] glucosamine into reovirus proteins was also blocked by 2DG. Mill Door/79 virus glycosylation appeared to be unaffected by TM. Therefore Mill Door/79 virus polypeptides, like reovirus polypeptides, probably contain small amounts of glucosamine (and possibly galactose) attached by an O-type glycosidic linkage [2DG blocks the formation of O-glycosidic bonds (Krystal et al., 1976)]. In addition Mill Door/79 virus also contains small amounts of mannose combined to polypeptides by a TM and 2DG resistant bond.

The mechanisms of glycosylation vary between rotaviruses,

reoviruses and Mill Door/79 virus. The presence of either O-type or N-type glycosidic linkages indicates significant differences in the modification mechanisms of viral proteins. The resistance of the mannose containing glycoproteins of Mill Door/79 virus to inhibitors of N and O-type glycosidic linkages suggests that a third mechanism of glycosylation may exist.

C. Purification of Mill Door/79 virus

Central to the study of a virus is the ability to purify it from cell culture or some other source. Attempts to purify Mill Door/79 virus were largely unsuccessful and the data presented in this thesis represents only the more successful experiments (Chapter 4, Section J). Approaches to the purification of orbiviruses, such as bluetongue virus, have been largely based on those devised for reoviruses (Smith et al., 1969; Martin & Zweerink, 1972). However, viruses of the Kemerovo serogroup may not be assembled in a manner identical to bluetongue viruses (Gorman et al., 1984) and they are not as stable with some of the physicochemical treatments necessarily involved in the purification procedure. Gorman et al. (1984) demonstrated that Nugget virus, a member of the Kemerovo serogroup, remained associated with cellular material. They also showed that the virus infectivity was lost during attempts to separate it from the cellular material using detergents and lipid solvents. Like Mill Door/79 virus, Nugget virus is sensitive to the treatments required for purification. It may be that the removal of cellular material is responsible for the fragmentation of the virus particles. Attempts to purify Kemerovo viruses have been made using both sucrose and CsCl gradients, and although core structures, in various states of fragmentation, have been

observed, no complete virion was seen (S.M. Eley, unpublished observations). If the separation of virus particles from cellular material requires the removal of the outer shell then it may not be possible to obtain complete purified virions.

D. Serological Reactions

One of the features most members of the Reoviridae have in common is the possession of a double-layered capsid. Important serological reactions, such as neutralization, are associated with capsid components, and reoviruses, rotaviruses and orbiviruses display clear similarities in the manner of their serological reactions. It was shown with members of these genera that group specific antigens were located on the inner capsid, whereas type-specific antigens were located on the outer capsid (Bridger, 1978; Huismans & Erasmus, 1981; Lee et al., 1981a).

Hayes et al. (1981) using monoclonal antibodies, identified three proteins in the reovirus capsid that were responsible for neutralization: these were σ3 an outer capsid polypeptide, σ1a minor outer capsid component, and λ2, a core polypeptide and the sole protein comprising the reovirus spikes. Of these σ1 was the type specific protein, whereas σ3 and λ2 displayed group-specific neutralization activity. Bridger (1978) demonstrated that group-specific antigens were present in the rotavirus core and type-specific antigens were contained in the outer capsid. Bluetongue viruses display this same arrangement; the surface polypeptide, P2, is implicated in the type-specific reactions, whereas the core polypeptides, P7 and possibly P3, are involved in group-specific reactions (Huismans et al., 1979; Huismans & Erasmus, 1981).

The structure of the Mill Door/79 virus capsid is not known,

although it is possible that it contains polypeptides p93, p69, p53/51, p37, p21 and p20. Immune precipitation of Mill Door/79 virus-specified polypeptides from cell culture demonstrated that 3 or 5 polypeptides (p93, p53/51, p37 and possibly p65 and p36) reacted with the ascitic fluid used. Since this ascitic fluid was capable of neutralizing Mill Door/79 virus it was probable that one of the polypeptides with which it reacted was responsible for neutralization reactions.

E. In vitro translation and gene coding assignments

In vitro translation of the genomes of members of the Reoviridae has been used to identify primary gene products and to assign proteins to the individual genome segments. Figure 1.2 shows the gene coding assignments for reovirus type 3, simian rotavirus, CPV of B.mori, and bluetongue virus type 1. The provisional coding assignments for Mill Door/79 virus are shown in Fig. 6.12. It is apparent in each case that there is no absolute correspondence between relative gene and protein size, as judged by electrophoretic migration rates. This means that untranslated regions must be present on some genome segments (e.g. segment 1 of reovirus). The presence of untranslated regions has been demonstrated on the reovirus S2 gene (segment 8) (Cashdollar et al., 1982). These workers cloned and sequenced the S2 gene and showed that, whereas it contained the coding capacity for a protein of 443 amino acids, its major open reading frame extended for only 331 codons [producing a protein the same size as σ2, which was the protein coded for by S2 (McCrae & Joklik, 1978)]. A second open reading frame was present at the 3' end of the gene capable of coding for a protein of 10,000 daltons. However, there was no evidence that this protein was

produced in reovirus infected cells. Incomplete translation of the genome segments means that quite substantial changes in genome size need not involve translated regions, and therefore have no effect on the protein product.

Additional polypeptides have been identified in the products of individually translated genome segments (McCrae & McCorquodale, 1982; Grubman et al., 1983; Mason et al., 1983; McCrae & Mertens, 1983; Mertens et al., 1984). Grubman et al. (1983) suggested that the additional polypeptides could be either unique products, incomplete translation products, or products modified post-translationally. Both Grubman et al. and Sangar and Mertens (1983) detected the additional in vitro products in vivo. In contrast, the additional products synthesized on the translation of individual Mill Door/79 virus genome segments were not detected in vivo. The synthesis of an in vitro product (migrating between p93 and p75) was only detected in some lysate preparations, suggesting that the production of some polypeptides was directed by the translation system (e.g. post-translational cleavage). Products from either incomplete translation or post-translational cleavage could be distinguished from unique (different/out of phase reading frame) products by partial proteolysis. For example, two proteins, produced by the in vitro translation of genome segment 7 of type 1 CPV, were shown to give similar digestion profiles, indicating that they were products of the same reading frame (McCrae & Mertens, 1983).

A polypeptide (p75) was detected by in vitro translation of Mill Door/79 virus dsRNA and provisionally assigned to genome segment 3. However, it was only detected in vivo when the cells were treated with raised levels of NaCl. Thus the failure of segment 3 to translate in vivo was possibly due to a control

mechanism which was inactivated by high NaCl concentrations. An alternative suggestion would be that it was a precursor of the putative cleavage product p30, since p30 was not detected *in vitro* and appeared rapidly *in vivo*. However, inhibition of p30 synthesis *in vivo* failed to produce a concomitant appearance of p75.

Care must be taken when extrapolating gene coding assignment from one virus to another. Segments 4, 5 and 6, from the genome of Mill Door/79 virus, migrate at almost the same rates in PAG, therefore minor changes in their rates of migration could alter their relative positions and therefore appear to change the gene coding assignments. This may have occurred with bluetongue virus serotypes 1 and 4 for which the genome coding assignments for segments 2 and 3, and 5 and 6 were reversed (Mertens *et al.*, 1984). The relative migration rates of some virus polypeptides can be altered by changing the electrophoresis buffer system (McCrae & Joklik, 1978). Various workers have presented conflicting data on the identity of bluetongue virus serotype specific antigen. Huismans and Erasmus (1981) and De Villiers (1974) implicated P2, whereas Grubman *et al.* (1983) proposed that it was P3. For this reason Grubman *et al.* (1983) suggested that comparisons between bluetongue virus proteins separated using different gel buffer systems were not reliable.

IV. AREAS OF FUTURE RESEARCH

This thesis has demonstrated that aspects of the replication of Mill Door/79 virus (and possibly other Kemerovo serogroup orbiviruses) can be studied with relative ease under well defined conditions. The efficient shutting off of host-cell protein

synthesis facilitated a detailed study of Mill Door/79 virus protein synthesis. Immediate future studies on Mill Door/79 virus should aim to further characterize the virus-specified proteins, to determine their structural and/or intracellular roles. An understanding of the functions of the virus proteins, in conjunction with the assignments of proteins to genome segments would help to determine the significance of reassortment and/or mutation of genome segments. However, before one draws any conclusions as to the significance of reassortment, the possibility that the gene coding assignments of different isolates vary should also be investigated, since Mertens et al. (1984) demonstrated that the assignments of two bluetongue virus isolates differed. This could be done by either in vitro translation of separate single segments of each isolate, or RNA-RNA hybridization, oligonucleotide fingerprinting or Northern blotting individual genome segments, after first having determined the assignments for one isolate. The latter method would be of greatest value when corresponding genome segments display homology.

Antigenic and genetic diversity were discussed in Section II in which it was shown that Kemerovo serogroup viruses displayed a high degree of antigenic diversity. However, little work has been done to determine the degree of genetic diversity within the Kemerovo serogroup. The dsRNA profiles of each of the Isle of May isolates, and of other Great Island Complex viruses, were distinct (Chapter 7 and F. Black, unpublished observations), although all retained the 2 + 4 + 3 + 1 separation pattern. Genome profile variations are common among all the genera of the Reoviridae (Chapter 1). It has been suggested, by various workers, that genome profiles can be used to type virus isolates;

an electropherotype system has been devised by Payne and Rivers (1976) for CPVs. However, it is still not clear what significance genome profile variations have. Viruses of the Great Island Complex (Kemerovo serogroup) offer a useful source of viruses in nature that display both antigenic and electropherotype diversity, since each virus can be defined both geographically (seabird colony or site) and temporally (date of tick collection). Great Island Complex viruses are also easy to isolate and cultivate in the laboratory. A detailed comparison of both serological data and dsRNA profiles may indicate whether, if at all, these two virus properties can be related. Since the basis of genome profile variation is not known, parallel studies, concentrating on a few virus isolates, should also be made to compare virus genomes more closely. The method I would choose would be Northern blot hybridization, since the stringency of the reaction between the probe and the virus RNA can be altered to suit the requirements of the particular experiment. Results from such studies should indicate whether (1) serology and electropherotyping can be related and (2) genome profile variation reflects genome sequence variation.

Research on the Great Island Complex viruses can also be used to formulate models for virus evolution. The restricted habitat of seabird colonies in which these viruses circulate may lead to the appearance of distinct serotypes (Main et al., 1973). The possible mechanisms responsible for generating new serotypes were discussed in Section IIA. Using Northern blot hybridization one could study isolates to detect the presence of genetic "shift" and "drift". A comprehensive picture of virus evolution within seabird colonies may provide insights into both the mechanisms and kinetics of variation within the family

Reoviridae and possibly other segmented genome viruses and the factors that influence this variation.

Reassortment occurs between bluetongue virus serotypes (Gorman et al., 1978); these experiments have not been carried out on members of the Kemerovo serogroup. Since viruses belonging to the Kemerovo antigenic complex (Kemerovo and Tribec) have been implicated in cases of mild febrile illness and various neuropathies in humans (Chumakov et al., 1963; Malkova et al., 1980) it would be of value to determine whether Kemerovo serogroup viruses can reassort. However, the demonstration of reassortment between Kemerovo Complex and Great Island Complex viruses in vitro does not indicate that it occurs naturally. If the virus populations are isolated from each other exchange of genetic information cannot take place. Therefore, if reassortment between these two complexes was demonstrated it would then be necessary to investigate the ways in which these groups might possibly be able to interact. The seabird hosts of the Great Island viruses migrate from seabird colonies in Great Britain, such as the Isle of May, to northern Europe and Russia (Mead, 1974). Viraemic birds, or birds carrying virus-infected ticks, could transport viruses of the Great Island Complex to areas where viruses of the Kemerovo complex occur. However, the tick vectors of Tribec and Kemerovo viruses are I.ricinus and I.persulcatus respectively, whereas the tick vectors of the Great Island Complex viruses are I.uriae and I.signatus. Studies should therefore be made to determine the vector ranges of these viruses in order to determine whether mixed infections (inter-complex), and therefore reassortment, can occur naturally.

The infection of arthropod vectors with orbiviruses should be studied to determine how long they can harbour virus. Two

isolates from the Isle of May, Mill Door/79 and Mill Door/81 viruses, were made from pools of male I.uriae indicating transtadial survival of the virus in the tick, since male I.uriae are not known to take blood meals. This would suggest that the virus can remain in the tick for a significant period of time, so it would be of value to determine whether viruses can reassort within the ticks, since extended periods of virus infection would increase the opportunities for dual infections. Hence the integration of ornithology, acarology, and molecular biology, in the study of viruses of the Great Island Complex, would provide valuable information with regard to virus evolution, and the generation and maintenance of virus strains and serotypes in nature.

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