Analyses of RNAs and proteins of rotaviruses with rearranged genomes: A study of molecular variability.

by

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SUMMARY

Genome heterogeneity among cocirculating human rotaviruses (hrv) has been described in different parts of the world and in this project was exemplified by a small collection of 100 specimens collected from infected children in Britain between 1975 and 1983. Pedley et al. (1984) described protracted hrv infections and virus shedding in children who suffered from severe combined immunodeficienc (SCID). The viruses isolated from the children showed abnormal genomes carrying extra bands of dsRNA which were found to have arisen by rearrangement of normal RNA segments to covalently linked concatemers. Rotaviruses with similar genomes were obtained after serial passage in-vitro at high m.o.i.: Hundley et al. (1985) isolated mutants D, A_4, B_4 and B_5 of bovine rotavirus (brv) whose genomes had lost RN segment 5 and instead carried additional RNA bands A-H.

The main part of the project was to further characteri these mutants. The additional RNA bands migrated betwee genomic segments 1 and 7, and were not integral multiples c segment 5 length. Further characterisation by RNase T_1 oligonucleotide mapping (Follett and Desselberger, 1983b) showed that band A of brv mutant D (Hundley et al., 1985) and band E of brv mutant A_4 (this thesis) consisted of segment 5-related sequences. The brv mutants D, A_4, B_4 and B_5 were not replication-defective as they could be passaged through multiple rounds of plaque-to-plaque purification without requiring parent virus (standard brv). Reduction

in the degree of genome transcription and replication, plaque formation, suppression of host cell protein synthesis and development of CPE was observed in cells infected with the brv mutants A_4, B_4 and B_5 when compared to standard brv. However, brv mutant D was similar to standard brv in all these parameters. Standard brv and the brv mutants grew to different titres at 31°C, 37°C and 39.5°C and the difference in infectivity titres was not dependent on changes in the ratios of virus particle count to plaque forming units (titre). None of the mutants was temperature-sensitive (ts).

Measurements of [^{32}P] labelled RNA synthesised in infected cells showed that non-equimolar amounts of RNA transcripts were produced but the incorporation of [^{32}P] was on the whole inversely proportional to the size of the RNA template. The rearranged bands A and E of brv mutants D and A_4, respectively, were transcribed in proportions similar to normal genomic segments of corresponding size. The sizes of rearranged RNA bands A-H were between 0.90 and 1.94-fold of the size of segment 5 (=1.00) and sequences in these bands represented an excess of 3.9 to 6.7 and possibly 30.5% of total RNA. Replication of standard brv and the brv mutants D and A_4 in infected cells was inhibited by 20ug/ml cycloheximide. During continued RNA synthesis, ssRNAs were more predominant at early time (6 hours pi) and gave way to abundance of dsRNAs at 16 hours pi in cells infected with standard brv and brv mutant D. The transition from ssRNA to dsRNA synthesis was slower in cells

infected with brv mutant A_4. Genomes of the brv mutan), A_4, B_4 and B_5 carrying rearranged bands A, E, F, and G'G and H were fully transcribed in-vitro.

Virus-specific proteins VP1-VP12 were observed in cells infected with standard brv and the brv mutants with the exception that none of the brv mutants did synthesise VP5. The brv mutant A_4 carrying the rearranged band E synthesised a novel protein called VP5A of 74.6Kd MW, and was considered a product compatible with the coding capacity of RNA band E. The protein profiles of brv mutants D, B_4 and B_5 did not contain any viral protein that could be attributed to the rearranged RNA bands. Comparative two-dimensional oligopeptide mapping showed that the VP5A was very similar to the normal VP5.

Relative abundancies of the proteins of standard brv and the different mutants were measured at m.o.i.'s of 1, 5 and 40 pfu/cell, and in this respect no major differences were detected for corresponding proteins. For each virus, the VP1-VP12 were present in unequal proportions independent of m.o.i.s. All viruses produced VP6 in largest amounts averaging 30%. For the brv mutants, VP9 was synthesised in quantities which exceeded that of standard brv. The brv mutants A_4, B_4 and B_5 were remarkably less efficient in suppressing host cell protein synthesis than standard brv and brv mutant D.

Gradient-purified virions of brv A_4 and standard brv were not different in shape, size and surface appearance. The virions of both standard brv and brv mutant A_4 contained

VP1, VP2, VP3, VP4, VP6, VP7 and VP10 of which the VP7 and VP10 were outer capsid proteins. VP5, VP5A, VP8 and VP9 were found not to be associated with purified virions.

In time course experiments VP1-VP12 were detected at the same time (6 hours pi) in cells infected with standard brv and the brv mutants. The glycoprotein VP10 reached peak synthesis at 8 hours pi and suddenly declined 30 fold at 16 hours pi. "Pulse-chase" experiments showed that the VP1-VP10 were stable and no changes in mobility were found.

Radio-labelling of intracellular viral proteins with mannose and glucosamine showed that VP2, VP6, VP7 and VP10 were glycoproteins. It was striking to find that brv mutants D, A_4 and B_4 incorporated abnormally low amounts of mannose in the VP7 and VP10 but not in the other glycoproteins. VP7 and VP10 were N-linked glycoproteins as indicated by the sensitivity of glycosylation to tunicamycin. VP2 and VP6 glycosylation was not influenced by tunicamycin treatment of infected cells. The infectivity titres of standard brv and the brv mutants D and A_4 were reduced by 1 $\log_{10}$ unit when grown in the presence of 1ug/ml or 5ug/ml tunicamycin.

In a plaque reduction assay using polyvalent hyperimmune sera raised against standard brv and the brv mutants D and A_4 it was found that the brv mutants were serotypically very similar to standard brv. Immunoprecipitation reactions using the same antisera revealed that VP1, VP2, VP3, VP4, VP6, VP7, VP8 and VP10 were common to both standard brv and the brv mutants. VP5, VP9, VP11 and VP12 were not

immunoprecipitated.

The rearranged RNA bands of hrv isolates (Pedley et al., 1984) had been found to replace normal standard brv genome segments structurally and functionally in reassortants (Allen and Desselberger, 1985). Gene reassortment studies were performed using mixed infection of the brv mutant D (containing RNA band A) and hrv A59. Selection and plaque purification of reassortants was done without exerting deliberate external selective pressures. Out of 36 plaque isolates, five were reassortants corresponding to 14% frequency of reassortment. Two reassortants had acquired hrv A59 segment 5 and lost the rearranged band A, showing that rearranged band A was genetically equivalent to normal hrv A59 segment 5. The other three reassortants had acquired segment 8 from hrv A59, and not lost RNA band A. When studied for plaque size, no significant changes were found and plaque reduction tests using antiserum raised against brv mutant D indicated that no changes in serotype were introduced by the reassorted hrv A59 genes. The genotypes of the reassortants were found to be stably maintained through several rounds of plaque-to-plaque purification. Viral proteins synthesised in cells infected with reassortants B320-2 and B317-1 contained a VP5 differing in size from that of the brv VP5 and likely to be coded for by the reassorted RNA segment 5 of hrv A59.

ABBREVIATIONS

ATP	adenosine triphosphate
brv	bovine rotavirus
bp	base pair
Bq	Becquerel (37MBq = 1mCi)
BSA	bovine serum albumin
Bis	N, N'-methylene bisacrylamide
CPE	cytopathic effect
cpm	counts per minute
CTP	cytidine triphosphate
DMSO	dimethyl sulfoxide
ds	double stranded
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis-(-amino ethylether)-N, N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
GBq	giga becquevel (= 27·027 mCi)
GTP	guanosine triphosphate
hr	hour
hrv	human rotavirus
IP	immunoprecipitation
Kb	kilobase
Kd	kilodalton
M	molar
MBq	mega becquevel
mg	milligramme
min	minutes
ml	millilitre
mM	millimolar
ug	microgramme
moi	multiplicity of infection
mRNA	messenger RNA
NP40	nonidetP40
ONM	oligonucleotide mapping
PAGE	polyacrylamide gel electrophoresis
pfu	plaque forming unit
pi	post infection
PMSF	phenyl methyl sulfonyl fluoride

POPOP	1, 4-di[2(5-phenyloxazolyl)] benzene
PPO	2, 5-diphenyloxazole
RIP	radio immunoprecipitation
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SAM	S-adenosyl methionine
SAll	simian rotavirus
SDS	sodium dodecyl sulphate
ss	single stranded
TBq	tetrabecquerel (10^{12} Becquerels)
tc	tissue culture
TEMED	N, N, N', N'-tetramethylethylene-diamine
TLC	thin layer chromatography
TPCK	L-1-tosylamide-2-phenlyethylchloromethyl ketone
TRIS	tris (hydroxymethyl) amino methane
ts	temperature sensitive
UTP	uridine triphosphate
v/v	volume:volume ratio
w/v	weight:volume ratio

INTRODUCTION

1 HISTORICAL PERSPECTIVE

The earliest reports of what could have been rotavirus gastroenteritis (diarrhoea) were in 1943 and 1949 by Light and Hodes who isolated a filtrable agent from diarrhoeic infants; they were able to demonstrate that the filtrable agent caused diarrhoea in newborn calves. This "agent" was later confirmed to have been a bovine rotavirus (Hodes, 1976). Subsequently, the viruses causing epizootic diarrhoea in infant mice (EDIM), (Cheever and Mueller, 1947; Kraft, 1957; Adams and Kraft, 1967; Banfield, 1968), in monkeys (simian SA 11 virus; Malherbe and Strickland-Cholmley, 1967) and calves (calf diarrhoea virus; Mebus 1969) were identified by electron microscopy of faecal samples. In Australia, Bishop et al. (1973) reported a virus similar to that found in calf diarrhoea (Mebus, 1969) in thin sections of duodenal biopsies taken from children who had gastroenteritis. From the United Kingdom (Flewett et al., 1973), Canada (Middleton et al., 1974), the United States (Kapikian et al., 1974) and Australia (Holmes et al., 1974; Bishop, et al. (1974) came reports which described rotaviruses as a major cause of infantile gastroenteritis .

Neonates are known to be susceptible to rotavirus infection but many infected children shed rotaviruses asymptomatically (Madeley and Cosgrove, 1975; Chrystie et al., 1975, 1978; Murphy et al., 1977; Madeley et al., 1978; Banatvala and Chrystie, 1978; Champsaur et al., 1984a,1984 b; Hoshino et al., 1985a; Besselaar et al., 1986).

Rotavirus infections occuring in premature infants (van Renterghem et al., 1980) took the course of mild gastroenteritis (Rocchi et al., 1981; Thomson et al., 1981). Special care babies were noted to develop symptomatic virus infections more than full term babies (Bishop et al., 1979). Children of school age (Hara et al., 1977) and those aged < 6 months to > 24 months (Follett et al., 1984) were found to suffer most from acute gastroenteritis. Adults could be experimentally infected with rotavirus (Middleton et al., 1974; Kapikian et al., 1983, 1985; Clark et al., 1986). Rotavirus gastroenteritis in adults can also occur under natural conditions (Ostavik et al., 1976; Zissis et al., 1976; Kim et al., 1977; Wenham et al. 1979; Hildreth et al., 1981; Echeverria et al., 1983; Hung et al., 1983, 1984) and epidemics of rotavirus diarrhoeal infection in normal adult populations have been described (von Bonsdorff et al., 1978; Lycke et al., 1978. Hung et al., (1983 , 1984) also reported epidemics of diarrhoea but caused by non-group A human rotavirus in adult populations of China. Rotavirus diarrhoea was reported in 70-90 year olds residing in nursing homes (Halvorsrud and Ostavik, 1980) and in cardiac patients (Holzel, 1980). The non-immune South American Indians living in isolated communities were found to be susceptible to rotavirus infections in all age groups (Linhares et al., 1981).

Rotaviruses are members of the Reoviridae family together with five other genera: orthoreoviruses, orbiviruses, phytoreoviruses Fiji viruses and cytoplasmic polyhedrosis viruses (Matthews, 1979; Joklik, 1983). Viruses reported in

the literature as EDIM (Kraft, 1957; Banfield et al., 1968), as Neonatal and Nebraska calf diarrhoea virus (Mebus et al., 1969, 1971), as orbivirus-like (Middleton et al., 1974), as duovirus (Davidson et al., 1975a), as reovirus-like (Kapikian et al., 1974; Flewett et al., 1974), and as infantile gastroenteritis virus (Petric et al., 1975) are all rotaviruses. The nomenclature commonly used to name rotaviruses after the host source does not strictly imply virus-host species specificity (Woode et al., 1976; Todd et al., 1976; Sato et al., 1982; Bridger and Brown, 1984 reviewed by Estes et al., 1983, 1985).

2 ROTAVIRUS MORPHOLOGY.

Morphology of the rotaviruses has been described from observations made by electron microscopy (E.M.) of negatively stained virus preparations (Flewett et al., 1973, 1974; Middleton et al., 1974). The E.M. appearance of rotaviruses isolated from human infants, calves, foals and pigs, lambs, mice and monkeys, were indistinguishable from each other (Kapikian et al., 1976a; Woode et al., 1976; McNulty et al., 1976b). The most common morphological forms of rotavirus particles were the double capsid and the single capsid virions. Complete rotavirus particles have a double layer capsid structure of 65-75nm diameter (Flewett et al., 1975; Woode et al., 1976; Palmer et al., 1977; Cohen et al., 1979) and appear "smooth" (Flewett et al., 1974; Holmes et al., 1975; Holmes, 1983). The single capsid virus particles appear "rough" because the virus particles lack the outer structural protein layer and

measure on average 55-65nm in diameter. Some preparations of tissue culture grown virus contained particles of varying size in addition to the double and single capsid virions (Chasey, 1977; Chasey and Labram, 1983). Tubular forms of rotavirus particles (rotatubes) of diameters 20 and 80nm were sometimes found in faecal samples (Holmes et al., 1975; Kimura and Murakami, 1977; Chasey and Labram, 1983) as well as in the tissue culture-grown virus samples (Banfield et al., 1968; Adam and Kraft, 1967; Altenburg et al., 1980; Suzuki et al., 1981; Chasey and Labram, 1983). The rotatubes were found to be made of inner capsid proteins (Kimura, 1981; Chasey and Labram, 1983). The exact geometry of the capsomeres making up the viral capsid layers has not been definitely worked out, but the available studies (Martin et al., 1975; Palmer et al., 1977; Stannard and Schoub, 1977; Esparza and Gil, 1978; Kogasaka et al., 1979; Roseto et al., 1979; Palmer and Martin, 1982) suggested that there are 132 capsomeres built or organised in trimeric/triangular units. The most recent studies (Gorziglia et al., 1985) suggest that there may be 132 holes surrounded by 260 trimeric morphological units resulting in a total of 780 protomeric molecules of VP6 per virion. Studies describing further structural proteins of the rotavirus particle are given below.

3 PHYSICO-CHEMICO PROPERTIES OF ROTAVIRUSES.

Rotavirus particles can be purified and separated by ultracentrifugation on CsCl gradients into the light (L) double capsid and the dense (D) single capsid form banding

at buoyant densities of 1.36 g/ml and 1.38 g/ml, respectively (Petric et al., 1975; Bridger and Woode, 1976; Kapikian et al., 1976a; Elias, 1977a; Cohen et al., 1979). The L particles have a sedimentation coefficient of 500-530S (Petric et al., 1975; Newman et al., 1975) and the D particles of 380-400S (Tam et al., 1976). The icosahedral cores which were described in human faecal extracts (Palmer et al., 1977) and were later found in tissue-culture grown bovine rotaviruses (Bican et al., 1982) had a buoyant density in CsCl of 1.44 g/ml and a sedimentation coefficient of 280S.

Heat treatment of rotavirus at 56°C for 30 minutes did not destroy virus infectivity or cause alteration in structure of the virus particles (Steinhoff, 1980). Estes et al. (1979a) found that SA11 lost infectivity by 10-90% per hour of incubation at 50°C, and this decline depended on virus diluent. Shirley et al. (1981) established that virus infectivity was stabilised in low concentrations of calcium chloride 0.15-1.5mM and of 1.5mM strontium chloride but not in 1mM magnesium chloride. Degradation of virion structure occurred by treatment with chaotropic agents such as sodium thiocyanate (Almeida et al., 1979), and 2M calcium chloride (Bican et al., 1982). Calcium chloride (1.5mM) was used to produce virus cores (Bican et al., 1982). Low concentration of 51mM EDTA converted the L to the D particle form by degrading the outer capsid layer through a chelating effect on calcium ions (Palmer et al., 1977; Cohen et al., 1979; McCrae and Faulkner-Valle, 1981), and the infectivity of the simian SA11 rotavirus was lost. Rotaviruses were shown

to be stable at room temperature after treatment with chloroform, freon, freeze and thaw, sonication and maintaining at pH 4-9 (Palmer et al., 1977; Estes et al., 1979a) also when exposed to 5-10mM EDTA and EGTA and at pH $\geq$ 10. While heating the SA11 at 50°C in the presence of high salt 2M magnesium chloride destroyed virus infectivity, similar heat treatment in 2M magnesium sulphate caused no observable changes in the virus infectivity (Estes et al., 1979). Both simian SA11 and bovine rotavirus (brv) were degraded by treatment with 95% alcohol (Bishai et al., 1978; Bastardo and Holmes, 1980). Tan and Schnagl (1981) found in addition that phenol rapidly inactivated simian SA11 rotavirus. Lamb rotaviruses were rendered non-infectious after 2 hours in 4-10% formalin (Snodgrass and Herring, 1977).

Treatment of rotaviruses with pancreatic enzymes trypsin, pancreatin, elastase was associated with enhancement of virus infectivity as a result of enzyme cleavage of the outer capsid protein VP3 in SA11 (Graham and Estes, 1980; Estes et al., 1981) or the VP4 in brv (Clark et al., 1981; Espejo et al., 1981; Novo and Esparza, 1981). Elias (1977a) and Rodger et al. (1977) used amylase and α-glycosidase treatment which degrade the capsid glycoproteins thereby reducing the double (L) to single capsid particles and showed that rotavirus infectivity was dominantly in the L particles.

4 ROTAVIRUS GENOME

The genomes of brv (Welch, 1971), EDIM (Munch and Zajac,

1972) and human rotavirus (Petric et al., 1975) were the first described to contain ribonucleic acid (RNA). The genomic RNAs were double stranded (ds) (Welch and Thompson, 1973; Petric et al., 1975; Rodger et al., 1975; Todd and McNulty, 1976) and further studies using electrophoretic separation through polyacrylamide gels (RNA-PAGE) demonstrated that the rotavirus genome consisted of 11 segments of dsRNA (Newman et al., 1975; Rodger et al., 1975; Kalica et al., 1976, 1978; Verly and Cohen, 1977). The patterns exhibited by the rotavirus genomic segments were named electropherotypes (Lourenco et al., 1981; Schnagl et al., 1981; Rodger et al., 1981; Follett et al., 1984; Flores et al., 1985, reviewed; Estes et al., 1984b). The distribution of these segments after gel electrophoresis (RNA profile) is highly variable but certain size classes can be distinguished (Kalica et al. 1976; Barnett et al. 1978; Lourenco et al., 1981). The four size classes, I-IV, represent RNA segments 1-4; 5 and 6; 7, 8, 9; and 10 and 11 (Kalica et al, 1976).

Soon it was realised that this formulation only applied to the group A rotaviruses (Pedley et al., 1983, 1986), and heterogeneity among rotavirus genomes gained yet another dimension with the emergence of so called atypical or pararotaviruses (McNulty et al., 1981; Bohl et al., 1982; Bridger et al., 1982; Rodger et al., 1982; Dimitrov et al., 1983; Nicolas et al., 1983; Hung Tao et al., 1983, 1984; Snodgrass et al., 1984a; Chasey and Banks, 1986). The genomes of the atypical rotaviruses exhibit RNA electropherotypes which differ in profile to those of the

conventional group A (Pedley et al., 1983, 1986) if considered in the grouping format of Kalica et al. (1976) and Barnett et al. (1978). Genomes of rotaviruses studied to date have been grouped into groups A to E (Pedley et al., 1983; Pedley et al., 1986) using serological criteria and the one-dimensional terminal fingerprinting technique (Clarke and McCrae, 1981, 1982). McCrae and McCorquodale, (1983) showed that group A rotavirus genomes were conserved in the terminal 40 nucleotides, irrespective of electrophoretic mobility shifts or species of origin of the virus. In this technique, RNase-T_1 was used to cleave the in-vitro 3' end-labelled dsRNAs at the guanine residues followed by separating the fragments by gel electrophoresis. When the rotavirus group B and C were compared with virus group A using the same terminal fingerprinting technique (Pedley et al., 1983) it was found that there was a terminal 10 nucleotides region of absolute conservation and that this region was unique for each of the viruses in group A, B, C and group D (Pedley et al., 1986). The region spanning 10-40 nucleotides from the termini was diagnostic and conserved for all 11 dsRNAs segments of viruses within groups A, C and D. The terminal fingerprint patterns of RNA segments of rotaviruses in groups D and E differed from those of the corresponding RNA segments of group A viruses and from each other (Pedley et al., 1986). The group B virus genome did not reveal any confirmed conservation in the terminal region of 10-40 nucleotides (Pedley et al., 1983). Gel electrophoretic mobility of RNA segments was also used to classify group A rotavirus genomes as "long" and "short" electropherotypes

with respect to the relative migration of RNA segment 10 and 11 (Espejo et al., 1979, 1980a; Dyall-Smith and Holmes, 1981a; Kalica et al., 1981; Rodger et al., 1981; Schnagl et al., 1981; Dimitrov et al., 1984; Follett et al., 1984).

Extensive genomic diversity was described in group A rotaviruses isolated from man in various parts of the world: Australia (Schnagl and Holmes, 1976; Rodger and Holmes, 1979; Rodger et al., 1981); France (Lourenco et al., 1981; Nicolas et al., 1983, 1984); USA (Kalica et al., 1978; Dimitrov et al., 1984); UK (Clarke and McCrae, 1981; Beards, 1982; Follett and Desselberger, 1983a; Follett et al., 1984; Pedley et al., 1984); Latin America (Espejo et al., 1979, 1980a, 1980c; Spencer et al., 1983; Pereira et al., 1983); New Zealand (Croxson and Bellamy, 1979; Street et al., 1982); Japan (Matsuno and Nakajima, 1982; Konno et al., 1984). Genomic heterogeneity/polymorphism was also found in field isolates of brv (Verly and Cohen, 1977; Sabara et al., 1982a; Schroeder et al., 1982), of avian rotaviruses (Todd et al., 1980) and of simian SA11 rotavirus (Pereira et al., 1984).

Rotaviruses possessing genomes with apparently more than 11 genomic segments were isolated from two chronically infected children who suffered from severe combined immunodeficiency [SCID] (Pedley et al., 1984). In this study rotaviruses were isolated from one child over a 7 week period and from another over $8^1/_2$ months. In one case the RNA profile was missing a genomic segment 11 and extra bands of dsRNAs were distributed between the normal RNA segments 1

and 7. The abnormal RNA bands when hybridised to segment-specific cDNA probes (McCrae and McCorquodale, 1982), showed that some of the extra bands contained sequences derived from genomic segments. Dolan et al. (1985) documented another case of chronic hrv infection in an immunodeficient child. The genomes of isolates contained extra bands of dsRNA which were lost except one when the virus was adapted to tissue culture. Similar observation were reported by Eiden et al. (1985). Recently, rotaviruses sharing the common group A antigen, but possessing an abnormal RNA profile were isolated from asymptomatic neonates born in Johannesburg over a period of 4 months: RNA segment 11 was missing from the profile but an additional band of genomic RNA was found migrating near RNA segment 5 (Besselaar et al., 1986).

Recently Hundley et al. (1985) described genome rearrangements of the genome of the tissue culture adapted brv after serial passage at high m.o.i.. After each passage the virus yield was analysed by RNA-PAGE and silver staining. Mutants were recovered and plaque-purified which had lost the genomic RNA segment 5 but carried segment 5-specific sequences in concatemeric form migrating between segments 1 and 6 (Hundley et al., 1985).

The size of the rotavirus genome was initially estimated from gels using RNA segments of the reovirus genome as size markers: the molecular weights of the 11 rotavirus RNA segments were found to be between 0.2 x 10^6 and 2.2 x 10^6 daltons, resulting in a size of 11 x 10^6 to 12 x 10^6 daltons for the total genome (Welch, 1971; Much and Zajac, 1972; Petric et

Table A

Molecular weights of rotavirus RNAs obtained by gel electrophoresis of RNAs and cDNAs, and by electron microscopy.

Molecular weight x 10^6 (number of bp in column 6, 7 & 8 are in parenthesis)

Segment Number	brv 1 Newman et al. 1975	brv 2 Verly & Cohen 1977	hrv 3 Kalica et al. 1978	brv 4 Todd et al. 1980	SAll 5 Rodger & Holmes 1982	SAll 6 Both 1982	brv 7 Holmes 1983	brv 8 Rixon et al. 1984	Nucleotide sequences
1	2.2	2.2	1.89	2.19	2.05	2.46(3720)	2.17(3300)	2.25(3409)	
2	1.9	1.8-1.85	1.72	1.79	1.68	2.15(3650)	1.72(2600)	1.81(2737)	
3	1.9	1.70-1.73	1.65	1.79	1.60	2.08(3150)	1.68(2550)	1.75(2653)	
4	1.7	1.55-1.60	1.61	1.59	1.60	1.87(2830)	1.56(2370)	1.56(2359)	2244a
5	0.9	0.44-1.00	1.18	0.97	0.98	1.07(1620)	1.52(1550)	1.00(1519)	
6	0.8	0.78-0.82	1.10	0.75	0.81	0.89(1350)	0.88(1340)	0.89(1351)	1356b
7 }							(1050)		1059c
8 }	0.5	0.51	0.86	0.49	0.50	0.73(1100)	0.69(1050)	0.73(1099)	1062d
9 }							(1050)		1076e
10	0.3	0.26	0.63	0.26	0.30	0.50 (765)	0.50 (760)	0.48 (721)	751f
11	0.2	0.20	0.52	0.20	0.20	0.44 (660)	0.45 (650)	0.42 (637)	663g
Total Mol Wt	11.4		12.88	11.01	10.72	13.65	12.08	12.35	

(a) Lopez et al. (1985; strain SAll), partial sequence
(b) Cohen et al. (1984; strain brv, RF); Estes et al. (1984a; strain SAll)
(c) Dyall-Smith et al. (1983a; strain brv)
(d) Ellerman et al. (1983; strain brv)
(e) Ward et al. (1984a; strain brv)
(f) Both et al. (1982; SAll); Baybutt and McCrae (1984; strain brv) Ward et al. (1985); Okada et al. (1984; strain Wa)
(g) Imai et al. (1983; hrv, Wa strain); Ward et al. (1985; strain brv)

al., 1975; Rodger et al., 1975; Newman et al., 1975; Verly and Cohen, 1977; Todd et al., 1980). Under the denaturing conditions of methyl mercury-agarose gels, Bernstein and Hruska (1981) measured the genomic transcripts of brv to range from 0.39 x 10^6 to 1.4 x 10^6 and those of the simian rotavirus genome from 0.40 x 10^6 to 1.7 x 10^6; in both estimates ribosomal RNAs were used as a size marker. Rodger and Holmes (1982), using reovirus RNA size markers, found RNA segment sizes ranging from 0.2 x 10^6 to 2.05 x 10^6 daltons for the SA11 virus with a total genome size of 10.72 x 10^6 daltons. The genome size of hrv was 12.89 x 10^6 daltons, the genomic segments ranging between 0.52 x 10^6 and 1.89 x 10^6 daltons (Kalica et al., 1978). Further variations in genome size measurements were evident in the study of Both et al. (1982) in which cDNA copies of the simian rotavirus genome were estimated against pBR322 fragments as size markers: the size of the total genome was 13.65 x 10^6 daltons and the genomic segment sizes were between 0.44 x 10^6 and 2.46 x 10^6 daltons. Slightly different results were reported by Holmes (1983). Rixon et al. (1984) determined the genome size of brv (UK Compton strain) by electron microscopy using ØX174 RF DNA as a standard and found that individual RNA segments measured between 0.42 x 10^6 daltons and 2.25 x 10^6 daltons and the total brv genome size was 12.35 x 10^6 daltons. A detailed comparison of the different results is shown in Table A, which also contains sizes obtained from direct sequencing.

The observed differences in the reported sizes of rotavirus RNA segments is less a reflection of sequence

heterogeneity and the size differences of corresponding segments than of various techniques and different size markers used for the estimates. Rotavirus genomic RNA segments are monocistronic and when translated produce proteins of size corresponding in order to the molecular sizes of the coding segments. There are exceptions of inversions in the region of RNA 7, 8 and 9 (Table B); and of the reversed coding potential of RNA segment 10 and 11 of the "short" electropherotypes (Dyall-Smith and Holmes, 1981a).

Homology of rotavirus RNA sequences is known to conform to functional similarities regardless of differences in RNA migration (RNA mobility) on polyacrylamide gels (Dyall-Smith et al., 1981a; McCrae and McCorquodale, 1982; Flores et al., 1982a, 1982c, 1985; Schroeder et al., 1982; Street et al., 1982) or the animal source (Dyall-Smith et al., 1983a; (Dyall-Smith and Holmes, 1984). Hence it has been shown that a cDNA copy of brv gene 8 when used as a probe, recognised genes 9 of hrv (Wa strain) and the SA11 on Northern blots (Dyall-Smith et al., 1983a). Vice versa, the cDNA clones of gene 9 from hrv (Wa strain) and SA11 rotavirus selected RNA segment 8 of brv and of hrv (HU/5 strain) as their homologous counterpart (Ellerman et al., 1983; Dyall-Smith et al., 1983a). Depending on the rotavirus strain under study, the serotype specifying gene may be either the RNA segments 8 or 9 which both code for a VP7.

The sequence of gene 8 coding for structural glycoprotein VP7 of brv [UK Compton strain] (McCrae and

McCorquodale, 1982) was 1062bp long and capable of coding for a protein of 326 amino acids (Ellerman et al., 1983). The predicted amino acid sequence of the brv VP7 contained three glycosylation sites of the form asparagine-X serine or asparagine-X-threonine in residues 69-71, 238-240 and 318-320. Overall the VP7 amino acid sequence had a preponderance of acidic to basic amino acid residues in a ration 32:24 (Ellerman et al., 1983). Hydropathy measurements displayed strong hydrophobic N-terminal region at amino acid residues 6-23 and 37-44 (Ellerman et al., 1983). The regions of hydrophobicity were thought to contain the signal sequences which play a part in translocation of the glycoprotein VP7 across cell membrane during maturation of virus particles (Petrie et al., 1982). The hydrophilic regions indicated possible locations of antigenic determinants at amino acids residues 95-100, 179-183, 251-256 and 311-316. However, an attempt to produce antibodies directed against these potential regions of antigenicity resulted in antipeptide sera of the neutralising titres close to 1:10 and in most cases no neutralising reactivity was demonstrable (Gunn et al., 1985). It appears that antigenicity predicted from linear amino acid sequence of VP7 does not influence the immunogenicity associated with the protein to any desirable degree.

A comparison of amino acid sequences predicted from the nucleotide sequences of genes coding for the glycoprotein VP7 of the brv (NCDV strain, serotype 6), hrv (Wa strain, serotype 1), hrv (HU/5, serotype 2) and the simian SA11

(serotype 3) displayed 75-86% homology (Glass et al., 1985). A notable divergence was identified at amino acids 82-102 which was thought to confer serotype specificity (Glass et al., 1985).

Potential glycosylation sites were found at amino acid residues 69-71 in SA11 gene 9 (Both et al., 1983a), three sites in HU/5 genome at residues 69, 146 and 238 (Dyall-Smith and Holmes, 1984) and three sites at amino acid residues 69, 238 and 318 were revealed in the sequence of brv (UK) glycoprotein gene (Ellerman et al., 1983 and Gunn et al., 1985). A comparison of the glycoproteins VP7 from brv (UK strain), simian SA11 and the hrv (Wa strain) by (endo H) endoglycosidase digestion appeared to support the view that each of the predicted glycosylation sites were used in the synthesis of mature glycoproteins (Kouvelos et al., 1984). All structural glycoproteins VP7 irrespective of virus strain and source, have been shown to carry serotype specific antigen responsible for eliciting virus neutralising antibodies (Kalica et al., 1981; Greenberg et al., 1981, 1983; Bastardo et al., 1981; Killen and Dimmock, 1982; Coulson et al., 1985; Offit and Clark, 1985; Hoshino et al., 1985b; Offit and Blevat, 1986).

The RNA segment 6 codes for the major inner capsid protein VP6 of 41-45K MW (Thouless, 1979; McCrae and Faulkner-Valle, 1981; Arias et al., 1982; McCrae and McCorquodale, 1982) which bears the rotavirus specific subgroup (common) antigens (Bridger, 1978; Greenberg et al., 1981; Thouless et al., 1982). The VP6 is a trimeric protein (Novo and Esparza, 1981; Bican et al., 1982; Gorziglia et al., 1985)

Monoclonal antibody directed against the common antigen VP6 was used to group the typical rotaviruses into subgroups 1 and 2 (Greenberg et al., 1983; Wyatt et al., 1983a; Follett et al., 1984; Hoshino et al., 1985a). However, the subgrouping did not apply to the atypical rotaviruses which lack the common group antigen and are now reported with increasing frequency (Saif et al., 1980; Bridger et al., 1982; Bohl et al., 1982; McNulty et al., 1981; Nicolas et al., 1983; Dimitrov et al., 1983; Pereira et al., 1983; Hung Tao et al., 1983, 1984; Espejo et al., 1984; Snodgrass et al, 1984).

A complete sequence of genomic RNA segment 6 of simian rotavirus was determined from cDNA copy of mRNA and was found to be 1357bp long (Estes et al., 1984a). This nucleotide sequence differs from that of the RNA segment 6 of brv (RF strain; Cohen et al. 1984) which is 1356bp long by one nucleotide at 1323bp in the 3'-non coding region. Gene 6 codes for a polypeptide of 397 and 396 amino acids length in the SA11 virus (Estes et al., 1984a) and brv [RF strain] (Cohen et al., 1984), respectively. VP6 contains five hydrophilic antigenic determinants. The authors did not mention a potential glycosylation site located between 917-926bp covering amino acid residues 299-301. The SA11 and brv nucleotide sequences were 87% homologous while the amino acid sequences had 97% homology (Cohen et al., 1984; Both et al., 1984).

The sequence of the RNA segment 7 of brv [UK strain] (Dyall-Smith et al., 1983b) was found to be 1059bp long in one reading frame, coding for a protein of 33Kd size and was

identical in size to the protein coded by RNA segment 8 of the simian SA11 rotavirus (Both et al., 1982) which consisted of 317 amino acids. Sequence homology between the RNA segment 7 of brv (UK) and that of simian SA11 rotavirus RNA segment 8 was 88% and the amino acid homology between the two predicted proteins was 96% (Both et al., 1982). In view of the well documented phenomenon that genes coding for non-structural proteins are less likely to be influenced by host-immune system (Scholtissek, 1978) it is no wonder that conservation of such genes of two different viruses has been maintained. No biological function has yet been assigned to the non-structural proteins 33Kd.

The sequence of RNA segment 11 of hrv (Wa strain) was found to be 663bp long (Imai et al., 1983). The deduced amino acid sequence is that of a protein of 21.6Kd and contains a high proportion of serine (18%). There are 7 potential glycosylation sites in the amino acid sequence. No biological function is known for the gene product of RNA segment 11.

A full length ds cDNA copy of UK brv gene 9 which codes for a non structural protein, VP9, was found to be 1076 nucleotides long (Ward et al., 1984a). The segment 9 had a coding capacity of 313 amino acids corresponding to a protein of 36Kd and the VP9 was speculated to have functions of enzyme replicase[Ward et al 1984a].

The nucleotide sequence of the RNA segment 10 of the brv (UK strain) was found to be 751bp long (Baybutt and McCrae, 1984), and to code for a protein 175 amino acids long.

There is a striking feature of a long 184bp non-coding region at the 3' end. Hydropathy profile of the predicted amino acids revealed that half of the amino acids were in hydrophilic regions and the other half was hydrophobic. The RNA segment 10 of brv (UK Compton) was equivalent to the RNA segment 10 of SA11 rotavirus and both segments were identical in their coding capacities, sequence length and untranslated regions. Two sites of potential glycosylation of the form asparagine-X-threonine/serine were found to be located at amino acid residues 8 and 18 close to the N-terminus within the region containing the uncharged amino acids 7-21. The RNA segment 10 specifies the synthesis of NCVP5 (Arias et al., 1982) in SA11 rotavirus from its precursor pNCVP5 equivalent to the VP10 derived from precursor VP12 of brv (McCrae and McCorquodale, 1982). On tunicamycin treatment, the VP10 was shown to contain N-linked oligosaccharides (McCrae and Faulkner-Valle, 1981; Arias et al., 1982; Sabara et al., 1982b; Both et al., 1983b) which indicated that the N- terminal sequences 7-21 were not cleaved (like a signal sequence) during the translocation (transport) of NCVP5/VP10 through the cell membrane. The VP10 is an outer capsid protein (Thouless, 1979). Kinetics of virus adsorption were described for brv (Sabara and Babiuk, 1984) in which it was suggested that VP10 was a factor influencing and probably controlling virus adsorption. As it was found that virus-cell attachment was restricted by a monoclonal antibody directed against an epitope on VP7, it was assumed that tissue tropism mediated through virus adsorption was a shared property of two gene

products, the glycoproteins VP10 and VP7 (Sabara et al., 1985).

Biologically important proteins have been assigned functions, although their coding RNA segments are not sequenced. In this context, the RNA segment 4 which codes for the VP4 (88Kd) of brv (UK Compton strain) (McCrae and McCorquodale, 1982) and for VP3 of SA11 rotavirus (Mason et al., 1980, 1983; Smith et al., 1980) was thought to be responsible for promotion of growth in tissue culture (Kalica et al., 1981; Greenberg et al., 1981, 1982). The RNA segment 4 was also shown to specify for protease-enhanced plaque formation (Kalica et al., 1983); and is the basis of trypsin-enhanced virus infectivity described for rotavirus (Graham and Estes, 1980; Sato et al., 1981; Urasawa et al., 1981; Espejo et al., 1981; Estes et al., 1981; Clark et al., 1981). A partial sequence of the SA11 gene 4 product (VP3) was produced to show the trypsin cleavage site (Lopez et al., 1985) see below. In reassortant experiments RNA segment 4 of brv was found to segregate with the haemagglutination function (Kalica et al., 1983). Other studies demonstrated that some bovine (Inaba et al., 1977; Fauvel et al., 1978); simian (Kalica et al., 1978); simian and canine (Hoshino et al., 1983); and human rotaviruses (Kitaoka et al., 1984) also could agglutinate human and other animal erythrocytes. Gene 4 product specifies neutralisation antigens which can differ from the serotype specificity of the neutralising VP7 (Hoshino et al., 1985b; Offit et al., 1986; Offit and Blavat, 1986). Kalica et al. (1983) described a

ts mutant of brv with a lesion in RNA segment 1. Otherwise genomic RNA segments 1, 2, 3 and 5 have not been either sequenced or assigned any functions.

5 ROTAVIRION STRUCTURAL PROTEINS

The proteins comprising the structure of rotavirion were studied using purified rotaviruses derived from faecal samples of cattle (Rodger et al., 1975; Newman et al., 1975; Bridger and Woode, 1976), tissue culture grown brv (Cohen et al., 1979; Matsuno and Mukoyama, 1979; Thouless, 1979; McCrae and Faulkner-Valle, 1981; Novo and Esparza, 1981; Bican et al., 1982), lamb rotaviruses (Todd and McNulty, 1977), simian rotavirus SA11 (Kalica and Theodore, 1979; Mason et al., 1980; Estes et al., 1981) and faecal extracts of hrv (Obijeski et al., 1977; Thouless, 1979). All these studies agreed to a certain extent on the polypeptide composition of the different rotaviruses but there were discrepancies owing to differences in resolution of SDS-PAGE systems, to the use of heterogeneous virus population and/arising from exposure of virus to proteolytic enzymes before SDS-PAGE analysis. The nomenclature of viral proteins has presented difficulties because it is based on non-uniform methods of analysis. The use of molecular weights as measured from relative migration of proteins in polyacrylamide gels showed variations in the same protein of different rotavirus strains, or of identical strains if the findings came from different laboratories (Rodger et al., 1977; Thouless, 1979) (See Table 9.1)

The structural proteins make up the inner and outer

capsid layers of the rotavirions D and L respectively (Cohen et al., 1979) but it is not documented whether or not rotaviruses have RNA associated proteins.✳ In the case of the simian rotavirus, the inner capsid proteins include VP1, VP2 and VP6, and the outer capsid proteins were VP3 and VP7, of which only VP7 is glycosylated (Mason et al., 1981, 1983; Estes et al., 1983). The simian SA11 rotavirus proteins translated from RNA segments 4, 7, 8 and 10 were designated non-structural, NS (Ericson et al., 1982). There is some argument about the location of the simian SA11 rotavirus protein VP2 and VP3 of molecular sizes of 96Kd and 91KD, respectively (Smith et al., 1980): whereas Arias et al. (1982) reported that VP3 was an inner capsid protein coded on the RNA segment 3; Smith et al. (1980), Mason et al. (1983) reported VP3 as product of gene 4. Also Smith et al. (1980) found that VP4 (84Kd) was an inner capsid protein but later on Arias et al. (1982) working on the same simian SA11 rotavirus described a product of gene 4 as a non-structural protein NCVP1. Also from segment 4, a protein of a low molecular weight (13Kd) was synthesised in-vitro but this size of polypeptide could not be detected in infected cells (Arias et al 1982). For brv, the structural proteins included the inner capsid proteins VP1, VP2, VP3, VP6 and VP8 and the outer capsid proteins VP4, VP7, VP10 and VP11 (McCrae and Faulkner-Valle, 1981; Novo and Esparza, 1981). The status and localisation of the VP5, a product of RNA segment 5 (McCrae and McCorquodale, 1982) has continued to be controversial. Several authors (Bridger and Woode, 1976; Rodger et al., 1977; Matsuno and Mukoyama, 1979;

Thouless, 1979) report that brv VP5 was associated with the double capsid virus particles and was therefore an outer capsid (structural) protein. The observation contrasted sharply with the findings of Newman (1975), Cohen et al. (1979), Novo and Esparza (1981), Clark et al. (1981), McCrae and Faulkner-Valle (1981) who did not find the VP5 in either double or single capsid rotavirions but these authors did not designate VP5 a non-structural protein either. It has since been shown that VP3 (88Kd) of SA11, the analogue of VP4 of brv, is cleaved by trypsin to release polypeptides of ~ 62Kd and 28Kd (Lopez et al., 1985) and this is the mechanism for trypsin-enhanced infectivity (virus "activation"). There is general agreement on VP9 and VP12 being non-structural proteins. In studies of McCrae and Faulkner-Valle (1981), Killen and Dimmock (1982) the viral proteins VPX, an inner capsid, and VPY, an outer capsid protein were described. Both viral proteins were found migrating between VP4 and VP5 of the protein profiles of the virions which had been produced or exposed to trypsin prior to SDS-PAGE. The presence of these structural proteins VPX and VPY was not shown in brv infected cells nor has it been possible to identify the RNA segments coding for them (McCrae and McCorquodale, 1982). Post translational glycosylation of VP2, VP6, VP7 and VP10 was described in brv infected cells (Baybutt and McCrae, 1984), and that of VP7 was shown to occur in and around the structures of the endoplasmic reticulum [e.r.] (Petrie et al., 1982). During the process of glycosylation, the rotavirus particles acquired transient envelope which was thought to aid the

virus particles budding through the e.r. membranes (Saif et al., 1978; Altenburg et al., 1980).

Depending on the virus strain studied, the number of glycoproteins vary; for instance simian SA11 rotavirus in addition to VP7 has one non-structural glycoprotein 28K (VP10) (Estes, 1982; Ericson et al., 1982, 1983). The brv contained the VP7, on outer capsid protein according to Cohen et al. (1978), Matsuno and Mukoyama (1979), McCrae and Faulkner-Valle (1981), Sabara et al. (1982b) and Kouvelos et al. (1984). The brv also carries a glycosylated VP10 (Cohen et al., 1979; McCrae and Faulkner-Valle, 1981; Sabara et al., 1982b; Baybutt and McCrae, 1984) but there are brv strains which do not have VP10 glycosylated (Kouvelos et al., 1984). Viral proteins VP7 and VP10 of the simian SA11 rotaviruses (Petrie et al., 1983a) and of bovine rotavirus (Sabara et al., 1982b) were shown to influence virus replication, in a way that when N-linked glycosylation was inhibited with tunicamycin (Heifetz et al., 1979) the yield of infectious virus was reduced by 4 log units compared to the untreated virus. The VP11 of brv was found to be sensitive to tunicamycin (McCrae and Faulkner-Valle, 1981) and although no sugar labelling of the protein has been demonstrated, it may be a minor N-linked glycoprotein. However, VP2 and VP6 were insensitive to tunicamycin and should be considered to be glycosylated via O-glycosidic linkages.

The sensitivity of the viral glycoproteins to endo--N-acetylglucosidase H [Endo H] (Tai et al., 1977) was used to conclude that the glycoproteins were N- linked

mannose-rich oligosaccharides (Kouvelos et al., 1984). Glycosylation could be demonstrated using radiolabelled glucosamine and mannose but was not found when radioactive galactose or fucose were offered to infected cells (Ericson et al., 1982). Mannose had been suggested to form the terminal residue (Cohen et al., 1978) in a glycoprotein containing seven mannoses [$(Man)_7$]. In addition other forms ranging from $(Man)_5$ to $(Man)_9$ have been found in the simian SA11 rotavirus glycoproteins (Both et al., 1983b) and in brv (Kouvelos et al., 1984). The structural glycoprotein VP7 of all the rotaviruses studied to date bears the serotypic specific/neutralising antigens (Kalica et al., 1981; Bastardo et al., 1981; Greenberg et al., 1981, 1983; Killen and Dimmock, 1982; Coulson et al., 1985). Sabara et al. (1985) demonstrated that there was an epitope determining virus attachment to host cell located on the major neutralising antigen. Evidence is mounting that VP2 and VP6 of the brv [Compton strain] (Baybutt and McCrae, 1984) are glycosylated by a process not sensitive to tunicamycin, an inhibitor of N- linked glycosylation (McCrae and Faulkner-Valle, 1981; Sabara et al., 1982b; Ericson et al., 1982; Petrie, 1983a). At the time, Petrie (1983b) explained the incorporation of tritiated mannose in VP2 and VP6 as due to randomisation of label. Recent investigations (Baybutt and McCrae, 1984) revealed consistent specific sugar labelling in VP2 and VP6.

6 DETECTION OF ROTAVIRUSES

Much of the rotavirus diagnosis has been done by E.M.

and this method of directly visualising virus particles continues to be a standard technique for identification of rotaviruses (Flewett et al., 1973; Bishop et al., 1974; Middleton et al., 1974; Kapikian et al., 1974; Davidson et al., 1975a; Madeley et al., 1978; Brandt et al., 1981, 1983; Bridger 1980; Follett and Desselberger, 1983a), especially in the case of atypical rotaviruses. Immune electron microscopy (IEM) uses E.M. grids coated with protein A, and specific rotavirus antibody was used to bind rotavirus particles (Nicolaieff et al., 1980). The method was demonstrably useful and is an improvement on the direct E.M. in that it has a built-in specificity. Rotaviruses were identified by fluorescent virus precipitin test [FVPT] (Peterson et al., 1976; Watanabe et al., 1978) in which a specimen was reacted with rotavirus antibody-fluorescein conjugate, and the virus-antibody complexes (aggregates) were visualised by fluorescence microscopy. The method had a sensitivity similar to direct E.M. (Foster et al., 1975). Detection of rotaviruses in frozen sections of gut of experimental animals and in tissue culture cells was tried by indirect immunofluorescence (IF) (Barnett et al., 1975; Bridger and Woode, 1975; Banatvala et al., 1975; Davidson et al., 1975b; Snodgrass et al., 1977; Saif et al., 1978; Coelho et al., 1981) but the method had limited uses in diagnostic virology because the hrvs often failed to replicate in tissue culture. An improved IF method of Bryden et al. (1977) on microtitre plates was able to score 31 out of 35 E.M. positive specimens. At present, serological survey and studies utilise the enzyme-linked

immunosorbent assay (ELISA) kits currently available from commercial sources. The kits contain rotavirus IgG coupled to plastic beads which can bind virus from patient's specimen on tissue culture; the complex is detectable by an enzyme conjugate of anti rotavirus IgG in the presence of enzyme substrate. A radioimmunoassay (RIA) utilising iodinated antirotavirus IgG was described by Birch et al. (1979). When ELISA was compared with IF, E.M., RIA, it was found that ELISA offered more speed, simplicity and acceptable sensitivity (Cukor et al., 1978a; Birch et al., 1979; Brandt et al., 1981; Zissis and Lambert, 1980; Yolken et al., 1978; Grauballe et al., 1981; Yolken and Leister, 1981; Kalica et al., 1981; Rubenstein and Miller, 1982). Limitations found in using the enzyme immunoassays arise from the presence of protein A in faecal samples (Brandt et al., 1981, 1983) which contributes to false positivity of samples. ELISA negative but E.M.-positive samples mostly contain atypical rotaviruses (Bohl et al., 1982; Nicolas et al., 1983; Rodger et al., 1982; Pedley et al., 1983; Snodgrass et al., 1984a; Hung Tao et al., 1983, 1984; Theil and Saif, 1985; Chasey and Banks, 1986) which lack the group specific antigen detected in ELISA.

Rotavirus infections have been diagnosed by the complement fixation test (CFT). The test could detect rotavirus antibody (Kapikian et al., 1974, 1975, 1976b; Blacklow et al., 1976; Gust et al., 1977; Matsuno et al., 1977b) provided the viral infection was less than one year old (Madeley et al., 1983). CFT has false negative results because of anticomplementary activity of patient's

sera (Spence et al., 1975).

Agglutination of human 'O', guinea pig and one-day-old chicken red blood cells is mediated by antigenic epitopes specified by the product of gene 4 of rotaviruses (Spence et al., 1976; Inaba et al., 1977; Fauvel et al., 1978; Kalica, 1978, 1983; Bastardo and Holmes, 1980; Hancock et al., 1983; Hoshino et al., 1983; Kitaoka et al., 1984). Haemagglutination inhibition test (HAI) could distinguish bovine from simian rotaviruses (Spence et al., 1978) but type specificity carried on the VP7 (major structural glycoprotein) was not demonstrable for human rotaviruses in this test (Kitaoka et al., 1984).

More recently rotaviruses have been identified by genome analysis using gel electrophoresis (RNA-PAGE) (Espejo et al., 1979, 1980a; Lourenco et al., 1981; Rodger et al., 1981; Schnagl et al., 1981; Follett and Desselberger, 1983a; Follett et al., 1984; Dimitrov et al., 1984). RNA-PAGE is specific because the rotavirus genome is characteristic (Matthews, 1979) and avoids false positives. The silver staining technique has a high sensitivity: 10-20ng of total viral RNA can be visualised (Herring et al., 1982; Follett and Desselberger, 1983a).

7 VIRUS REPLICATION

Various tissue culture systems have been used to study growth characteristics of rotaviruses: SAll rotavirus (Malherbe and Strickland-Cholmley, 1967), brv (Fernelius et al., 1972; Mebus et al., 1971; McNulty et al., 1977; Welch and Twiehaus, 1973; Bridger and Woode, 1975), porcine

rotavirus (McNulty et al., 1976b; Theil et al., 1978) and avian rotavirus (McNulty et al., 1979). Initially, it was only possible to propagate human rotavirus by serial passages in piglets before continuing the virus adaptation on cell cultures as was the case for the Wa strain of hrv (Wyatt et al., 1980).

Direct isolation of virus from specimens was aided by recognition that pancreatic enzymes increased infectivity of rotaviruses (Babiuk et al., 1977; Babiuk and Mohamed, 1978; Theil et al., 1978; Bryden et al., 1977; Almeida et al., 1978; Barnett et al., 1979; Clark et al., 1979, 1981; Schoub et al., 1979; Begin, 1980; Graham and Estes, 1980; Theil and Bohl, 1980; Estes et al., 1981; Sato et al., 1981). Since then, hrvs have become amenable to isolation in cell cultures using trypsin-activated virus to infect cells and maintaining the infection in the presence of low concentration of trypsin in roller cultures at 37°C in an atmosphere of 5% CO_2 (Sato et al., 1981; Urasawa et al., 1981; Hasegawa et al., 1982; Ward et al., 1984b). At present, the most successful isolation of hrvs is reported to occur in primary African green monkey kidney cells, and to a lesser extent in a continuous cell line derived from foetal rhesus monkey kidneys [MA104 cells] (Murakami et al., 1981; Kutsazawa et al., 1982a; Taniguchi et al., 1982; Wyatt et al., 1983a; Birch et al., 1983; Albert and Bishop, 1984; Ward et al., 1984b; Hoshino et al., 1985a). Without proteolytic enzyme treatment, hrv and porcine, chicken and turkey rotaviruses only undergo a very limited replication whereas feline, canine, simian SA11 rotaviruses

and some strains of brv were observed to go through several rounds of replication but produced largely non-infectious virus particles (Hoshino et al., 1981; Clark et al., 1981). Growth curves constructed for brv (UK) at 39, 37 and 31°C showed that infectious virus was produced at these temperatures (McCrae and Faulkner-Valle, 1981).

During early stages of infection, the virus is first adsorbed, then endocytosed and sequestered into cytoplasm and cytoplasmic lysosomes where uncoating is said to take place. An eclipse phase of 2-3 hours pi occurs before a virus begins to be detected (Petrie et al., 1982; Suzuki et al., 1981, 1985). Replication of animal rotaviruses after infection at low m.o.i. showed that the replicated genomic RNAs maintained the same genotypes when analysed by RNA-PAGE. Studies on brvs (McCrae and Faulkner-Valle, 1981; Carpio et al., 1981) found that after infection at an m.o.i. of 5pfu/cell, novel genomic RNAs could be detected by 4 hour pi. In the same studies, the kinetics of RNA genome synthesis were constant over approximately 10-12 hours.

Transcription of rotavirus genomes was first demonstrated in-vitro with brv in a reaction catalysed by the virion-associated RNA dependent RNA polymerase [= RNA polymerase] (Cohen, 1977; Cohen et al., 1979). The virion associated RNA polymerase was also found in hrv (Hruska et al., 1978; Spencer et al., 1981; Spencer and Arias, 1981; Flores et al., 1982a) and in simian SA11 rotavirus (Mason et al., 1980, 1983). The RNA polymerase activity was exclusively found in single capsid virions. Thus, for polymerase "activation" the L (double shelled) particles

needed chemical treatment with EDTA, EGTA which chelate calcium ions or heat shock 60°C for 30 seconds to remove the outer capsid layer (Cohen, 1977; Cohen et al., 1979; Spencer and Garcia, 1984). When the inner capsid proteins were separated from virus cores and tested for RNA-polymerase activity in the absence of virus cores, no enzyme activity was detected; stripped core particles did not have polymerase activity and mixing of isolated inner capsid proteins with the stripped core particles did not reconstitute activity of the enzyme (Bican et al., 1982). To date it is not clear yet which RNA segment codes for the RNA polymerase. Other enzymes, guanyl transferase, methyl transferase and nucleotide phosphohydrolase which are essential for biosynthesis and maturation processing of the cap structure have neither been assigned to RNA segments nor clearly demonstrated; but are thought to be associated with the single capsid particles (Estes et al., 1983; Ward et al., 1984a).

Maximal activity of the RNA polymerase was described to occur after activation at 37°C in 1mM EDTA for 30 minutes, at reaction temperatures 45-50°C, at pH 7.5 in the presence of magnesium ions and the ribonucleosides ATP, UTP, CTP and GTP (Cohen et al., 1979; Mason et al., 1980, 1983; Spencer and Arias, 1981; Flores et al., 1982a). Genome transcription depends on ATP. ATP is necessary to initiate a magnesium ion-dependent first step of transcription (Spencer and Arias, 1981; Spencer and Garcia, 1984). S-adenosyl-methionine, (SAM), a substrate for methylation of cap structures (Monroy et al., 1978), was shown to stabilise

and promote greater efficieny of translation of the RNA transcripts; but SAM itself did not stimulate RNA polymerase activity or produce any differences in the transcripts (Mason et al., 1980; Spencer and Garcia, 1984). RNA transcription is not inhibited by actinomycin D, α-amanitin and rifampicin all of which inhibit cellular DNA-dependent RNA polymerases (Cohen, 1977; Spencer and Arias, 1981). In the presence of sodium pyrophosphate, rotavirus genome transcription in-vitro was undetectable. Rotavirus replication and transcription requires virus-coded RNA-dependent RNA polymerase and these events occur in cell cytoplasm (Altenburg et al., 1980; Carpio et al., 1981; Suzuki et al., 1981, 1985; Petrie et al., 1982). The nature of the in-vitro transcribed RNAs was studied by treatment with ribonuclease, hybridisation assays and by in-vitro translation: it was found that the rotavirus transcripts were ssRNAs, hybridised to all 11 genomic segments and were active in the mRNA dependent rabbit reticulocyte translation system (Cohen and Dobos, 1979; Mason et al., 1980, 1983; Arias et al., 1982; Flores et al., 1982a, 1982b, 1982c, 1985, 1986; McCrae and McCorquodale, 1982). Rotavirus mRNAs have so far all been found to be monocistronic.(See addendum on page 12)

By terminal sequence analysis, McCrae and McCorquodale (1983) showed that the products of in-vitro transcription of the brv genome were full length copies from the negative strand of genomic RNA. Bernstein and Hruska (1981) found that the in-vitro synthesised transcripts were of one polarity and carried no polyadenylation sequences. It is

now known that both rotavirus mRNAs and plus-strand genomic RNA do not have a polyadenylation tail at the 3' termini (Dyall-Smith et al., 1983a; Imai et al., 1983; Ellerman et al., 1983; Baybutt and McCrae, 1984; Cohen et al., 1984; Estes et al., 1984a; Ward et al., 1984a, 1985). The positive sense genomic RNA strands corresponding to mRNAs carry a methylated cap at their 5' ends (Imai et al., 1983; McCrae and McCorquodale, 1983). The genomic RNA of negative polarity is only synthesised in the infected host cell and is produced uncapped at its 5' terminal (Imai et al., 1983).

An enzyme poly A polymerase (oligoadelate synthetase) was found in association with L particles of hrv (Gorziglia and Esparza, 1981). The role of the poly A polymerase in the transcription of the rotavirus genome could not be established yet; in reoviruses the poly A polymerase constitutes an alternative form of the RNA polymerase (Joklik, 1974; Silverstein et al., 1974).

Rotavirus polypeptides have been synthesised in mRNA-dependent in-vitro translation assays using either the virus specific RNA transcripts of simian rotavirus (Mason et al., 1980, 1983) and hrv (Flores et al., 1982a) or denatured genomic dsRNA of simian rotavirus (Smith et al., 1980; Dyall-Smith and Holmes, 1981a; Arias et al., 1982), brv (McCrae and McCorquodale, 1982) and hrv (Dyall-Smith and Holmes, 1981a). The results obtained so far have shown that the genomic RNA segments are monocistronic, i.e. each of the 11 RNA segment translated one polypeptide. However, in reoviruses which have for long been known to have

monocistronic segments (McCrae and Joklik, 1978; Gaillard and Joklik, 1985) it was recently discovered that S1 segment transcribes two mRNAs which code for O_1 and p14 or p12 (Ernst and Shatkin, 1985; Jacobs and Samuel, 1985). Besides, Cashdollar et al. (1985) have found two reading frames in the sequence of S1 gene of all three reovirus serotypes. The relationship between gene and product is illustrated in experiments in which in-vitro translated viral polypeptides were immunoprecipitated by polyclonal monospecific antisera (Killen and Dimmock, 1982; Flores et al., 1982a) as well as by the similarity of tryptic peptide maps of proteins separated from purified virions and the polypeptides obtained from in-vitro translation assays (Dyall-Smith and Holmes, 1981a). Furthermore, viral proteins synthesised in the in-vitro translation assays were compared with corresponding polypeptides found in infected cells (Flores et al., 1982a).

Gene coding assignments for simian rotavirus based on the translation of denatured genomic RNA segments indicated that each RNA segment encoded a primary gene product of molecular sizes ranging from 125Kd to 26Kd (Mason et al., 1980) and were designated VP1 to VP7, NCVP1, NCVP2, NCVP3, NCVP4 and NCVP5 in order of decreasing molecular weight (Arias et al., 1982). From the simian rotavirus RNA segments 9 and 10, non-glycosylated precursors of VP7 and VP10 respectively were synthesised in-vitro and this finding was documented by other authors studying the processing of viral proteins in infected cells in the presence of the glycosylation inhibitor tunicamycin (Smith et al., 1980; Dyall-Smith and

Table B

Gene coding assignments of RNA segments 7, 8, 9 of different rotavirus strains.

	Coding assignment of RNA segment in rotavirus strain			
Viral protein	brv[a]	SA11[b]	HU/5[c]	Wa[d]
7 ---------->	8	9	8	9
8 ---------->	7	8	7	8
9 ---------->	9	7	9	7

a McCrae and McCorquodale (1982)

b Arias et al. (1982)

c Dyall-Smith and Holmes (1984)

d Dyall-Smith et al. (1983a)

Holmes, 1981a; Arias et al., 1982). For brv, McCrae and McCorquodale (1982) found that each RNA segment produced one polypeptide which they designated VP1-VP9, VP11, VP12 (using part of the nomenclature of Mason et al., 1980). The RNA segment 10 in-vitro translates into a VP12 which undergoes glycosylation to become the mature VP10 observed in infected cells (McCrae and Faulkner-Valle, 1981). There were inversions of coding assignments for genome segments 7, 8 and 9. For brv (UK Compton strain) the RNA segment 8 codes for precursor of the glycoprotein VP7 and RNA segment 7 codes for the VP8 (McCrae and McCorquodale, 1982). Gene coding assignment by genetic reassortment (Kalica et al., 1981) revealed that a hrv strain specified the serotype/neutralising antigen VP7 on the genomic RNA segment 9 similar to the simian SA11 rotavirus (Table B).

Identical gene protein assignments do not hold true for different rotavirus strains and for instance, proteins encoded on the RNA segments 10 and 11 of the "long" electropherotype simian SA11 and hrv (Wa) were NS_3 and O_4 respectively (Dyall-Smith and Holmes, 1981a). In contrast, the "short" electropherotype hrv (strain M) had the gene coding assignment for segments 10 and 11 in reverse order (Dyall-Smith and Holmes, 1981a). In addition, the neutralising antigen VP7 of the "short" electropherotype hrv DS-1 was assigned to genomic RNA segment 8 (Greenberg et al., 1983) and RNA segment 8 also codes for the neutralising antigen in the "short" electropherotype hrv HU/5 (Dyall-Smith et al., 1983a; Dyall-Smith and Holmes, 1984). Inversions of gene coding assignments are depicted in Table B.

Protein synthesis during virus infection was found to be maximal at 4-6 hours pi for brv (McCrae and Faulkner-Valle, 1981; Carpio et al., 1981) and SA11 (Ericson et al., 1982). The pattern of viral polypeptide synthesis in infected cells reported for brv (Matsuno and Mukoyama, 1979; Thouless, 1979; Carpio et al., 1981; McCrae and Faulkner-Valle, 1981; Arias et al., 1982) was similar to that of the simian rotavirus SA11 (Ericson et al., 1982) and that of the hrv [Wa strain] (Flores et al., 1982a). The viral proteins were produced in unequal amounts. However, no measurements of relative abundance of these proteins was reported. The viral polypeptides designated VP1 through VP12 observed in infected cells were of similar sizes when compared to those produced in the in-vitro translation assays. The exceptions were that of the proteins VP7 and VP10 which are derived from their precursors (VP7c and VP12 respectively) by post-translational glycosylation.

Posttranslational modification by glycosylation was demonstrated for the VP7 and VP10 proteins using radiolabelled mannose and glucosamine. Glycosylation was sensitive to tunicamycin, an N- linkage glycosylation inhibitor (McCrae and Faulkner-Valle, 1981; Sabara et al., 1982b; Ericson et al., 1982). Unglycosylated precursors to VP7 and VP10 were shown to vary in their migration patterns on gels; for instance the simian SA11 precursor of VP10 designated pNCVP5 (Arias et al., 1982), comigrates with the VP12 of brv. The precursor of VP7 migrates ahead of the VP9 while the VP12 (20Kd), the precursor to VP10 (28Kd), migrates faster than VP11 (McCrae and Faulkner-Valle, 1981;

Both et al., 1983a, 1983b). By following viral proteins through cellular processing, McCrae and Faulkner-Valle (1981) were able to describe fifteen virus specific polypeptides in brv-infected cells.

8 GENETICS

Genome heterogeneity of rotaviruses isolated from natural field infections was demonstrated for brv (Rodger and Holmes, 1979; Sabara et al., 1982b, 1983), hrvs (Kalica et al., 1978; Espejo et al., 1980a; Lourenco et al., 1981; Rodger et al., 1981; Schnagl et al., 1981; Kutsuzawa et al., 1982b; Beards, 1982; Street et al., 1982; Follett and Desselberger, 1983a; Follett and Desselberger, 1983b; Sanders et al., 1983; Pereira et al., 1983; Follett et al., 1984; Konno et al., 1984; Svensson et al., 1986). While cocirculation of different hrvs occurs in nature, the hrvs with similar RNA electropherotypes were shown to be related by sequential point mutations whereas hrvs differing in electropherotypes showed extensive variations in the large unique oligonucleotides (Follett and Desselberger, 1983b). Rotaviruses share with the other segmented RNA viruses (influenza viruses: Desselberger et al., 1978; Palese, 1984); reoviruses: (Brown et al., 1983) and non segmented RNA viruses (Holland et al., 1982 and Holland, 1984) the characteristic of frequent mutational changes of the genome. In search of a genetic system to study the functions of rotavirus genes, temperature sensitive mutations were induced in the genomes of brv (Greenberg et al., 1981; Faulkner-Valle et al., 1982) and of SA11

rotavirus (Ramig, 1982). Temperature sensitive mutants were isolated and characterised to provide genetic selection markers. Greenberg et al. (1981) described four recombination/reassortment groups but five recombination (reassortment) groups were later reported for SA11 rotavirus (Ramig, 1982) and brv [UK strain] (Faulkner-Valle et al., 1982).

Reassortment of rotavirus RNA segments has been shown to occur after mixed infections in-vitro with simian SA11 rotavirus and brv (Matsuno et al., 1980), ts mutants of brv and non-cultivatable hrv (Greenberg et al., 1981, 1982), two cultivatable hrvs (Garbarg-Chenon et al., 1984), brv subpopulations (Sabara et al., 1982a), and some poorly cultivatable hrv and brv (Allen and Desselberger, 1985). In-vivo reassortment has been described between simian SA11 and the rhesus RRV rotaviruses after mixed infection in mice (Gombold and Ramig, 1986). Analysis of reassortants isolated after mixed infection with hrv and brv ts mutants allowed Kalica et al. (1981) to assign the neutralisable, serotype-specific antigen to RNA segment 9 of hrv (Wa strain). In the short electropherotype hrv DS-1 strain the neutralizing antigen is coded for by RNA segment 8 (Greenberg et al., 1983). RNA segment 6 was observed to segregate with the common (subgroup-specific) antigen (Kalica et al., 1981; Greenberg et al., 1983). Furthermore RNA segment 4 was identified as coding for growth and plaque formation in tissue culture (Greenberg et al., 1983). In addition, studies using reassortants derived from brv ts mutants and rhesus monkey virus (MMV

18006) showed that RNA segment 4 determined haemagglutination activity and protease enhanced plaque formation (Kalica et al., 1983). Independent segregation of the antigenic specificities of VP3 and VP7 coded by segment 4 and 9, respectively, of SA11 were demonstrated to be involved in neutralisation of rotavirus infection. This determination was achieved by reassorting two rotaviruses differing in both electropherotype and serotype. Clones were selected which had only acquired either segment 4 or 9 from one parent virus (donor) while the rest of the segments were those of the recipient (second parent virus) (Hoshino et al., 1985b; Offit and Blavat, 1986).

9 CLINICAL SYNDROMES ASSOCIATED WITH ROTAVIRUS INFECTION.

Rotavirus gastroenteritis is a disease found largely in infants and young children of age group 6 to 24 months worldwide (reviewed in Flewett and Woode, 1978; McNulty, 1978; Holmes, 1979, 1983; Estes et al., 1983, 1984b). In hospital based studies from both developed and developing countries (WHO, 1980) rotavirus was detected in approximately 50% of diarrhoea cases, thus rotavirus was rated a major cause of viral gastroenteritis. This was revealed in epidemiological studies from England (Flewett et al., 1975), Australia (Holmes et al., 1974; Davidson et al., 1975a; Schnagl et al., 1978), USA (Kapikian et al., 1974, 1976b; Brandt et al., 1979, 1983; Rodriguez et al., 1978, 1980; Dimitrov et al., 1984), Canada (Tallett et al., 1977; Middleton et al., 1977; Gurwith et al., 1981), Scotland (Madeley et al., 1975, 1978, 1983; Follett and

Desselberger, 1983a, 1983b; Follett et al., 1984), and Japan (Konno et al., 1984; Oishi et al., 1985). Reports on rotavirus infections in developing countries were produced in India (Maiya et al., 1977; Paniker et al., 1982), Java (Soenarto et al., 1981), Bangladesh (Ryder et al., 1977; Black et al., 1980, 1981, 1982), Chile (Espejo et al., 1977, 1980a; Avendano et al., 1982), Thailand (Echeverria et al., 1981), Ethiopia (Stintzing et al., 1981), Mexico (Bolivar et al., 1980), Brazil (Sutmoller et al., 1982; Pereira et al., 1983), Kuwait (Al-Nakib et al., 1980), Kenya (Mutanda, 1980). All reports emphasise that rotavirus diarrhoea is a major health problem of the young. Rotavirus gastroenteritis follows a constant seasonal pattern in the temperate climates with peak incidence during the winter months (Bryden et al., 1975; Davidson et al., 1975a; Middleton et al., 1974; Kapikian et al., 1976b; Schnagl et al., 1978; Follett and Desselberger, 1983a; Brandt et al., 1983; Follett et al., 1984). Fluctuations in atmospheric humidity (Brandt et al., 1983) were thought to have influence on incidence of rotavirus infection; this influence was difficult to show in tropical and semi-temperate climates (Hieber et al., 1978; Mutanda, 1980; Robin-Browne et al., 1980; Soenarto et al., 1981).

Where epidemiological investigations have been done employing electropherotyping techniques (reviewed: Estes et al., 1984b), rotavirus of different genomic electropherotypes were shown to cocirculate (Lourenco et al., 1981; Schangl et al., 1981; Rodger et al., 1981; Avendano et al., 1982; Dimitrov et al., 1983, 1984;

Follett and Desselberger, 1983a; Pereira et al., 1983; Follett et al., 1984). A shift and/or replacement of one dominant rotavirus electropherotype by another during a course of epidemic have also been observed (Espejo et al., 1980c; Lourenco et al., 1981; Konno et al., 1984; Svensson et al., 1986). It is therefore not surprising that Pereira et al. (1983) and Spencer et al. (1983) reported double infections of the same host with two rotaviruses differing in their electropherotypes.

Rotavirus infections were observed to occur independent of factors such as sex, race (Brandt et al., 1983), nutritional status (Soenarto et al., 1981). Socio-economic status of parents of the sick children was significantly correlated to the high incidence of rotavirus infection in early in life black children (Brandt et al., 1983) and Indians living in the Southern Indian state (Maiya et al., 1977) but no such correlation was found in other studies (Soenarto et al., 1981). However Schoub et al. (1982) reported more frequent rotavirus infections in the white children than were found in the age matched black children living in segregated regions of South Africa.

Rotaviruses were also found in association with other human diseases with and without acute gastroenteritis. Intussusception with intestinal bleeding was found in association with rotavirus infections (Konno et al., 1978) but in a controlled study Mulcahy et al. (1982) did not demonstrate a causative role for the rotaviruses in intussusception. Rotaviruses were isolated from children suffering from Henoch-Schoenlein purpura, encephalitis and

Reye Syndrome (Salmi et al., 1978) and exanthema subitum (Saitoh et al., 1981). Other reports linked rotavirus infection to respiratory diseases (Brandt et al., 1979; Goldwater et al., 1979; Maki et al., 1981; Santosham et al., 1983). Lewis et al. (1979), reported in a prospective study that rotaviruses were found in 66% of cases of diarrhoea and respiratory illness and/or otitis media. Sudden infant death syndrome was reported to have occurred in the presence (Brandt et al., 1975) and absence (Yolken et al., 1982) of acute gastroenteritis in patients who had also symptoms of respiratory illness. Isolation of rotavirus from a patient with Crohn's disease (Rieman and Demling, 1979) suggested rotaviruses as a possible factor in the progression of Crohn's disease; but this association was disputed in a controlled study (Whorwell, 1981). Disseminated intravascular coagulation (DIC) and haemolytic ureamic syndrome were described in cases of rotavirus gastroenteritis (WHO, 1980). An epidemic of neonatal necrotising enterocolitis was described among patients who had rotavirus infections (Rotbart et al., 1983).

10 PATHOPHYSIOLOGY OF ROTAVIRUS GASTROENTERITIS

Histological studies have shown that rotavirus infections lead to assembly and accumulation of virions in the cytoplasm of mature villous epithelial enterocytes of intestinal mucosa. The virus infection was demonstrated in the epithelial cells of duodenum and upper jejunum; the infection involved neither immature cells of the crypts nor did it extend to the lamina propria (Middleton et

al., 1974; Davidson et al., 1975b; Snodgrass et al., 1979). No viraemic phase of rotavirus infection has been described. As infection continues, cellular metabolic activity (both cellular DNA and RNA synthesis) was observed to be severely inhibited (Carpio et al., 1981) and to be followed by cell lysis. The rotavirus infection lyses and destroys absorptive surface with concomitant loss of digestive enzymes, especially disaccharidases including lactase. The diarrhoea associated with rotavirus infection is thus thought to operate through a disturbance of osmotic balance in the intestinal lumen, where partially digested and undigested disaccharides accumulate (Graham et al., 1982).

The clinical signs of rotavirus infection are diarrhoea, vomiting, and mild fever followed by dehydration; the symptoms may start at 24 hours pi and last usually less than one week (Davidson et al., 1975a; Rodriguez et al., 1977; Middleton et al., 1975; Holzel et al., 1980; Stals et al., 1984; Dearlove et al., 1983). During symptomatic rotavirus infection, maximal virus shedding occurs at the peak of illness. And although high concentrations of virus particles of the order 10^{12} virus particles per gram of stool/faecal matter can be reached, there is no established correlation of virus particle counts to the severity of symptoms (Madeley, 1983; Stals et al., 1984).

Recovery from acute infection is usually rapid, 8-10 days after the onset of diarrhoea (Gurwith et al., 1981; WHO Report, 1980). Prolonged shedding of rotavirus for 5 weeks in symptomatic patients has been documented in a

retrospective cohort study in which repeated infections were reported to be common (Mata et al., 1983). Because the rotaviruses were not identified by electropherotyping (RNA-PAGE), the identity of the rotaviruses implicated in prolonged shedding or repeated infections could not be ascertained. Protracted diarrhoea and virus shedding for several months was reported in children suffering from severe combined immunodeficiency (SCID) (Saulsbury et al., 1980; Booth et al., 1982; Chrystie et al., 1982; Pedley et al., 1984; Dolan et al., 1985; Eiden et al., 1985). High virus particle counts have been found in stools of asymptomatic children (Madeley, 1983). In a prospective study over an 11 month period, involving children of neonatal to 24 months age, asymptomatic rotavirus infection (rotavirus shedding in the presence of seropositivity) affected 2% neonates, 20% of children of 1 to 6 months of age and 37% of those 7-24 months of age (Champsaur et al., 1984a, 1984b). In the same study, rotavirus carriage (virus shedding without seroconversion) affected 27% neonates, 19% of the 1 to 6 month olds and 14% of the age 7 to 24 months. There are also reports of asymptomatic rotavirus shedding in neonates, infants and older children for periods of unspecified lengths (Totterdell et al., 1976; Chrystie et al., 1978; Madeley et al., 1978; Scott et al., 1979; Murphy et al., 1977; Bishop et al., 1979; Gurwith et al., 1981; Hoshino et al., 1985a). Asymptomatic shedding of rotavirus was found in adults (Blacklow and Cukor, 1981) but no data were provided on their immunological status.

11 EPIDEMIOLOGY AND HOST RANGE OF ROTAVIRUSES

Rotavirus infection occurs via the oral route by contaminated food, drink, formites and hands. Laboratory experiments confirmed this for piglets (Hall et al., 1976; Middleton, 1975; Pearson et al., 1977; Torres-Medina et al., 1976), cattle (Mebus, 1971, 1977; Snodgrass et al., 1984b), lambs (Snodgrass et al., 1977, 1984b), monkeys (Wyatt et al., 1976; Mitchel et al., 1977), dogs (Tripori, 1976), mice (Adams and Kraft, 1967; Offit et al., 1984) and for man (Middleton et al., 1974; Kapikian et al., 1983; Clark et al., 1986). Transmission of rotaviruses by aerosols was suggested but the studies (Kraft, 1957; Foster et al., 1980) did not consider that rotavirus-laden aerosols could cause infection of the gut if swallowed. Consequently Maki (1981) in a controlled study could not show that rotavirus infection caused respiratory illnesses. Goldwater et al. (1979), were unsuccessful in attempts to isolate rotavirus from the nasopharynx. Intramuscular injection of purified rotavirus was followed by the development of a humoral immune response; no viral diarrhoea was recorded (Bohl and Saif, 1975; Offit et al., 1983).

Rotaviruses have been isolated from a wide range of animal hosts (pigs: Lecee et al., 1976; Woode et al., 1976; Lecee and King, 1978; sheep: McNulty et al., 1976b; Snodgrass and Wells, 1976: goats: Scott et al., 1978; rabbits: Bryden et al., 1976; apes: Ashley et al., 1978; deer: Tzipori, 1976: antelopes: Reed, 1976: Thomson

gazelles, Impala and addex: Eugster, 1978; dogs: Eugster and Sidwa, 1979 and cats; Snodgrass et al., 1979). The avian species harbouring rotaviruses include turkeys (McNulty et al., 1979), fowl (Flewett et al., 1975; Dickson et al., 1979) and chickens (Jones et al., 1979; McNulty et al., 1980). Although in nature there seems to be species specificity of rotavirus infection, this is of doubtful significance given that interspecies infections have been demonstrated under laboratory conditions: hrvs were shown to infect conventional (Middleton et al., 1975; Davidson et al., 1977) and gnotobiotic piglets (Middleton et al., 1975; Bridger et al., 1975; Torres-Medina et al., 1976), monkeys (Wyatt et al., 1976; Mitchel et al., 1977; Majer et al., 1978), lambs (McNulty et al., 1976b; Snodgrass et al., 1977), calves (Mebus et al., 1976, 1977), dogs (Tzipori, 1976) and mice (Gouvea et al., 1986). Bovine rotaviruses were used to infect piglets (Woode et al., 1974, 1975; Hall et al., 1976) and mice (Noble et al., 1981; Bridger and Brown, 1984). Simian rotavirus caused infection in rhesus monkeys, chimpanzees and baboons (Soike et al., 1980), and the virus was found to replicate in gnotobiotic piglets and young calves (Rodger et al., 1977) and mice (Gombold and Ramig, 1986). Pararotaviruses (rotaviruses by morphology but lacking the group specific antigen) have recently been described in piglets (Bridger et al., 1982; Bohl et al., 1982), lambs (Chasey and Banks, 1986), chicken (McNulty et al., 1981), children (Rodger et al., 1982; Dimitrov et al., 1983; Nicolas et al., 1983; Espejo et al., 1984; Snodgrass et al., 1984a), calves and lambs (Snodgrass et

al., 1984b). Bovine pararotaviruses were found to infect lambs and the lamb pararotavirus infected piglets (Snodgrass et al., 1984a). There is some evidence to suggest that rat pararotavirus infects man (Eiden et al., 1985) and rat or pig pararotavirus was responsible for epidemic diarrhoea among adults in China (Hung Tao et al., 1985).

12 IMMUNITY AND IMMUNOLOGY

Epidemiological surveys have often used serological methods as complement fixation (CF) test, immunofluorescent (IF) test and enzyme linked immunosorbent assays (ELISA) to indicate the prevalence of rotavirus infection. Based on seroconversion in infected hosts, Kapikian et al. (1974), Elias (1977b), Ghose et al. (1978) and Yolken et al. (1978) were able to show that over 60% of children aged 6 years had antibodies against rotavirus. Neutralising antibodies against rotavirus were detected in sera of 67% of people aged 50-59 years while 31% of those 70-79 years were seropositive (Elias, 1977b). The studies however, did not make a distinction between primary infection, booster antibody response resulting from recurrent infections or persisting rotavirus antibody arising from primary infection during childhood (Fonteyne et al., 1978; Rodriguez et al., 1978; Yolken et al., 1978; Gurwith et al., 1981; Black et al., 1981).

Oral administration of rotavirus to human volunteers (Kapikian et al., 1983; Clark et al., 1980) was associated with seroconversion in 50-90% of cases. Champsaur et al.

(1984a, 1984b) showed in a prospective study, that the presence or isolation of rotavirus in man was not correlated with the development of serological evidence of infection. In the same study, seroconversion to rotavirus antigens was detected in 36% whereas virus carriage was 20% (virus isolated but no serological markers of infection) for the population of children aged 0-24 months. In another prospective study Bishop et al. (1983) found that neonatal infection did not confer immunity to rotavirus infection later in life. Some children had rotavirus reinfections with the rotavirus of a serotype different from that which caused the primary infection. Against this background, it is hardly surprising that Kapikian et al. (1974, 1981, 1983) documented recurrent rotavirus infection which occurred in the presence of detectable serum antibodies.

The result of vaccination of children with oral live attenuated brv RIT 4237 (Vesikari et al., 1983, 1984) did not show any correlation between either serum or local antibody and serological protection against subsequent rotavirus infection. Although the vaccinated children were challenged unequally by natural infection, a success rate of approximately 80% was claimed in the population under study (Vesikari et al., 1984).

Given that antirotavirus antibodies persist for several months postpartum (Cukor et al., 1978b, 1979; Snodgrass et al., 1980) breast feeding babies on antirotavirus antibody rich colostrum did not significantly influence rates of rotavirus infection (Brandt et al., 1979; Murphy et al., 1977; Bishop et al., 1979; Crewe and Murphy, 1980;

Totterdell et al., 1980; Gurwith et al., 1981). However, other studies showed that secretory IgA (in milk) had a protective role against rotavirus infection (Banatvala and Chrystie, 1978; McLean and Holmes, 1981; Riepenhoff-Talty et al., 1981; Davidson et al., 1983; Ebina et al., 1983; Sheridan et al., 1983).

Snodgrass et al. (1976, 1984b), protected gnotobiotic lambs, normal lambs and rabbits by feeding the animals with colostrum and sera containing type specific antirotavirus antibody. It is important to note that because challenge rotavirus infection experiments could not be done in man, relying on natural infection as a means to test immunological response has led to dubious interpretations of the protective functions of neutralising antibodies. One factor contributing to the poorly defined immunology in rotavirus infection is the presence in nature of at least seven rotavirus serotypes (Hoshino et al., 1984), five of which are found in man (Beards et al., 1980; Thouless et al., 1978, 1982; Sato et al., 1982; Wyatt et al., 1983a; Beards and Flewett, 1980; Hoshino et al., 1984, 1985a). Infection with each of the serotypes induces type specific immunity (Thouless et al., 1977; Fonteyne et al., 1978; Rodriguez et al., 1978; Yolken et al., 1981; Simhon et al., 1981; Snodgrass et al., 1984b).

Rotavirus infection in children whose immune system is suppressed (Saulsbury et al., 1980) or in children suffering from SCID (Pedley et al., 1984; Dolan et al., 1985; Eiden et al., 1985) and the children undergoing bone marrow transplantation (Yolken et al., 1982) resulted in protracted

diarrhoea and virus shedding. While it is assumed that an active immune response (both humoral and cell-mediated) is required to clear patients of rotavirus infection, the mechanisms involved in the defence against rotavirus infection are not fully understood (reviewed: Estes et al., 1985). Animal models have been used to show that T-cell mediated immunity was not required in the defence against rotavirus infections (Eiden et al., 1985). In contrast, Pedley et al. (1984) had described protracted rotavirus diarrhoea and virus shedding in children with SCID, one of whom 4 months after receiving a successful bone marrow transplant, mounted a cell mediated immunity and was able to overcome rotavirus reinfection.

13 PROSPECTS OF VACCINATION AGAINST ROTAVIRUSES

Diarrhoeal disease is a major cause of morbidity and mortality in the developing nations of the world; and is also an important cause of morbidity among the infants and young children from developed countries. There are an estimated 0.7-1 billion episodes of diarrhoea and 4.6 million deaths from diarrhoea which occur in children under 5 years of age residing in the regions of Africa, Latin America and Asia excluding China (Snyder and Merson, 1982). Because third world countries are deficient in resources and often lack facilities to manage diarrhoeal disease, illnesses frequently lead to severe fatal dehydration (Black Besides improvement of hygiene conditions, efficient vaccination against rotaviruses is regarded as the main tool to reduce morbidity and mortality due to rotavirus diarrhoea (DeZoysa and Feachem, 1985),

Several approaches in the development of a live attenuated rotavirus vaccine for use in man have been envisaged and include the live attenuated rotavirus strains (Kapikian et al., 1980, 1983; Wyatt et al., 1983b) attenuated rotaviruses of animal origin, RIT 4237 strain of brv (Vesikari et al., 1984), the rhesus rotavirus strain RRV (Kapikian et al., 1985) and reassortant rotaviruses (Midthun et al., 1985; Clark et al., 1986). There are indications that immunity against rotavirus infection is serotype specific (Offit and Clark, 1985). Vaccine strains of rotaviruses can be expected to afford a homotypic protection. However, often a heterotypic rise in serum antibody against one other human rotavirus serotype 1, 2 or 4 occurs (Kapikian et al., 1985) and this seems to be the basis of the vaccine RIT 4237 (Vesikari et al., 1984). In the face of uncertainties surrounding the capacity of a rotavirus vaccine based on one serotype to protect against heterotypic rotaviruses, of which five hrv serotypes are known so far (Hoshino et al., 1984, 1985a), there is a move towards constructing attenuated rotavirus strains by gene reassortment: Midthun et al. (1985) have employed the genetic technique to construct single gene RRV/hrv reassortants which contain the ten genes of RRV and one serotype specifying gene from hrv. In this way RRV/hrv reassortants representing hrv serotypes 1, 2 and 4 have been constructed and hopefully when used as a combined vaccine should confer an efficient protection against the known hrv-serogroups. Assuming a rise in serum antirotavirus antibody as a measure of immunological protection, Clark et

al. (1986) reported that human volunteers given 10^6 pfu orally of bovine/human rotavirus reassortant carrying a human serotype 1 antigen (Wa strain) failed to develop virus-specific neutralising serum antibody.

In the vaccine trials in humans done to date (Vesikari et al., 1983, 1984; Kapikian et al., 1983; Clark et al., 1986) none showed a correlation between immunological/serological markers with protection and immunity against rotavirus.

14 AIMS OF PROJECT

A wide genome heterogeneity among cocirculating human rotaviruses possessing the standard genome of 11 segments of dsRNA was observed in different parts of the world (Espejo et al., 1980a, 1980c; Schnagl et al., 1981; Rodger et al., 1981; Beards, 1982; Follett and Desselberger, 1983; Follett et al., 1984). Another form of genome polymorphism of hrvs was detected in chronically infected children who were suffering from severe combined immunodeficiency (SCID) (Pedley et al., 1984). The genomes of these viruses were atypical in that normal RNA segments were missing from the PAGE profile but additional bands of dsRNA were found. Using Northern blotting techniques, the extra RNA bands were found to be derived from normal genome segments by concatemer formation. And although in one case the segment 11 disappeared during the time of follow up, the patient remained infected and continued to shed the abnormal rotavirus for several months. Against this background, it was thought that genome rearrangements described by Pedley

et al. (1984) could have arisen in-vivo under conditions which in SCID permitted prolonged infection at high multiplicity; very much similar to the mechanisms of generating defective interfering particles (Huang and Baltimore, 1977; Holland et al., 1980, 1982; Holland, 1984). Because at that time, there was no suitable tube roller tissue culture system as used in the adaptation of hrvs (Ward et al., 1984b), the tissue culture-adapted hrv (UK Compton strain) was taken as a representative virus strain. After serial passage of brv at high m.o.i. Hundley et al. (1985) obtained a number of brv mutants all of which lacked RNA segment 5 but contained extra RNA bands migrating slower than segment 5 and derived from segment 5 by concatemer formation. I was given four brv mutants which were to be characterised for specific infectivity, transcription and replication, for virus-specific protein synthesis including analysis of protein processing, and for antigenic relationships with the parent virus. Sequence relationships between the RNA band E and segment 5, were studied by T_1 oligonucleotide mapping technique (Follett and Desselberger, 1983b) while the proteins VP5 and VP5A were analysed by oligopeptide mapping (Cleveland et al., 1977; and MacDonald, 1980).

In other studies Allen and Desselberger (1985), having adapted the genomically rearranged hrv described by Pedley et al. (1984), to moderate growth in secondary rhesus monkey kidney cells (RMK), superinfected cultures with standard brv and isolated various reassortants. The authors showed that the extra RNA bands of the genomically rearranged hrv

(Pedley et al., 1984) were able to reassort and replace the normal segments in the brv genome structurally and functionally. I tried a similar experiment in which MA104 cells were mixedly infected as described by Garbarg-Chenon et al. (1984) with a brv mutant carrying a rearranged band A and a normal hrv. Reassortants were isolated and studied for genome electropherotypes, protein synthesis and gene coding assignments.

MATERIALS

Viruses.

The human rotaviruses (hrvs) were obtained in London between 1975 and 1983 from faeces of children suffering from acute gastroenteritis and were kindly supplied by Dr. Ian Chrystie, Department of Virology, St. Thomas Hospital, London. Samples 4934, 5485, 5655, 5574 were also collected from children with acute gastroenteritis during September/October of 1983 and were provided by Dr. Ray Sanders, Regional Virus Laboratory, East Birmingham Hospital, Birmingham. Sample 22941, collected in 1983, came from Dr. Edward A.C. Follett, Regional Virus Laboratory, Ruchill Hospital, Glasgow, and had been adapted to growth in tissue culture according to the procedure of Ward *et al.* (1984b) [Allen and Desselberger, unpublished results]. The tissue culture-adapted bovine rotavirus (brv UK Compton strain) (Bridger and Woode 1975, Follett and Desselberger, 1983a) was used as the standard brv; it had been 6x plaque-to-plaque purified. The brv mutants D, A_4, B_4 and B_5 were obtained after the standard brv had been serially passaged at high moi (Hundley *et al.*, 1985). These mutants were found to contain no genomic RNA segment 5 but had rearranged RNA bands labelled A-H which were partly known to be covalently-linked segment 5-specific sequences. Stocks of brv mutant viruses were raised in cell culture and had been twice plaque-to-plaque purified when the project started. Later on 5 and 6 times plaque-to-plaque purified brv mutants D and A_4 were used.

Cells and tissue culture.

M104 cells, an epithelial-like continuous cell line established in 1963 from explant cultures of minced rhesus embryo kidneys, were supplied by Microbiological Associates, USA, through Laboratory Dupex Ltd., Twickenham, UK. Secondary rhesus monkey kidney cells (RMK) were supplied through Flow Laboratories, U.K. The following tissue culture solutions and buffers were prepared and provided by the Media Department, Institute of Virology, Glasgow.

Eagle's Minimum Essential Medium (MEM; Eagle, 1959) contained: Salts: NaCl 6.8g, KCl 0.4g, $CaCl_2$ 0.2g, $MgSO_4.7H_2O$ 0.2g, $NaH_2PO_4.H_2O$ 0.14g, $NaHCO_3$ 7/5% 4.5ml, glucose 10g, 1% phenol red 2ml ; Amino acids 729mg/L of essential amino acids; vitamins 8.1mg/L; mixed to make to 1L in distilled water, adjusted to pH 7.0 and then supplemented with: 100 units/ml of penicillin, 100 ug/ml streptomycin, 2.5 ug/ml amphotericin B, 0.002% w/v phenol red.

For passaging of cells, Eagle's MEM was supplemented with 10% foetal calf serum.

PBS = phosphate buffered saline pH7.0, containing NaCl 8.0g, KCl 0.2g, $CaCl_2$ 0.132g, $MgCl_2.6H_2O$ 0.1g, $Na_2HPO_4.12H_2O$ 1.15g, $KH_2PO_4.3H_2O$ 0.2g, dissolved in 1 L of distilled water.

Versene = 0.006mM EDTA in PBS containing phenol red 0.015g/L.

Trypsin solution = 0.25% w/v (trypsin Difco 1:250) in

PBS containing 100ug/ml streptomycin and 100 units/ml penicillin.

Trypsin-versene = 4 volumes versene:1 volume trypsin.

Stain for plaques in infected cell cultures.

Giemsa stain = 1.5% v/v Giemsa solution in glycerol heated to 56°C for 90-120 minutes, then diluted in equal volume of methanol.

Laboratory Animals.

New Zealand white rabbits were purchased from Hyline Rabbit Farms, England.

Total RNA extraction buffers (Maniatis et al., 1982).

Buffer A = 0.15M NaCl, 10mM Tris-HCl pH 7.8, 1.5mM $MgCl_2$, 0.65% NP40.

Buffer B = 7.0M urea, 0.35M NaCl, 0.01M EDTA, 0.01 Tris-HCl pH 7.8, 1% SDS.

1xTBE buffer for electroelution of RNA .

10xTBE stock consisted of Tris base 108g; boric acid 55g, 0.5M EDTA 40ml; in 1 litre distilled water, adjust pH 8.3.

Urea-polyacrylamide gels.

2.8% polyacrylamide 6M urea gels (269 x 169 x 1.5mm) were prepared using the following recipe:

30% acrylamide:1.725% Bis 7ml, 10M urea 45ml, 10 x Loening's buffer 7.5ml, distilled water 14.3ml, 10% ammonium

persulphate 1.12ml, TEMED 75ul.

Ultrapure acrylamide and N,N'-methylene-bis acrylamide were purchased from Biorad, Watford, Herts.

10 x Loening's buffer (Loening, 1967).

0.36M Trizma base, 0.3M $NaH_2PO_4.2H_2O$, 0.01M EDTA.$Na_2.2H_2O$, dissolved in distilled water and adjusted to pH 7.8.

10M urea.

300g ultrapure urea (BRL) were dissolved in 500ml (final volume)in distilled water.

RNA samples were applied to gels in the following running mixture: 30% sucrose, 1 x Loening's buffer, 0.05% xylene cyanol FF, 0.05% bromophenol blue.

Silver staining solutions were prepared according to Herring et al. (1982) and Whitton et al. (1983) as follows.

Fixing solution I: 25% v/v ethanol, 10% v/v acetic acid in distilled water.

Fixing solution II: 10% v/v ethanol, 0.5% v/v glacial acetic acid, all in distilled water.

Silver nitrate solution: 0.95g dissolved in 500ml (= 0.19g%)

Reducing solution: 0.009g% sodium borohydride,3.0g% sodium hydroxide,0.3g% formaldehyde,dissolved in water.

Fixing solution III: 5% acetic acid in water.

Destaining solutions for overstained RNA gels (Merril et al., 1979).

Solution A: 37g cupric sulphate, 37g sodium chloride, made to 1L in distilled water.

Solution B: 458g sodium thiosulphate made to 1L distilled water.

Composition of gels for oligonucleotide mapping (ONM) (Follett and Desselberger, 1983b; Hundley et al., 1985)

First dimension (1D) gel (450x230x1.5mm)

Acrylamide 40%-Bis 1.3%, 50ml; urea 84g (final concentration 7M); 1M citric acid, 5ml; distilled water to 200ml; ferrous sulphate 25mg/10ml, 0.8ml; ascorbic acid 1g/10ml, 0.8ml; hydrogen peroxide 30%,0.08ml.
40% acrylamide + 1.3% N,N'-methylene bis-acrylamide in ratios 30.8:1; 1.5% mixed bed resin; 0.5% charcoal, and filtered through Whatman 3MM.

First dimension running buffer (1D)

0.025M citric acid pH 3.5

Second dimension buffer 10X

0.5M Tris; 0.5M boric acid; 0.01M EDTA, pH 8.3.

Second dimension (2D) gel (200mm x 400mm x 1.5mm).

Acrylamide 40%-Bis 1.3%, 82ml; 2D buffer I0X, 15ml; distilled water, 52ml; TEMED, 0.1ml; ammonium persulphate, 0.9ml.

Buffers used to produce RNase T_1 resistant oligonucleotides.
T_1-digestion buffer: 20mM Tris HCl pH 7.5; 2mM EDTA.
Kinasing buffer: 10mM Tris HCl pH 8.0; 10mM $Mg(OAC)_2$; 1mm dithiothreitol.

Stop mixture: 2mg/ml yeast RNA in 0.6M ammonium acetate

Oligonucleotide running mixture: 7M urea, 10% w/v sucrose, 0.05M EDTA, 5.0mg/ml T_1 digested and undigested yeast RNA, 0.15% w/v xylene cyanol, 0.15% w/v bromophenol blue.

CF11 chromatography (Franklin 1966):

TSE (0.05M Tris pH 6.8, 0.1M sodium chloride, 0.001M EDTA) was used as buffer. For washing the cellulose CF11 columns, the buffer was adjusted to contain 1% β-mercaptoethanol and 0.01M EDTA. Fractionation was done in TSE adjusted to 35% and 15% ethanol respectively.

Solutions and buffers for polyacrylamide gel electrophoresis of proteins (SDS-PAGE) (Palfreyman et al., 1984).
Lysis buffer: 0.1M Tris pH 8.0, 10% glycerol, 0.5% NP40, 0.5 sodium deoxycholate, 0.2mM PMSF in 80% methanol.

Protein dissociation buffer: 2% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.125M Tris-HCl pH 6.8, 0.05% bromophenol.

Tank buffer: 0.05M Tris, 0.055M Glycine, 0.1% SDS, pH 8.3.

Protein gel fixer: methanol:distilled water:glacial acetic

acid, 50:50:7.

Stacking gel buffer: 1M Tris-HCl pH 6.7 adjusted with concentrated HCl.

Resolving gel buffer: 1M Tris-HCl adjusted to pH 8.0.

Immunoprecipitation (IP) wash: 0.5M lithium chloride in 0.1M Tris-HCl pH 8.0 buffer containing 1% v/v β-mercaptoethanol.

Peptide mapping buffers and solutions.

Peptide running buffer: 6.2g Tris, 28.6g glycine, 2.0g SDS, pH 8.3 to 2,000ml distilled water.

Peptide elution sample buffer.

0.5M DTT, 2ml; SDS, 0.2g; 1M Tris-HCl, pH 6.8, 0.8ml; Glycerol, 1.5ml; Bromophenol blue (0.2% in ethanol),0.2ml; distilled water, 5.5ml; add carrier protein BSA, 100ug/ml.

Electrophoresis buffer: 640ml glacial acetic acid and 160ml formic acid was made up to 8L with distilled water, pH 2.1.

Chromatography buffer: 30ml butan 1-ol, 20ml pyridine, 24ml water, 6ml glacial acetic acid mixed together in one tank.

Scintillation fluids: PPO 7.0g, POPOP 0.5g made in IL toluene. Aquasol was diluted 5ml with 1ml distilled water

before use.

STANDARD CHEMICALS

Analytical grade chemicals were obtained from the following suppliers:

BDH Chemicals Limited, Poole, England

Ascorbic acid, hydrogen peroxide, Bis, acrylamide (biochemical grade), formic acid, bromophenol blue, citric acid, charcoal, EDTA, formic acid, glycine, hydrogen peroxide, hydrochloric acid, magnesium chloride, NP40, SDS, sodium borohydride (NaH_4B), sucrose, sodium pyrophosphate, triethylene.

Sigma Chemical Company Ltd. Poole, Dorset.

Freund's adjuvant, mixed bed resin, bentonite, cycloheximide, pancreatin, PMSF, pyruvate kinase, phosphoenol pyruvate, SAM, sodium deoxycholate, trizma base, tunicamycin, nucleoside triphosphates ATP, CTP, UTP, GTP; pyridine, Cleland's reagent (DTT).

Biorad Laboratories, Watford, Herts.

Analytical grade acrylamide, ammonium persulphate, TEMED.

Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Biochemical grade acrylamide, boric acid, caesium chloride, chloroform, diethylether, glacial acetic acid, glycerol, PPO, POPOP, sodium hydroxide pellets, toluene

Gibco, Paisley, Scotland.

Foetal calf serum, sodium bicarbonate solution (7.5%), 10X Glasgow modified Eagle's MEM.

Merck, Sharp and Dohme International Inc., New Jersey.

Actinomycin D.

Johnson, Matthey Chemicals Ltd., Herts, England.

Silver nitrate.

TAAB Laboratories Ltd.

Aqueous glutaldehyde EM 25%.

Kodak (London) Ltd., Kirkby, Liverpool.

X-ray film Kodak X-Omat S; XH1; TLC chromatograms.

Agfa Gevaert Belgium.

X-ray films Scopix CR3 NIF.

New England Nuclear, Boston, MA, USA.

En^{3}Hance, Aquasol.

Calbiochem, Stortford, Herts.

RNase T_1 Sankyo

New England Biolabs. CP Laboratories, Bishops Stortford, Herts.

T_4 E.coli polynucleotide kinase

Boehringer Mannheim GmbH, East Sussex, England.

Tris, tRNA, rRNA.

James Burroughs Ltd., London.

Absolute ethanol (analytical grade ethanol 100).

Becton Dickson UK Ltd., Oxford, U.K.

Falcon centrifuge tubes, Falcon plastic roller bottles, Linbro plates, syringes 1ml, 5ml, 10ml; needles gauge 26, 21, 19.

Sterilin, Teddington, Middlesex, England.

Petri dishes 50 and 90mm.

Flow Laboratories (UK), Ayrshire, Scotland.

Microfilters 0.22um and 0.45um pore size.

Millipore (UK) Ltd., London.

Whatman filter paper 3MM, Whatman CF11 cellulose powder Difco Laboratories Ltd., Molesey, Sussex.

Agar, trypsin 1:250.

Agar Aids Ltd., Essex, England.

Polystyrene beads stock 1.4 x 10^{12}/ml of diameter 0.091uM.

BRL (UK) Cambridge, England.

Immunoprecipitin.

Pharmacia Fine Chemicals Ltd.

Sephadex G25.

Fluorochem Ltd., Glossop, Derbyshire.

Formaldehyde.

Radiochemicals were obtained from Amersham International PLC, Bucks., England.

Adenosine 5'-α [^{32}P] triphosphate and Guanosine 5'-α [^{32}P] triphosphate at specific activity $\geq$ 14.8 TBq/mmol. 5'-γ [^{32}P]-ATP was supplied at specific activity $\geq$ 13.5 TBq/mmol. L-[^{35}S] methionine, specific activity $\geq$ 30 TBq/mmol. D-(2-^{3}H) mannose, specific activity 370-740 GBq/mmol. D(6-^{3}H) glucosamine, specific activity 0.74-1.5 TBq/mmol. (5,6-^{3}H) uridine specific activity 1.5-22TBq/mmol. (^{14}C) methylated protein mixtures for molecular weight markers (CFA 626) consisted of: myosin 200Kd, phosphorylase b 92.5Kd, BSA 69.0Kd, ovalbumin 46.0Kd, carbonic anhydrase 30.0Kd and lysozyme 14.3Kd.

[^{32}P]-inorganic orthophosphate carrier free was obtained from Western Infirmary, Glasgow: supplied by Amersham PLC.

METHODS

Growth of cells.

The procedures followed were those published by Thouless, 1979; Ward et al., 1984b; Hundley et al., 1985. MA104 cells were raised from a stock of cells kept frozen at -70°C. $2x10^6$ cells were thawed and seeded in 150ml of 1 x Glasgow MEM (modification of Eagle's Minimum Essential Medium) containing 10% foetal calf serum for two 200ml falcon flasks. The cells were incubated overnight in an atmosphere of air and 5% carbon dioxide at 37°C. The medium was changed the next day. Subsequently, confluent cell monolayers were passaged using 30ml versene:trypsin (4:1), then dispersed into 200ml 1 x Eagle's MEM containing 10% foetal calf serum and seeded into two 2 litre burlers. The burlers were gassed with CO_2 and kept rolling at 37°C until cells were fully confluent. Tissue culture petri dishes and 24 well Linbro plates were kept in a humidified incubator at 37°C with circulating 5% CO_2.

Propagation of viruses in MA104 cells.

Having recognised that proteolytic enhancement of virus infectivity occurs in all rotaviruses irrespective of animal/host source (Babiuk et al., 1977; Almeida et al., 1978; Graham and Estes, 1980; Clark et al., 1981; Sato et al., 1981; Estes et al., 1981), it has become possible to produce rotaviruses in high titres. Confluent monolayers of MA104 cells in roller burlers were twice washed in sterile PBS to remove foetal calf serum. Rotavirus was

"activated" in 20ug/ml trypsin (Difco 1:250) for 30 minutes at 37°C, diluted to 5ug/ml trypsin concentration and adsorbed onto the cells for 60 minutes. The inoculum was removed and infected cells incubated under 30ml 1 x Glasgow MEM containing 1ug/ml trypsin and 5% CO_2 per roller burler, or under 5ml of 1 x Glasgow MEM + 1ug/ul trypsin per 50mm petri dish kept in a humidified 37°C incubator with 5% CO_2. To raise virus stocks, infection at 0.1 pfu/cell was allowed to proceed to full CPE and the resulting virus suspensions were frozen and thawed once and stored at -20°C in 1ml aliquots.

Virus titration.

Virus stocks were titrated using the procedure of Matsuno et al. (1977a). Serial tenfold dilutions of trypsin activated virus were made in sterile PBS. Inoculum of 0.3ml virus suspension was adsorbed on each 50mm petri dish for 1 hour at 37°C, then washed off and replaced with 5ml Eagle's MEM medium without phenol red and containing 5ug/ml pancreatin and 0.5% agar. The infected dishes were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Plaques became visible usually between 5-7 days pi, cells were then fixed in 2.5% glutaraldehyde in PBS for at least 60 minutes, the agar removed and cells stained with Giemsa solution for at least 20 minutes at room temperature. After washing off the Giemsa stain with cold tap water, dishes were left to dry and plaques scored to determine the virus titre in pfu's/ml.

Purification of rotavirus.

Rotaviruses grown in 2L burlers of MA104 cells were harvested after two rounds of freezing and thawing. The crude virus-cell suspension was first clarified by low speed centrifugation (at 1,500g for 10 minutes), and from the resulting supernatant virus was concentrated by ultracentrifugation through a 30% w/v sucrose cushion at 83,000g for 2 hours using the AH627 rotor in Sorvall centrifuge. The viral pellet was resuspended in 0.5ml PBS per burler equivalent of MA104 infected cells.

Banding of virions was done by isopycnic centrifugation in a CsCl-virus mixture of density 1.4g/ml at 18°C for 16 hours (Novo and Esparza, 1981) on a Beckman AH650 rotor at 115,000g. Visible bands containing the double capsid virions (d) and the heavier single capsid virions (s) were harvested by puncturing the centrifuge tube just below the virus band and approximately 0.5ml fractions were collected. The empty virus particles (p) fraction was collected from just below the meniscus of each tube.

Refractive index of the fractions (virus-CsCl mixture) was measured on an Abbé refractometer and used to work out the density of the virions according to Bruner and Vinograd (1965) and Cohen (1977). After dialysis against PBS at 4°C overnight, virus was recovered by ultracentrifugation at 115,000g on an AH650 Beckman rotor at 4°C for 90 minutes and resuspended in 50-100ul of PBS.

The hrv's obtained directly from clinical specimens were semi-purified by preclearing 10% fecal suspensions in PBS at low speed (1500g) for 10 min. The supernatant was

ultracentrifuged through 30% w/v sucrose cushions as described above (Follett and Desselberger 1983a). The virus pellets were resuspended in 0.3ml of PBS per 5ml crude suspension.

Electron microscopy.

Procedures for electron microscopic visualisation and counting of rotaviruses have been described previously (Madeley et al., 1978; Esparza and Gil 1978; Brandt et al., 1981; Follett and Desselberger 1983a).

Rotavirus suspensions (5ul aliquots of concentrates after ultracentrifugation) were mixed with equal volume of 1.5% sodium phosphotungstate pH 7.2, and of marker polystyrene beads of 91nm diameter and at a concentration 1.4×10^{11} beads/ml. One drop of the mixture was placed on a 400 mesh copper grid which had been coated with formvar. After allowing 2-5 minutes for the mixture to stick to the grid, excess fluid was blotted off with filter paper. The grids were examined at 80KV using the JEOL 100B electron microscope. The number of virus particles counted in a field were expressed as ratio of the number of polystyrene beads (included for reference) of known concentration seen in the same field and the concentration in virus particles (v.p.)/ml determined assuming direct proportionality of counts.

Rotavirus genome analysis by polyacrylamide gel electrophoresis (RNA-PAGE).

Electrophoretic separation of rotavirus genomes on

polyacrylamide gels has been used to identify and differentiate rotavirus isolates (Kalica et al., 1976; Espejo et al., 1980a; Schnagl et al., 1981; Rodger et al., 1981; Flores et al., 1982a; Follett and Desselberger 1983a; Follett et al., 1984).

a. Extraction of virion RNA. The RNA-PAGE method used was as described by Follett and Desselberger (1983a). Genomic RNA of virus recovered from either infected cells and/or 10% fecal suspensions was extracted by adding SDS and sodium acetate to final concentrations of 1% and 0.3M, respectively and then mixing with an equal volume of chloroform:phenol (1:1). Three rounds of extraction in phenol:chloroform were carried out until the aqueous phase and interphase were clear. Samples were then ether extracted twice and ether removed by blowing nitrogen gas over the tube under a fume hood. RNA was precipitated with 2.5 volumes of cold absolute ethanol for 1 hour at -70°C or overnight at -20°C and pelleted at 11,000g on a bench microfuge Microcentaur MSE for 15 min., lyophilised on a Vacuum centrifuge (1725rpm) and dissolved in ten microlitres of distilled water.

b. Gel electrophoresis. Unless specifically indicated, 2.8% acrylamide (acrylamide:bis 17:1) 6M urea slab gels as described under Materials were used to analyse the RNAs. Gels were run with continuous circulation of 1 x Loening's buffer (Loenig, 1967). The RNA sample was mixed with equal volume of 5ul of RNA running buffer, heated to 56°C and loaded onto the gel. Electrophoresis was at 130-150 V and room temperature for 16 hrs and was stopped when the

bromophenol dye marker had reached the bottom of the gel.

c. RNA-PAGE silver staining procedure. The silver staining procedure of Sammons et al. (1981) was adopted to visualise genomes of hrv's and of brv's (Herring et al., 1982; Follett and Desselberger, 1983a). Briefly, the RNA gel was soaked in fixing solutions I and II for 1 hour each. Gel was transferred to a 0.19g% solution of silver nitrate for 90-120 minutes, washed 2-3 times in cold tap water and then placed in the reducing solution for 5-10 minutes until the RNA bands became distinctly visible. Immediately the gel was soaked in fixing solution III to retain the desired colour intensity. Results of silver stained gels were recorded by taking photographs of transilluminated gels.

d. Destaining of overstained RNA gel.

In the event of overstaining, the destaining procedure of Merril et al. (1979) was used to adjust the gel colour to the appropriate intensity. Gels have to be photographed immediately afterwards, because the loss of silver stain continues rapidly. The RNA gel was soaked in mixtures of equal volumes of destaining solutions A and B which had been diluted 1:3, and left for 10-30 minutes at room temperature.

In-vivo RNA synthesis (Clark et al., 1981).

a. To investigate the kinetics of viral RNA synthesis in MA104 cells, confluent cell monolayers of MA104 cells in 24 well Linbro plates were pretreated for 1 hour with actinomycin D 5ug/ml in 1 x Eagle's MEM to inhibit cellular RNA synthesis. The cells were then infected at an m.o.i of 5 pfu/cell in the presence of actinomycin D 5mg/ml, trypsin 1ug/ml in 0.5ml of 1 x Eagle's MEM containing 0.037 MBq of

(5,6-^{3}H) uridine. The infected Linbro plates were incubated at 37°C in humidified atmosphere of 5% CO_2 for various times. The samples were solubilised at time intervals between 30 minutes pi to 24 hours pi in 100ul solubilization buffer containing 1% SDS, 8M urea and 2% β--mercaptoethanol (Clark et al., 1981). Ten microlitres of each sample were prepared in duplicate for TCA precipitable counts according to Szilagyi and Pringle, 1979 (as adopted from Bollum, 1968).

b. In experiments in which the viral dsRNA synthesis was monitored by RNA-PAGE and silver staining (Herring et al., 1982; Follett and Desselberger 1983a) large 50mm petri dishes of MA104 cells were infected at 5 pfu/cell under the conditions described above (a). At various times, the earliest being 6 hr. pi, the infected cells were frozen and thawed once to release virus, which was pelleted by ultracentrifugation and the dsRNA was extracted using the phenol:chloroform method (Kalica et al., 1978; Follett and Desselberger 1983a). Confluent cell monolayers on 50mm petri dishes were maintained overnight in phosphate free medium and then infected in the presence of actinomycin D 5ug/ml. For an appropriate labelling period, 8 hours and 16 hours post-adsorption, the cells were incubated in phosphate free Eagle's medium containing 7.4 MBq (200uCi)/ml [^{32}P] orthophosphate. To investigate viral RNA synthesis in the absence of viral protein synthesis, cycloheximide (20ug/ml) was added to the labelling medium containing actinomycin D 5ug/ml as described by Nonoyama et al. (1974).

RNA synthesis in brv infected cells was followed by

monitoring the incorporation of (^{3}H) uridine (Clark et al., 1981) in MA104 cells (24 well Linbro plates) which had been pretreated with 5ug/ml actinomycin D and infected in the presence of actinomycin D 5ug/ul and 0.3MBq (5,-6^{3}H) uridine. At hourly intervals post infection, the cells were washed in PBS and harvested in the RNA solubilisation buffer (Clark et al., 1981).

Cells infected with standard brv (UK strain), or the brv mutants D and A_4 in the presence of actinomycin D and either [^{32}P] orthophosphate or (5,6-^{3}H) uridine were scraped from petri dishes into PBS and pelleted at low speed, 1,500g for 5 minutes. The cell pellet was kept on wet ice for 5 minutes to swell in buffer A and followed with vortexing in ice to disrupt cells and release RNA. The samples were cleared of cell debris by low speed, 1,500g centrifugation for 5 minutes on the bench centrifuge MSE, supernatants combined with equal volume of buffer B and extracted twice with equal volumes of phenol:chloroform 1:1. Total RNA was recovered after ethanol precipitation and pelleting at 11,000g for 15 minutes as in (a).

Cellulose CF11 chromatography of rotavirus ribonucleic acid.

Because neither rotavirus genomic dsRNAs nor the mRNAs contain 3' polyadenylation sequences (McCrae and McCorquodale 1983; Imai et al., 1983), separation of the constituent RNA species from a mixture of total RNA was not possible on oligo dT cellulose columns. Smith and Hay (1982), Herring et al. (1982) and Snodgrass et al. (1984a) have used CF11 cellulose chromatography to purify rotavirus

genomic dsRNAs according to Franklin (1966), with modifications from Bevan et al., 1973.

The method I used was that of Franklin (1966). Whatman CF11 cellulose was prewashed with 35% ethanol in TSE and packed in a 10ml graduated pipette to the 6ml mark, and washed again with TSE containing 1% β-mercaptoethanol. The washing buffer was changed and washing continued using 35% ethanol 65% TSE until the optical density at 260nm of the eluate was that of the washing solution. Samples of [^{32}P] labelled RNA were dissolved in 35% ethanol in TSE and eluted in the same buffer; 500ul fractions were taken until the radioactivity registered on the Geiger counter was near zero. The buffer was changed to 15% ethanol in TSE and fractions taken in the same way. The last elution step was done in TSE alone. At each elution step, the volume of buffer applied and the number of fractions depended on how fast peaks of radioactive counts (registered on the Geiger counter) were eluted.

All fractions were counted on the Intertechnique SL 4,000 scintillation counter using tritium channel. Peak values of each elution step were precipitated in 2.5 volumes of ethanol and 5ug of tRNA overnight. The RNA in each fraction group was pooled and analysed by PAGE.

In-vitro transcription of brv genomes.

The study of rotavirus RNA synthesis in-vitro was based on the finding that virion associated RNA polymerase (RNA dependent RNA polymerase) of calf rotavirus (Cohen 1977; Cohen et al., 1979; Bernstein et al., 1981) simian SA11

rotavirus (Mason et al., 1980) and the hrv (Hruska et al., 1978; Spencer and Arias 1981; Flores et al., 1982a) could be activated in-vitro to synthesise ssRNA (mRNAs). The in-vitro transcription assay conditions used in this study were as described by Hundley et al., 1985 and had been described by Flores et al., 1982a. The brvs were concentrated and partially purified from infected cell suspensions, incubated for 30 minutes with 10mM EDTA in 0.1M Tris-HCl pH 7.4 at 37°C to "activate" the RNA polymerase associated with the double capsid virions (Cohen 1977, 1979). Aliquots of 15ug of the activated virus were added to a transcription reaction mixture, 400uL, consisting of 2.5mM of unlabelled triphosphate nucleotides ATP, GTP, UTP and CTP, 0.5mM SAM, 0.1% bentonite, 10MM Tris-acetate pH 7.8, the nucleotide regenerating system made of pyruvate kinase 50ug/ml and 8mM phosphoenol pyruvate. In synthesising [^{32}P] labelled ssRNA, either α-[^{32}P] ATP or α-[^{32}P] GTP at 2.2MBq per reaction mixture were used while the corresponding unlabelled nucleotides were reduced from 2.5mM to 0.8mM concentration. The transcription was initiated by addition of 12mM magnesium chloride. At appropriate time intervals (60 min.to 6 hours) twenty microlitre aliquots were taken for measurement of TCA precipitable [^{32}P] counts. At the end of transcription reaction at selected temperatures, 42°C, 37°C and 31°C, the bentonite and virus particles were partially removed by centrifugation at 11,000g for 10 minutes to yield the ssRNAs in the supernatant which were then extracted in phenol:chloroform 1:1. The resulting ssRNAs were separated

by RNA-PAGE (Methods) and gels autoradiographed on Kodak XS films with image intensifying screens.

Scintillation counting.

Five microlitres of sample labelled with [^{32}P], [^{3}H] or [^{35}S] were spotted and left to dry on Whatman no. I discs 2.5cm diameter in duplicate. The discs were washed in TCA 10% twice followed by a single wash in 5% TCA and two washes in absolute ethanol. The discs were dried and counted in 3ml of PPO-toluene scintillation fluid on Intertechnique SL 4000 scintillation counter (Szilagyi and Pringle 1979).

The tritium channel of the Intertechnique SL 4000 scintillation counter was used to measure directly total radioactivity in samples labelled with [^{32}P].

[^{35}S] methionine labelled peptides were counted in 1ml of aquasol:water 5:1.

RNase T_1-resistant oligonucleotide mapping.

Oligonucleotide mapping technique has been applied to investigate genome composition of influenza viruses (Desselberger et al., 1978; Nakajima et al., 1978; Young and Palese 1979) retroviruses (Pedersen and Haseltine 1980) poliovirus (Nottay et al., 1980; Kew et al., 1981) and the rotaviruses (Follett and Desselberger 1983b; Hundley et al., 1985).

Genomic dsRNA segments were separated by RNA-PAGE on a quantitative RNA gel. The RNA gel was stained with 4ug/ml ethidium bromide in 1 x TBE for 30 minutes. The genomic segment 5 and the rearranged RNA segment E was sliced out of

the gel under U.V. lighting. A photographic record was made before and after slicing the RNA bands in order to confirm accuracy of RNA segment isolation. The gel pieces were packed into dialysis bags with a small volume of 1 x TBE. Avoiding introduction of air bubbles, the bags were clipped and placed in a horizontal tank of 1 x TBE. Electroelution was performed at 150-200V constant voltage (30-40mA) for 1 hour and then the current was reversed for 90 seconds (Maniatis et al., 1982; Hundley et al., 1985). The electroeluted RNA was made in 0.3M sodium acetate and recovered by ethanol precipitation at -20°C overnight, followed by pelleting at 11,000g for 15 minutes.

To generate RNase T_1-resistant oligonucleotides, approximately 1ug of segmental RNA in 3ul of distilled water was denatured by boiling for 2 minutes and chilling on ice. The dsRNAs were digested with 3ul RNase T_1 containing 3 unit/ml of 2 x digestion buffer at 37°C for 30 minutes. The T_1-digests were incubated in a final volume 50ul of kinasing buffer containing [^{32}P]-ATP of specific activity $\geq$ 140TBq/mmol in the presence of 1ul T_4 polynucleotide kinase (= 1 unit). The labelling reactions were terminated by adding in 50ul of stop mixture, the mixtures were then extracted in 100ul redistilled, buffer saturated phenol. The top aqueous phase was ethanol precipitated at -70°C for 1 hour and the oligonucleotides recovered by 15 minute spin at 11,000g were dried and mixed with 20uL of ID buffer. The RNAse T_1-resistant oligonucleotides were separated on a 10% polyacrylamide gel which was made as described under Materials. Edges were sealed with 3.6% agar and then the

gel was placed in a buffer tank carrying the (+) electrode. Ten microlitres of each sample were loaded per gel slot and the gel connected by wicks with the tank carrying the electrode. Electrophoresis was set to run at 600V for 16 hours in 0.025M citric acid until the marker dye bromophenol blue reached the 19cm mark below the loading slot. The gel was then autoradiographed wet at 4°C for 2-3 hours. From the 1D gel, 20cm long gel pieces were cut between 8cm above and 12cm below the xylene cyanol spot. They were placed between the glass plates of the 2D gel and sealed at both sides and bottom to allow casting the 20% polyacrylamide gel which was composed as described under Materials. Having marked the 18cm on 2D gel above the bromophenol dye spot seen in the ID gel strip, the gel was placed in the (-) electrode tank containing 1x2D buffer. Whatman filter paper wicks soaked in 2D buffer were used to connect the gels with the (+) electrode tank. Electrophoresis was at 10mA per gel for 16 hours and until the bromophenol dye marker moved through the 18cm. Gels were exposed wet at 4°C against Kodak XH1 film using intensifying screens.

Pulse labelling of intracellular viral proteins.

Confluent cell monolayers on 50mm petri dishes were washed in sterile PBS and infected at moi 5 pfu/cell with trypsin-activated standard brv and the brv mutants. Virus adsorption was allowed to proceed at 37°C for 1 hour, the virus inoculum removed and the infection maintained in 5ml of 1 x Eagle's MEM containing lug/ml trypsin. Cell monolayers to be labelled were treated for 15 minutes with

hypertonic methionine-free Eagles MEM containing additional 0.15M sodium chloride; under these conditions host cell mRNAs synthesis is suppressed and viral mRNA is preferentially translated (Nuss et al., 1975; Racaniello and Palese, 1979). The medium was then changed to contain 7.4 MBq/ml L-[^{35}S] methionine, trypsin lug/ml and additional 0.15M sodium chloride. Labelling was performed for 2 hours at 37^{o}C using 2ml of medium per 50mm petri dish. Cells were scraped into 250ul of protein dissociation buffer for each 50mm petri dish of cells. Before separation of proteins by SDS-PAGE, the cell lysates were boiled for 3-5 minutes and then loaded onto the gels.

Pulse-chase labelling of viral proteins.

Cells on 50mm petri dishes were infected with rotaviruses in duplicate and were pulse labelled for 2 hours with 7.4MBq/ml of L-[^{35}S] methionine in 2ml of methionine-free Eagles MEM containing extra 0.15M NaCl and trypsin lug/ml. The chase samples were washed once and incubated for a further two hours in 2ml of 1 x Eagle's MEM containing an excess 300X unlabelled L-methionine, (1x = 0.015 mg/ml L-methionine) and trypsin lug/ml but lacking radioactive label.

Continuous labelling of rotavirion proteins.

In this case rotavirus infection in roller culture burlers was maintained in the presence of 1.85MBq/ml of L-[^{35}S] methionine in 30ml of methionine free Eagle's MEM containing lug/ul trypsin from 1 hour post adsorption. When complete CPE was observed, the culture burlers of 2x10^{8}

cells, were frozen and thawed once to release virus particles.

The virus from these cultures was subjected to CsCl gradient purification as described above.

Labelling of viral glycoproteins in infected cells.

Cell monolayers on 50mm petri dishes ($2x10^6$ cells) were infected at a m.o.i. of 5pfu/cell. The infection proceeded in the presence of either 3.7MBq/ml of [2-^{3}H] mannose or 3.7MBq/ml of D[6-^{3}H] glucosamine in 1 x Eagle's MEM containing lug/ml trypsin for the 16 hours of infection. Cells were washed in cold PBS, care being taken not to lose cell monolayers where CPE was marked. Cells were then harvested in 250ul of dissociation buffer per sample and stored at -20^{o}C before use.

Polyacrylamide gel electrophoresis of proteins (SDS-PAGE).

Polyacrylamide gel electrophoresis of proteins was carried out in the discontinuous system of Laemmli(1970). Gels were prepared using clean glass plates 154x165mm separated by 1.5mm thick spacers. Although a variety of gel concentrations, (9, 12, 15 and 17%) were used according to the requirements of enhanced separation of particular polypeptides, the gel most commonly used in the studies was 12% and prepared as described under Materials (Elliott et al., 1984; Hundley et al., 1985). The gel was overlaid with a thin layer of resolving gel buffer:water 1:4 to allow the gel to polymerise forming a straight separating boundary. A 3cm deep stacking gel (as described under

Materials) was poured with a teflon comb in place to form sample wells. Precaution was taken to avoid trapping air bubbles. Samples were heated to 100°C for 2 minutes in protein dissociation buffer and loaded onto the gel. Electrophoresis was carried out at 4°C or room temperature at 40 mA constant current (approximately 300V) for 4 hours or an overnight run at 70V constant voltage until the bromophenol dye marker reached the bottom of the gel. With the exception of gels which were used for tryptic peptide mapping, all gels were fixed in water:acetic acid:methanol 50:7:50 for 30 minutes, washed in water for another 30 minutes and dried. When [^{3}H] labelling was used, gels were washed in EN^{3}HANCE after the fixation step for one hour at room temperature and washed in cold water for 60 minutes. The gels were dried and autoradiographed on Kodak XS film at -70°C.

Estimation of molecular weights.

Apparent molecular weights of the viral proteins VP1-VP12 and of VP5A were calculated from a standard curve in which the logarithm of molecular weights of (^{14}C)-methylated protein size markers (myosin 200Kd, phosphorylase 92.5Kd, BSA 69.0Kd, ovalbumin 46.0Kd, carbonic anhydrase 30.0Kd and lysozyme 14.3Kd) were plotted against the distance of migration of these proteins on the gel. The unknown molecular weights were then estimated from their distance of migration on the same gel. Determination of sizes of genomically rearranged RNA bands of the brv mutant D, A_4, B_4 and B_5 respectively was done in a similar way.

Finding the distances migrated in polyacrylamide gels by each RNA segment and reading the corresponding molecular size from a standard curve relating reference molecular weight of brv (UK Compton) segments (Rixon et al., 1984) and their mobility in gel the sizes of these rearranged bands of genomic RNA segments were estimated.

Densitometry (Whitton et al., 1983).

Quantitation of genome transcription, genome replication and protein synthesis was carried out by measuring optical densities of peaks from autoradiographs using a Joyce Loebl microdensitometer 3CS. Autoradiographs of gels which contained the bands of RNA or protein to be evaluated were randomly scanned through a range of calibrated wedge densities. A suitable wedge was that which would record a measurable peak of the most faint band and the heaviest/darkest band without overshooting the size of A4 paper.

Computation.

The densitometer tracings were placed on a digitising tablet (Summagraphic ID) and each peak was outlined with a stylus. The areas were calculated and compared by the computer programme DENS (written for the PDP-11 laboratory minicomputer by P. Taylor, MRC Virology, Glasgow).

One-dimensional peptide mapping (Cleveland et al., 1977).

Limited (partial) proteolytic analysis of rotavirus proteins was done as described by Dyall-Smith and Holmes (1981b) and Sabara et al. (1985), using the procedure previously reported by Cleveland et al. (1977). The [^{35}S] labelled proteins were separated on a single concentration 12% resolving gel (acrylamide:bis 75:1, Elliott et al., 1984). The dried gel was marked with dots containing [^{32}P] at three points on the periphery and autoradiographed. The dots on the autoradiograph were superimposed with the marks on the gel and the desired proteins bands cut out. The template gel was exposed before and after protein bands were cut out to confirm accuracy of protein band isolation. The gel slices were rehydrated in 0.125uM Tris-HCl pH 8.0 containing 1% SDS, for 40 minutes, trimmed to suitable sizes, loaded on a 3% stacking gel and topped up with 20ug V8 protease in protein dissociation buffer per slot. The samples were electrophoresed at 40 mA to half way in the stacking gel and the current turned off to allow partial digestion of the proteins to occur. The peptides were then resolved through a 15% acrylamide gel by all night electrophoresis at 50V and room temperature. Subsequently, the gel was fixed, treated with En^{3}Hance for 60 min., washed for 30 min. in cold water, dried and autoradiographed on Kodak X-Omat S film.

Two-dimensional oligopeptide mapping (MacDonald 1980).

To compare the VP5 and the mutant protein VP5A, the two-dimensional oligopeptide mapping technique described by

MacDonald (1980) was adopted. Gel pieces containing proteins to be compared were cut out of the template 12% polyacrylamide SDS-gel under guidance of an autoradiograph as described above. Then, the gel pieces were packed into a column 8cm long by 0.8cm diameter with 50ul of sample elution buffer containing 100 ug/ml of unlabelled BSA as carrier protein. Samples were electroeluted in the peptide running buffer at 100V constant for 16 hours at 4°C into a small dialysis bag containing 300ul of electrophoresis buffer attached at the bottom of the column. The eluates were adjusted to 2.5ml each sample and desalted by passing over a Sephadex G25 column bed volume of 9ml. Allowing the sample to sink in the column, 3.5ml of sterile distilled water per sample was added, and an equal volume was collected in three fractions: the pellets were three times washed in 1.5ml of acetone:acetic acid:triethylamine:water 17:1:1:1. After the pellets had been allowed to dry in air, trypsin digestion was started and left to proceed for 16 hours at 37°C using 10ug of TPCK treated trypsin per sample in 100ul ammonium bicarbonate buffer. Another round of trypsin digestion was continued the next day by adding fresh TPCK treated trypsin 5ul of 1mg/ml stock and incubated for a further 4 hours at 37°C. Pellets from this step obtained by lyophilisation were dissolved in 100ul of performic acid and kept at 4°C for $2^1/_2$ hours in order to oxidise cysteine and methionine residues. Subsequently samples were resuspended in 5ul of running buffer containing 1% xylene cyanol as a dye marker. Half of the sample in 0.5ul aliquots were spotted to a point 4cm from each of the

adjacent edges of the thin layer cellulose (TLC) chromatography plates of 20cmx20cm size. After each application the spot was dried in a current of cold air. Electrophoresis in one dimension was at 550V (constant voltage) for 45 minutes using acetic acid:formic acid:water 4:1:45 (pH 2.1) as running buffer. The chromatograms were then air dried at room temperature. Ascending chromatography in the second dimension (perpendicular to the direction of initial electrophoresis) was performed under a fume hood in a tank containing buffer acetic acid:butanol:water:pyridine 3:15:12:10 for 3-4 hours. The TLC chromatograms were air dried, treated three times with En^{3}Hance spray and autoradiographed against Kodak XOmat S film for 6 days at -70^{o}C. The remaining half of sample was used to produce maps of oligopeptide mixtures of the proteins under study by the same procedure as detailed above.

Raising antisera against brv.

New Zealand white rabbits were immunised according to the protocol of Thouless et al. (1982). Bovine rotavirus antigens were prepared from brv N and the brv mutants D and A_4 grown in burlers. The cells were disrupted by freezing and thawing and precleared by low speed (1500g) 10 min. centrifugation. The supernatants were concentrated at 115,000g through 30% w/v sucrose cushion and the virus pellets were resuspended in a small volume of sterile PBS. The genotype of each virus to be used in the immunisation was first checked by RNA-PAGE as described above after

Follett and Desselberger, (1983a). The volume of the virus suspension was adjusted to 2ml with PBS, then mixed with an equal volume of Freund's complete adjuvant and 2ml aliquots injected intramuscularly into the anteromedial side of both thighs. After 3 weeks, two intravenous injections of the same antigen without adjuvant were given via the earlobe vein in 4 weekly intervals. The rabbits were bled through the earlobe vein one week after boostering and the blood collected in 20ml universal bottles and left at 4°C for clotting. The clear serum was removed with a Pasteur pipette into a new universal, the remaining clot was centrifuged at 1,500g for 5 minutes. The supernatant serum was combined with the first harvest and centrifuged again to remove any cellular debris. Serum was aliquoted and stored at -20°C.

Radioimmunoprecipitation (RIP) of the brv antigens.

MA104 cells infected with standard brv and brv mutants D, A_4, B_4 and B_5 were labelled intracellularly with 7.4MBq/ml of [^{35}S] methionine in Eagle's MEM lacking L-methionine. Cells were gently washed in 3 one-minute changes of PBS, scraped into 1ml of lysis buffer, vortexed and cleared of cell debris at low speed (1,500g for 5 minutes). Before RIP the labelled antigens were reacted with half volume of normal rabbit serum 1:20 in order to remove any non specific reaction. In a 1.5ml eppendorf tube, 2 volumes (50ul) of precleared antigen were mixed with 1 volume of hyperimmune antiserum at 1:20 dilution and incubated for 3 hours or overnight at 4°C.

A suspension of formalin-fixed Staphylococcus aureus (Cowan strain 1) carrying the immunoprecipitin protein A was added (0.1ml for 0.02ml of antigen) to bind to the Fc portion of the antibody IgG thereby increasing the size of the antibody/antigen complexes. The mixtures were kept at 4°C for a further hour. Immunoprecipitates were collected by spinning samples at 11,000 g for 20 seconds and discarding the supernatant. The pellets were washed three times in 0.6ml IP wash buffer. The final pellet was taken up in 50ul of protein dissociation buffer, vortexed and heated to 100°C for 5 minutes. The sample was then centrifuged at 11,000g for 5 minutes and the supernatant was used for SDS-PAGE analysis.

RESULTS

1. Genome heterogeneity among cocirculating human rotaviruses (hrv).

Stool samples of children presenting with diarrhoea at St. Thomas's Hospital, London, East Birmingham Hospital and Ruchill Hospital, Glasgow, were examined by electron microscopy and those found to be positive for rotaviruses were extracted for RNA; the RNAs were analysed by RNA-PAGE and visualised after silver staining (Herring et al., 1982, Follett and Desselberger, 1983[a]). One hundred (100) samples of rotavirus positive faeces collected between 1975 and 1983 were analysed. In Figure 1.1, RNA profiles of the human rotaviruses (hrv's) representing fifteen different electropherotypes are shown and were designated Long (L) and Short (S) electropherotypes with variants denoted by a small letter subscript according to Follett et al., 1984. Figure 1.2 presents a schematic diagram of the RNA electropherotypes shown in Figure 1.1 [and the electropherotypes L, Lg and Sd.] With the exception of electropherotypes Ld, S and Sa the majority (10 out of 13) of hrvs showed electropherotypes differing from those observed by Follett et al. (1984). The distribution of hrv's in the 8 year period was such that those of "long" electropherotypes were more prevalent than those of "short" electropherotypes in proportion 4:1 as shown in Table 1.1. The various long and short electropherotypes cocirculated during each of the 8 year period studied as previously reported in Glasgow epidemics (Follett and Desselberger, 1983a, Follett et al., 1984). The hrv's of Ld

Figure 1.1.

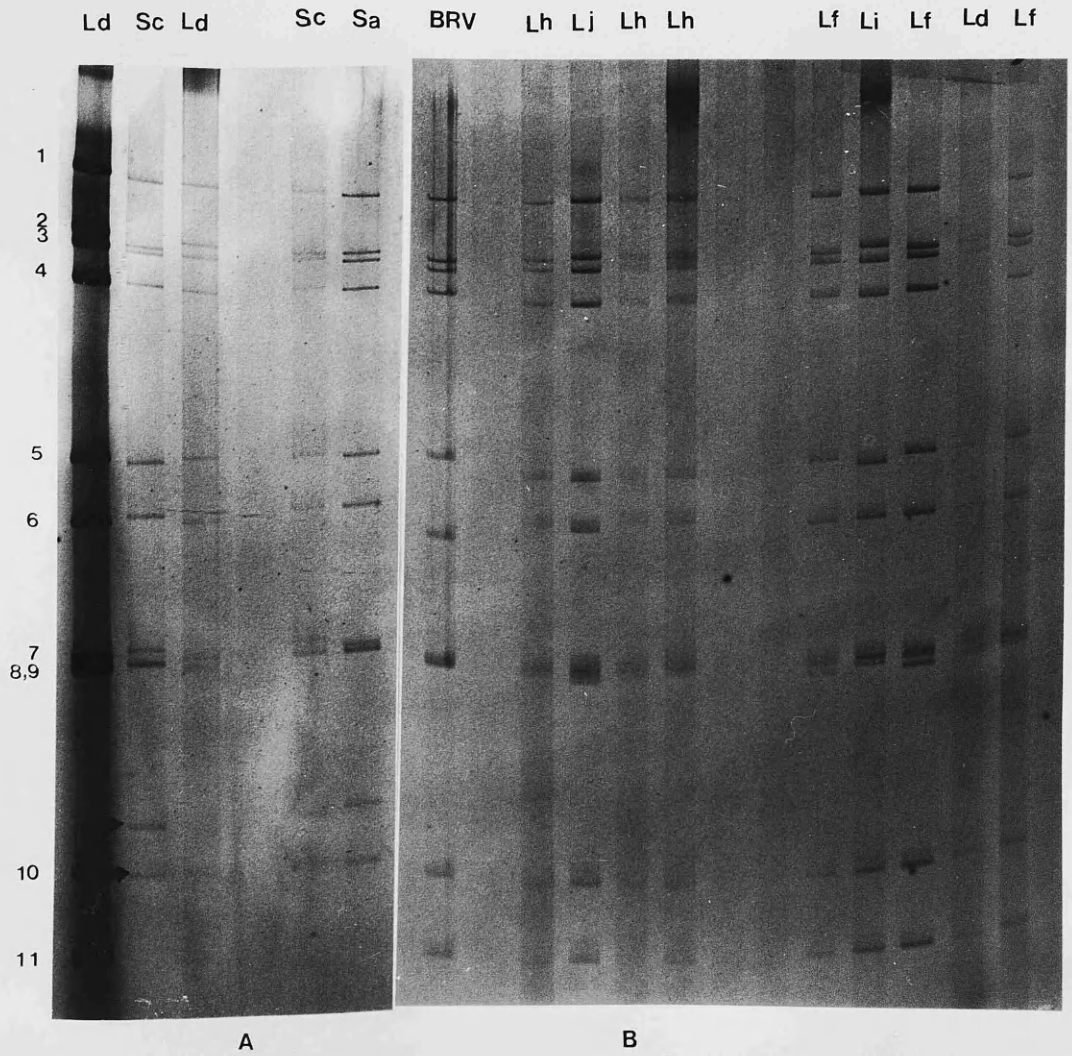
Heterogeneity of hrv genomes.

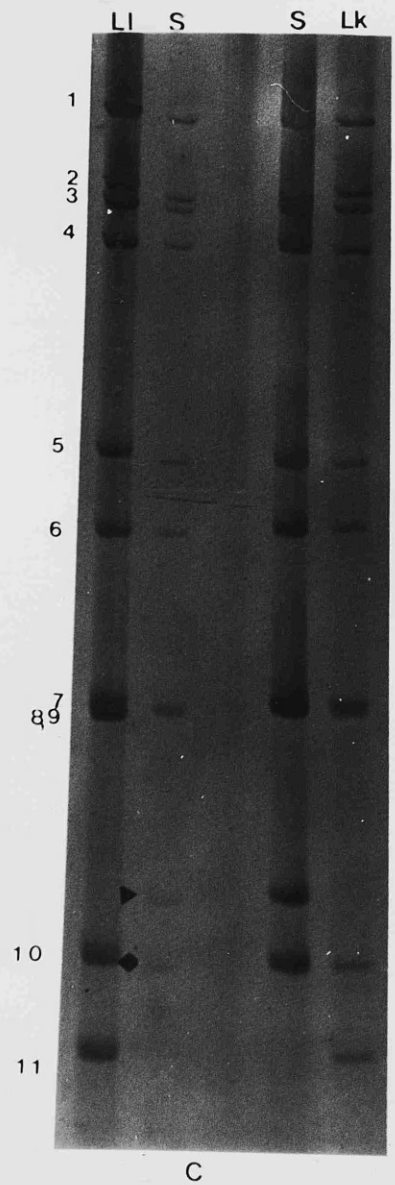
The 2.8% polyacrylamide 6M urea gels show the RNA profiles (electropherotypes) of hrvs obtained from children suffering from acute diarrhoea at the St. Thomas Hospital, London, Regional Virus Laboratory, East Birmingham Hospital and the Regional Virus Laboratory, Ruchill, Glasgow.

The RNAs shown in panels A, B, C and D are representative hrv electropherotypes observed over the period 1975-1983. The RNAs were extracted and the segments visualised after RNA-PAGE and silver staining as described in Methods. Genomic RNA segments are numbered 1-11 on the left, letters L and S denote "long" and "short" electropherotypes and the small letter subscript denotes a variant of the main RNA electropherotype. The designation of the electropherotypes was taken and extended from that described by Follett et al. (1984).

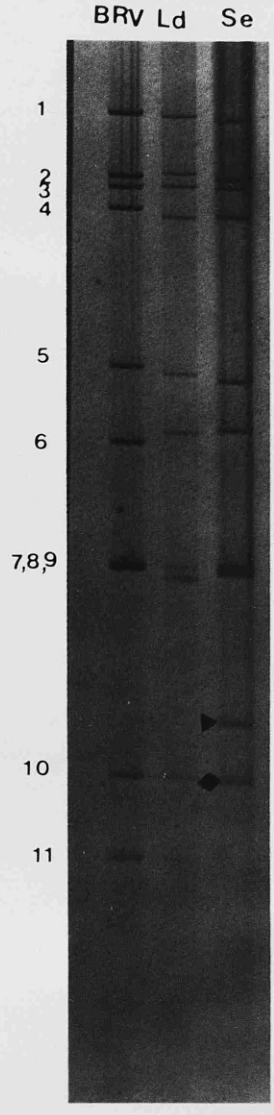
The arrow (▶) indicates RNA segment 10 and the diamond (◆) RNA segment 11 of the short electropherotype.

BRV denotes RNA profile of the reference bovine rotavirus.


Ld
Sc
Ld
Sc
Sa
BRV
Lh
Lj
Lh
Lh
Lf
Li
Lf
Ld
Lf
1
2
3
4
5
6
7
8,9
10
11
A
B



C



D

Figure 1.2.

Diagram of RNA electropherotypes which were observed among the genomes of hrvs obtained at St. Thomas Hospital, London, Regional Virus Laboratory, East Birmingham Hospital, Regional Virus Laboratory, Ruchill Hospital, Glasgow between 1975 and 1983. The letters L and S denote long and short electropherotypes, respectively, and the small letter subscript indicates the variation from the reference electropherotypes. The designation of RNA electropherotypes is according to Follett et al. (1984) and in extension of that publication. L was the reference (prototype) long electropherotype (Follett et al., 1984). BRV denotes bovine rotavirus electropherotype.

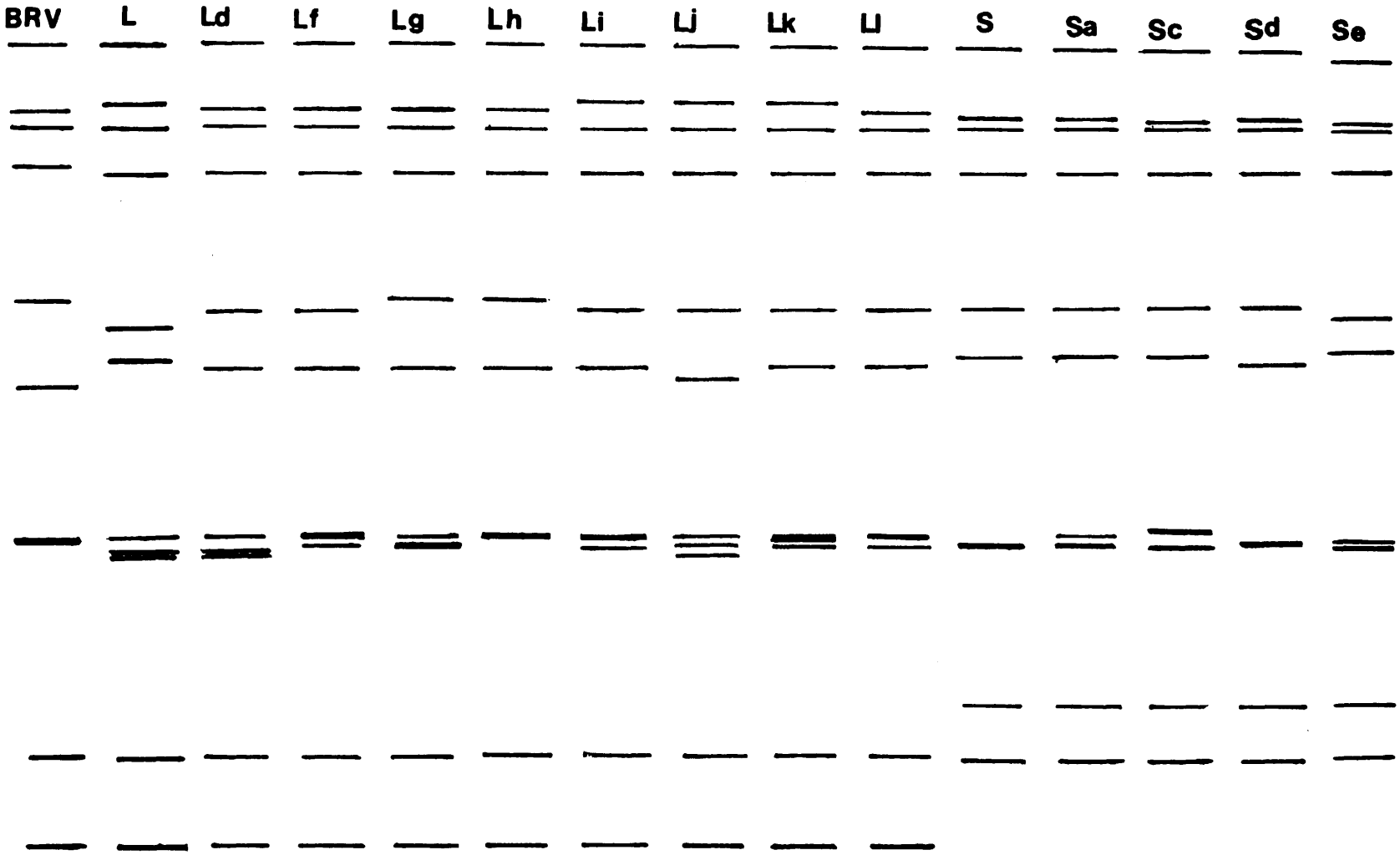

BRV
L
Ld
Lf
Lg
Lh
Li
Lj
Lk
Ll
S
Sa
Sc
Sd
Se

Figure 1.3.

RNA profile of human rotavirus isolate 5485.

RNA extraction, electrophoresis and silver staining were as described in Legend to Figure 1.1. The arrow points to the extra genomic RNA segment in panel A. An enlargement of the bracketed area on panel A is shown in panel B below.

Included on the gel were RNAs of standard brv (track BRV), hrv isolates 4934, 5655, 6574 and M83 as indicated on top of the gel.

The RNA segments (1-11) were numbered from top to bottom to the left of panel A.

A
M83
5574
5655
5485
4934
BRV
1
2
3
4
5
6
7
8
9
10
11
B

Year	RNA Electropherotype --> Ld	Lf	Lg	Lh	Li	Lj	Lk	Ll	S	Sa	Sc	Sd	Se	Positive by: RNA-PAGE	E.M.
75														0	2
77	1	1	1											3	4
78			1	7					1					9	17
79	3	3		3	1	1	1	1					2	15	23
80	5	1		1				1		1	2			11	16
81	6													6	7
82	2													2	2
83	7								6			1		14	29
TOTAL	24	5	2	11	1	1	1	2	7	1	2	1	2	60	100

Table 1.1. Electropherotypes of human rotaviruses

The genomes of hrvs found in diarrhoea cases admitted to hospitals in London and Glasgow between 1975 and 1983 were analysed by RNA-PAGE and silver staining techique (Herring et al., 1982; Follett and Desselberger, 1983a; Follett et al., 1984). The letters L and S denote the "long" and "short" eletropherotypes and small subscripts indicate a variant of either L or S eletropherotypes according to Follett et al. (1984).

electropherotype were most represented (24% of total) and appeared to have been more persistently maintained in the population than any other variant electropherotype. The electropherotypes Lf, Lg, Lh, Li, Lj and Lk appeared between 1977 to 1980, and were not seen during the period 1980-1983. The short electropherotype Viruses were found to be distributed inconsistently over the years; unlike the long electropherotype Viruses which were regularly found. The variants Sa and Sc; S and Sd, appeared together in 1980 and 1983 respectively and during 1983 the ratio of short to long reached 1:1. Detecting rotavirus by RNA-PAGE and silver staining was 60% (60 out of 100) sensitive compared with electron microscopy, Table 1.1.

Among the stool samples studied, only one sample, no. 5485 (obtained in Birmingham in 1983 from a patient with diarrhoea), was found to contain rotavirus the genome of which separated into apparently twelve RNA segments as shown in Figures 1.3 A and B. Hereby we confirmed an observation originally made by Dr. R. Sanders , Regional Virus Laboratory, Birmingham, who had sent us this specimen. An interpretation of this finding is attempted under Discussion.

2. RNA profiles of the genomically rearranged brv mutants.

The RNA profiles of bovine rotavirus mutants on which most of the investigations of this thesis were done are shown in Figure 2.1. The mutants were produced after serial passage of the standard bovine rotavirus (brv; UK Compton strain) at high multiplicity of infection ($\geq$ 100 pfu/cell) as described previously by Hundley et al., (1985).

Figure 2.1.

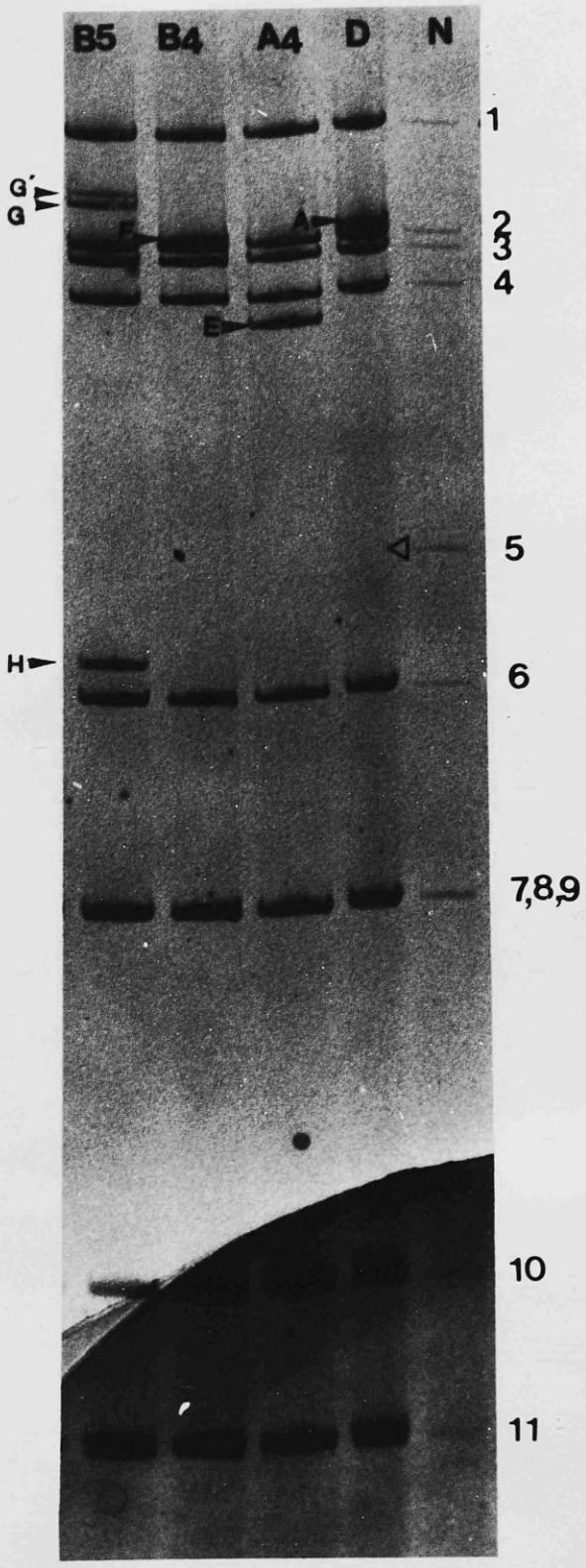
RNA genomes of plaque purified bovine rotaviruses obtained after serial passages at high m.o.i. (Hundley et al., 1985).

Track N shows the genotype of the standard brv (UK Compton strain) used in the study as reference electropherotype.

Track D, A_4, B_4 and B_5: Genome of twice plaque purified brv mutants D, A_4, B_4, B_5, respectively.

The brv mutants purified by plaque purification were amplified to raise virus stocks by growing plaque isolates in cells under liquid overlay. The RNA of purified viruses was extracted and separated on 2.8% polyacrylamide gel using Loening's buffer system (Loening, 1967; Follett and Desselberger, 1983a). Electrophoresis was from top to bottom at 120V for 16 hours. The gel was silver stained (Herring et al., 1982; Follett and Desselberger, 1983a). The numbering of the genomic RNA segments (1-11) was in order of decreasing size.

Arrows ► point to the rearranged RNA bands A in brv D, E in brv A_4, F in brv B_4, G', G and H in B5. The missing genomic RNA segment 5 in brv mutants D, A_4, B_4 and B_5 is shown by an open arrow ▷


B5
B4
A4
D
N
G'
G
F
A
E
H
1
2
3
4
5
6
7,8,9
10
11

Plaque purified brv (= standard brv) was used to infect MA104 cells, the infection allowed to proceed to complete CPE and the virus concentrated by ultracentrifugation of the preclarified culture supernatant through a sucrose cushion at 115000g for 90 minutes. The virus pellets were resuspended into small volume PBS and used to infect new cells on 50mm petri dishes. After nine to eleven such passages, the brv genome showed complete disappearance of RNA segment 5 and the emergence of new RNA bands A-H in the profile. The variants were called mutants D (possessing band A), A_4 (band E), B_4 (band F) and B_5 (bands G, G', H) [all Figure 2.1] were further investigated after several rounds of plaque-to-plaque purification which had shown that the new variants are not defective in replication (Hundley et al., 1985; and see below). The RNA bands were all distributed between segments 1 and 5 except for band H which appeared just before segment 6. When measured for size, the RNA bands were in order A = 1.82, E = 1.48, F = 1.76, G' = 1.94, G = 1.91 and H = 0.90 times the size of RNA segment 5 (= 1). In a standard brv genome of 18,683bp (Rixon et al., 1984) the rearranged RNA bands A-H of brv mutants represented amounts of RNA to the extent of 6.7, 3.9, 6.1 and possibly 30.5% of the size normally packaged. The high percentage of 30.5% needs further investigations (see Discussion).

3. Evidence for genome rearrangements.

Genome characterization of brv mutant D, Figure 2.1, indicated that RNA band A was dsRNA as judged from its

Figure 3.1.

Preparative polyacrylamide gel for isolation of RNA pieces.

Electrophoretic separation of the genomic RNA of brv (UK Compton strain) and the brv mutant A_4 was achieved on a preparative 2.8% polyacrylamide 6M urea gel. The gel was stained in 1xTBE buffer containing 4ug/ml ethidium bromide. The positions of the RNA segment 5 and the rearranged RNA segment E are indicated by arrows in panel A, tracks N and A_4, respectively. Panel B shows the same gel after cutting out gel pieces containing RNA segment 5 and band E.

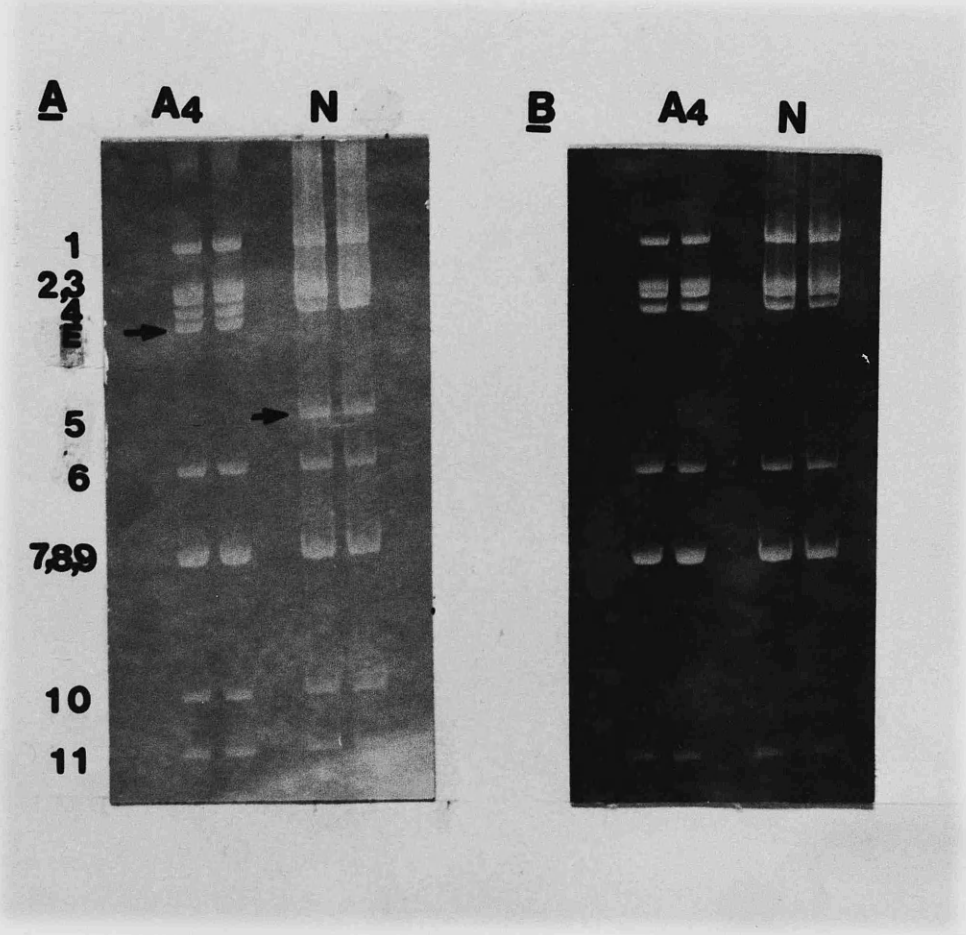
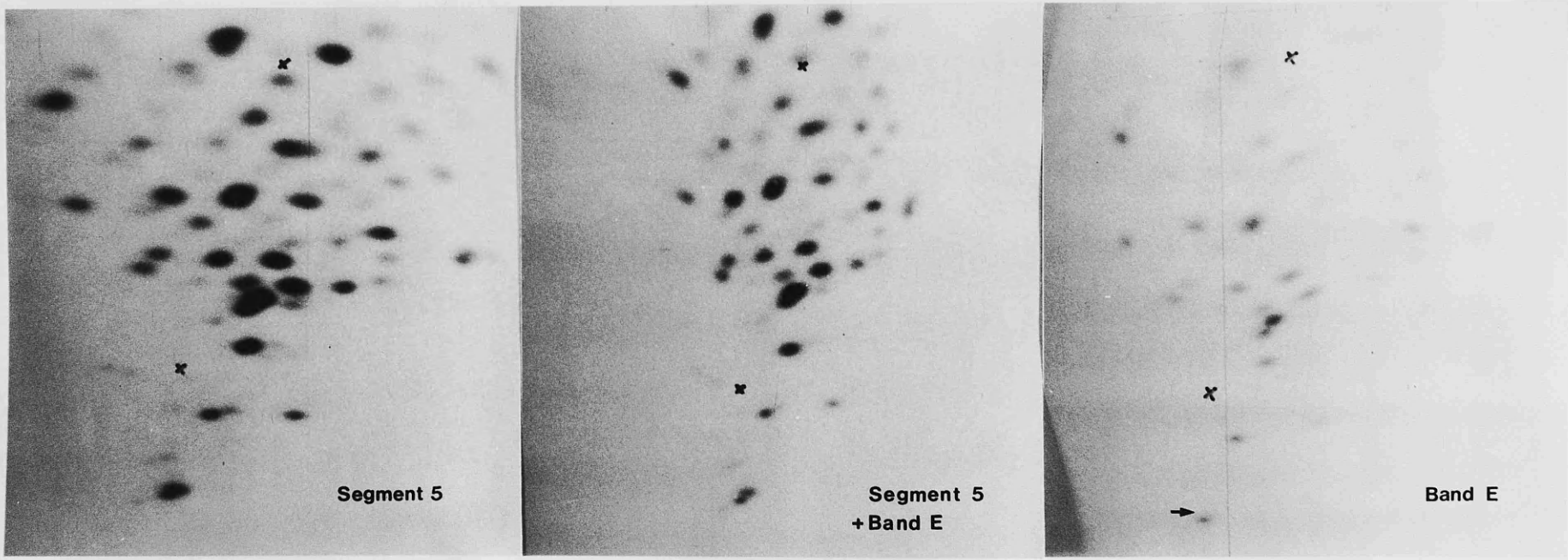

A
A4
N
B
A4
N
1
2
3
4
5
6
7,8,9
10
11

Figure 3.2.

T_1-oligonucleotide maps of the RNA segment 5 of standard brv and the rearranged RNA segment E of brv mutant A_4.

The two-dimensional maps of the RNAse T_1-resistant oligonucleotides generated from RNA segment 5 and band E are shown. The mixture of the two is presented in the middle. The RNAs were electroeluted from the gel slices (Figure 3.1) and were recovered by ethanol precipitation.
Two-dimensional oligonucleotide maps were prepared as described in Methods. The separation of [^{32}P]-labelled, T_1-resistant oligonucleotides was from left to right in the first and from bottom to top in the second dimension. The dye markers xylene cyanol FF and the faster migrating bromophenol blue are indicated by crosses (X).


Segment 5
Segment 5
+Band E
Band E

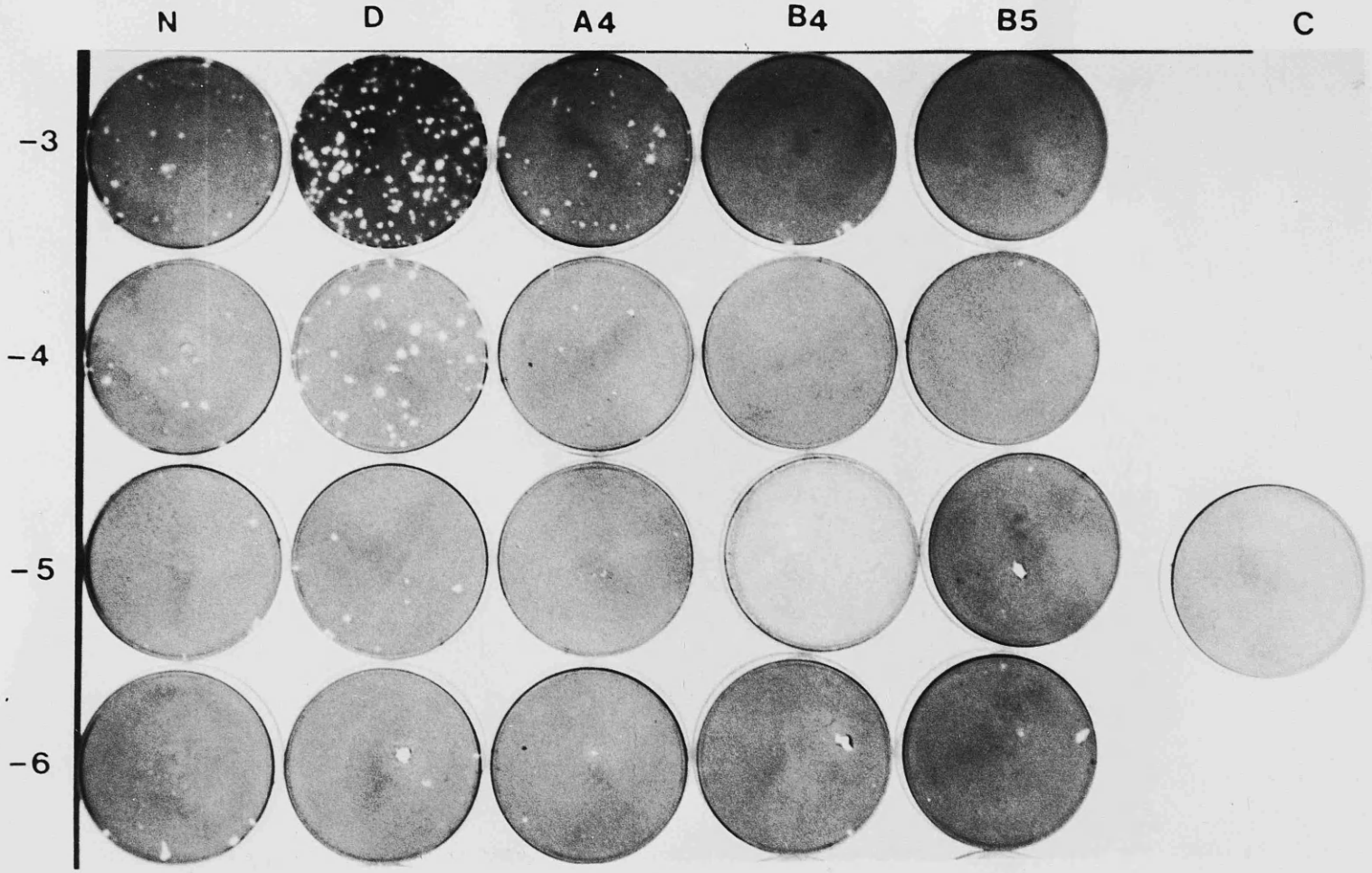
reaction with deoxyribonucleases and RNase T_1 with and without prior denaturation of the RNA (Hundley et al., 1985). The relative position of bands A and E was unchanged when the RNAs were electrophoresed under denaturing conditions in methyl mercuric hydroxide agarose gels (Hundley et al., 1985). Comparative mapping of RNase T_1 resistant oligonucleotides had shown that band A of brv mutant D and RNA segment 5 of the standard brv were very similar in their large oligonucleotides. The data thus had indicated that band A of mutant D was rearranged from RNA segment 5 of standard brv by concatemer formation (Hundley et al., 1985).

The RNase T1-resistant oligonucleotides of RNA band E of brv mutant A4 and of RNA segment 5 of standard brv were produced from isolated RNAs which were obtained by electroelution from gel pieces (Figure 3.1) and compared by two-dimensional gel electrophoresis (Figure 3.2). The resulting oligonucleotide maps were very closely related in the large unique oligonucleotides: there was a notable absence of only one large oligonucleotide in the T_1 map of RNA segment E when compared to the map of RNA segment 5 (arrowed in figure 3.2). The observation provides further evidence that RNA band E of brv mutant A4 had arisen by genome rearrangement of the normal RNA segment 5. The RNA rearrangements produced high molecular weight RNA molecules which however retained RNase T_1- cleavable RNA sequences in common with RNA segment 5 of standard brv.

Figure 4.1.

Plaque morphology of the brv mutants.

The virus stocks of plaque purified standard brv and of brv mutants D, A_4, B_4 and B_5 were used in a plaque assay at dilutions 10^{-3} to 10^{-6} (shown on the left). The inoculum was 0.3ml of virus suspension on 50mm dishes of MA104 cells, and the plaque assay set up as described in Methods. Plaques were counted after 7 days of incubation at 37°C and after cells were fixed with glutaraldehyde (2.5% in PBS) and stained with Giemsa solution as described in Methods.


N
D
A4
B4
B5
C
-3
-4
-5
-6

Virus	Log pfu/ml at temperature 31°C	37°C	39.5°C	Log VPC/ml	Specific infectivity at 37°C
N	7.00	7.30	7.52	9.29	1.99
D	5.82	6.78	6.82	8.17	1.39
A_4	5.82	6.52	7.08	8.38	1.86
B_4	4.52	5.82	6.52	7.52	1.70
B_5	4.82	5.82	6.52	7.67	1.85

Table 4.1. Effect of temperature on infectivity of brv mutants.

The infectivity of standard brv (N) and of brv mutants D, A_4, B_4 and B_5 was measured in a plaque test as described in Methods and is given as $\log_{10}$ values of pfu/ml at temperatures 31°C, 37°C and 39.5°C. The numbers of virus particles (VPC/ml) were counted by direct E.M. according to Follett and Desselberger (1983a). Specific infectivity was calculated by subtracting the log pfu/ml values obtained at 37°C from the log VPC/ml values.

4. Infectivity at different temperatures, specific infectivity and plaque morphology of brv mutants.

The infectivity of the brv mutants and whose genomes are shown in Figure 2.1 was determined in plaque assays using six times plaque-purified brv D and brv A4 and twice plaque-purified brv mutants B4 and B5; standard brv was titrated for comparison. The virus titres (pfu/ml) recorded at 31°C, 37°C and 39.5°C are shown in Table 4.1. For all brv mutants, the titre was lowest at 31°C. imtermediary at 37°C and was highest at 39.5C. Using the criteria of Greenberg et al (1981) whose ts mutants differed by >2.7 log units in titres between 31C and 39C our brv mutants were not temperature-sensitive. (See Discussion p 127)

Specific infectivity (i.e. the virus particle (vp) : infectivity ratio) for brv mutants and standard brv was determined. In Table 4.1, the $\log_{10}$vp/pfu values are given (using the pfu titres obtained at 37°C). The brv mutants showed specific infectivities ranging from 1.39 to 1.99 log units (corresponding to 25 to 95 virus particles per pfu) and were very close to the vpc/pfu ratio of the standard brv. Thus, the lower infectivity observed with some of the genomically rearranged brv mutants was not dependent on vpc/pfu ratios.

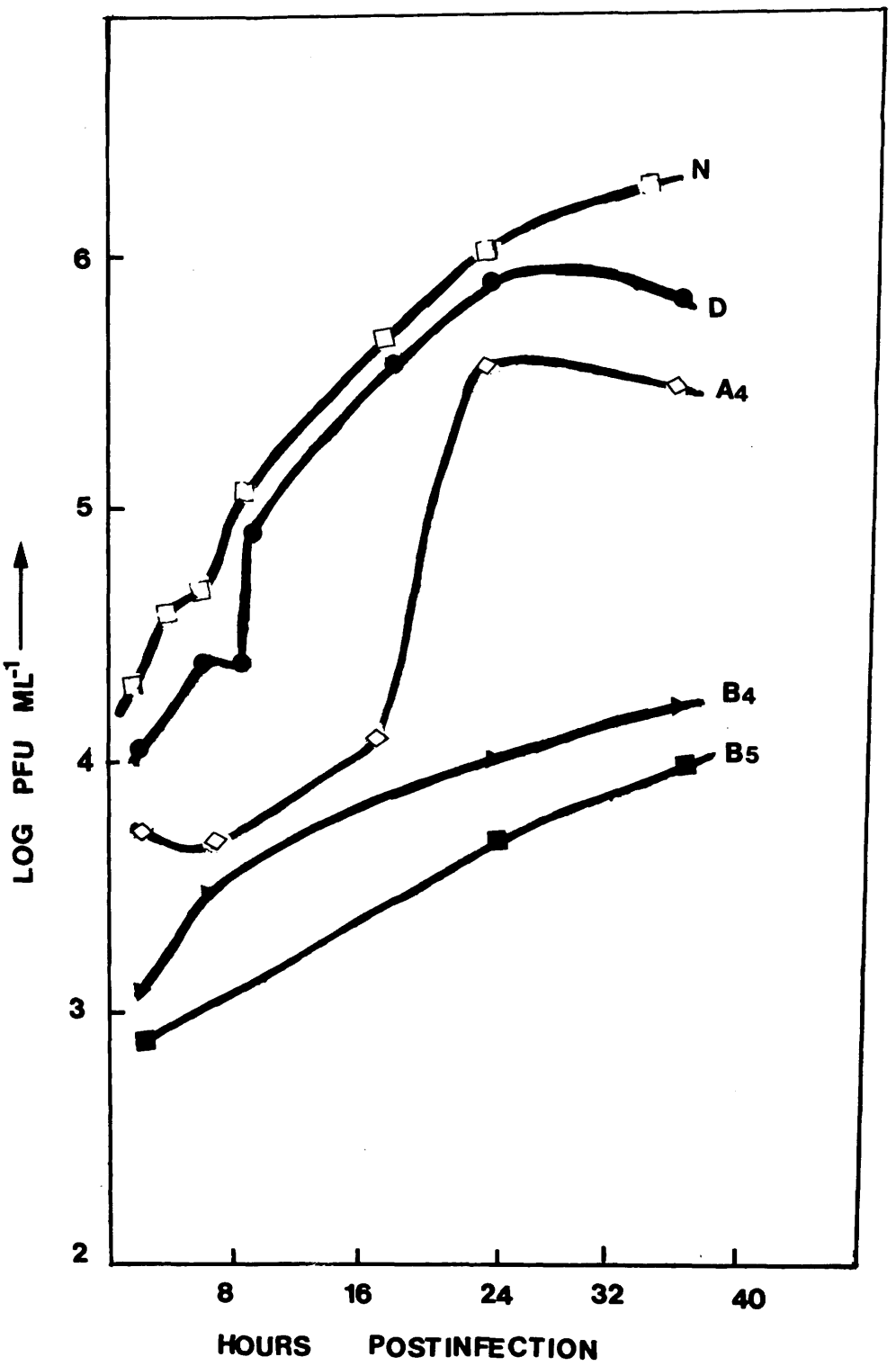
The brv mutants differed in plaque size (Figure 4.1). Plaques formed by the brv B_4 and B_5 mutants were pin point ($\leq$ 0.5mm) and difficult to visualise with the naked eye; the plaques of brv mutant A4 measured 1-2mm diameter and were smaller than those of standard brv and the brv mutant D which measured 2-4mm.

Figure 5.1.

Virus growth curves for standard brv and of brv mutants D, A_4, B_4 and B_5.

Standard brv and the virus mutants D, A_4, B_4 and B_5 were plaque titrated at 37°C using virus suspensions obtained from harvested cell cultures at intervals of 2, 4, 8, 16, 24 and 36 hours post infection. Plaques were counted after 7 days of incubation and infectivity titres were scored in $\log_{10}$ pfu/ml. The curves are marked N, D, A_4, B_4 and B_5 to indicate the growth pattern produced by standard brv N and the respective brv mutants.

The growth cuve of brv mutant A4 looks atypical on this figure but was found to be similar to that of brv mutant D in other experiments.


N
D
A4
B4
B5
6
5
4
3
2
LOG PFU ML-1
8
16
24
32
40
HOURS POSTINFECTION

5. Growth curves.

Cell cultures were infected at m.o.i. of 5-10 pfu/cell and growth curves obtained at 37°C for the brv mutants D, A4, B4 and B5 as for standard brv. The infected cells were harvested at intervals of 2, 4, 8, 16 and 24 hours pi and kept frozen. The viruses were plaqued out on 50mm petri dishes of MA104 cells under 0.5% agar overlay containing 5ug/ul pancreatin and infectivity titres were scored. Infectivity (pfu/ml) was plotted against the time of infection (Figure 5.1). The rate of production of infectious virus progeny indicated that at no time was the growth of brv mutants greater than that of standard brv. Whereas the growth curves of brv mutants D and A_4 were similar to that of standard brv, the brv mutant B4 and B5 produced infectious virus less efficiently than either standard brv or the brv mutants D and A4.

6. RNA synthesis in infected cells.

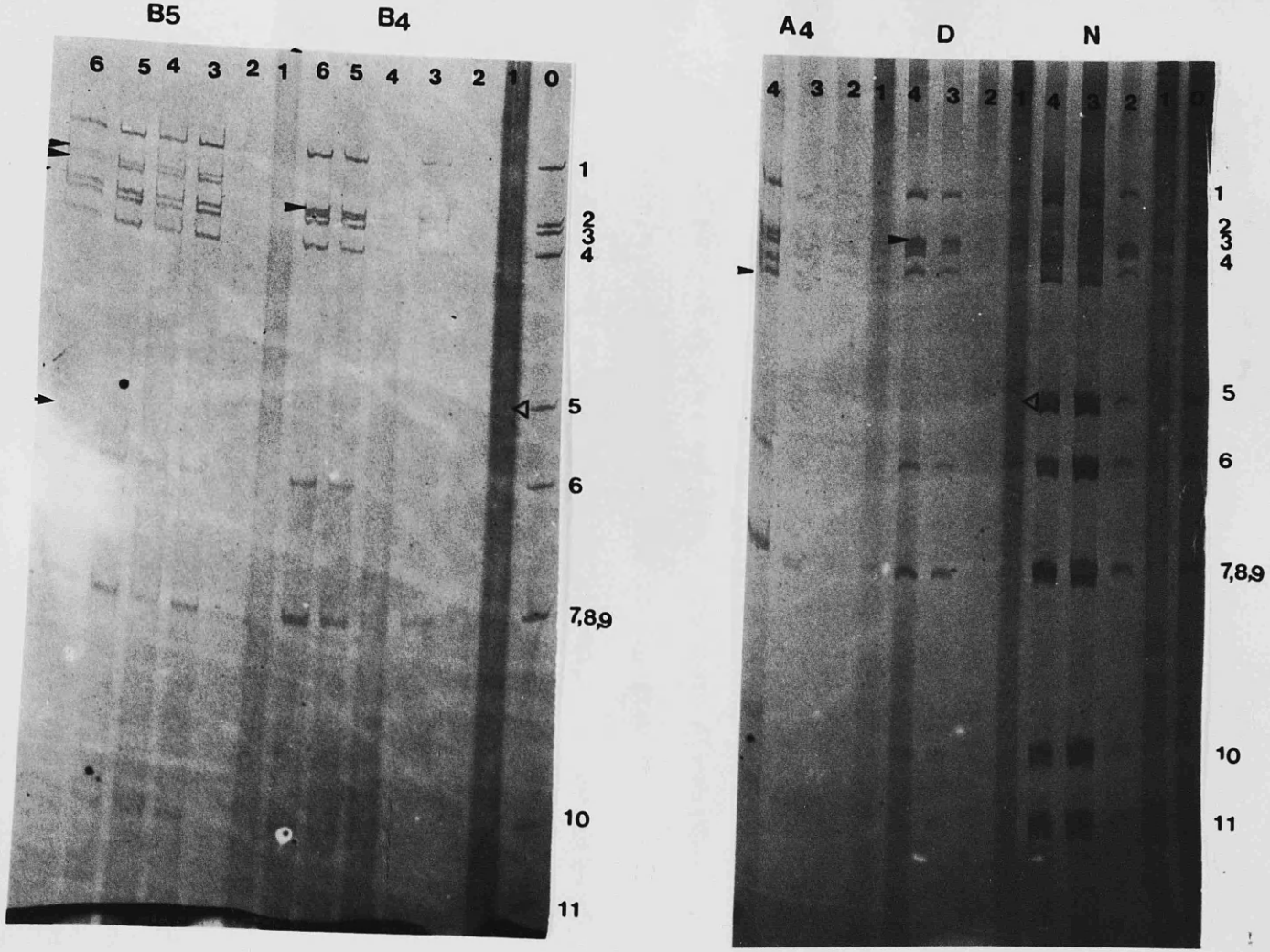
Confluent cell monolayers on 50mm petri dishes were infected at m.o.i.s of 5-10 pfu/cell. After 4, 8 16, 24 and 36 hours, the infected cells were frozen and thawed once. Viral RNA was extracted from supernatants after cell debris was cleared by low speed centrifugation and separated on 2.8% polyacrylamide 6M urea slab gels which were silver-stained. Genomic RNAs were detected on silver stained gels at 8 hours pi for all brv mutants as well as for standard brv (Figure 6.1) and in every case RNA synthesis was observed to increase with the duration of infection. At 8 hours pi both the standard brv and brv

Figure 6.1.

RNA synthesis in infected cells.

Confluent cell monolayers were infected at 5pfu/cell with the standard brv and the brv mutants D, A_4, B_4 and B_5 indicated on top of the gels. The RNAs extracted from samples harvested at 4, 8, 16 and 24 hours post infection (tracks 1, 2, 3 and 4, respectively) were analysed by RNA-PAGE and silver stained. Cells infected with the brv mutants B_4 and B_5 were allowed to continue to complete CPE at 72 hours pi. The RNAs were extracted and are shown in tracks 5 and 6 of panels B_4 and B_5 representing 32 and 72 hour time points.

The arrows indicate the positions of rearranged RNA bands G', G and H_l (very weak) in B_5; F in B_4; E in A_4; and A in D. The open triangles (▷) show the position of the missing genomic RNA segment 5. Track N: RNA of the standard brv. Genomic RNA segments were numbered from top to bottom to the right of gels.


B5
B4
6 5 4 3 2 1 6 5 4 3 2 1 0
1
2
3
4
5
6
7,8,9
10
11
A4
D
N
4 3 2 1 4 3 2 1 4 3 2 1 0
1
2
3
4
5
6
7,8,9
10
11

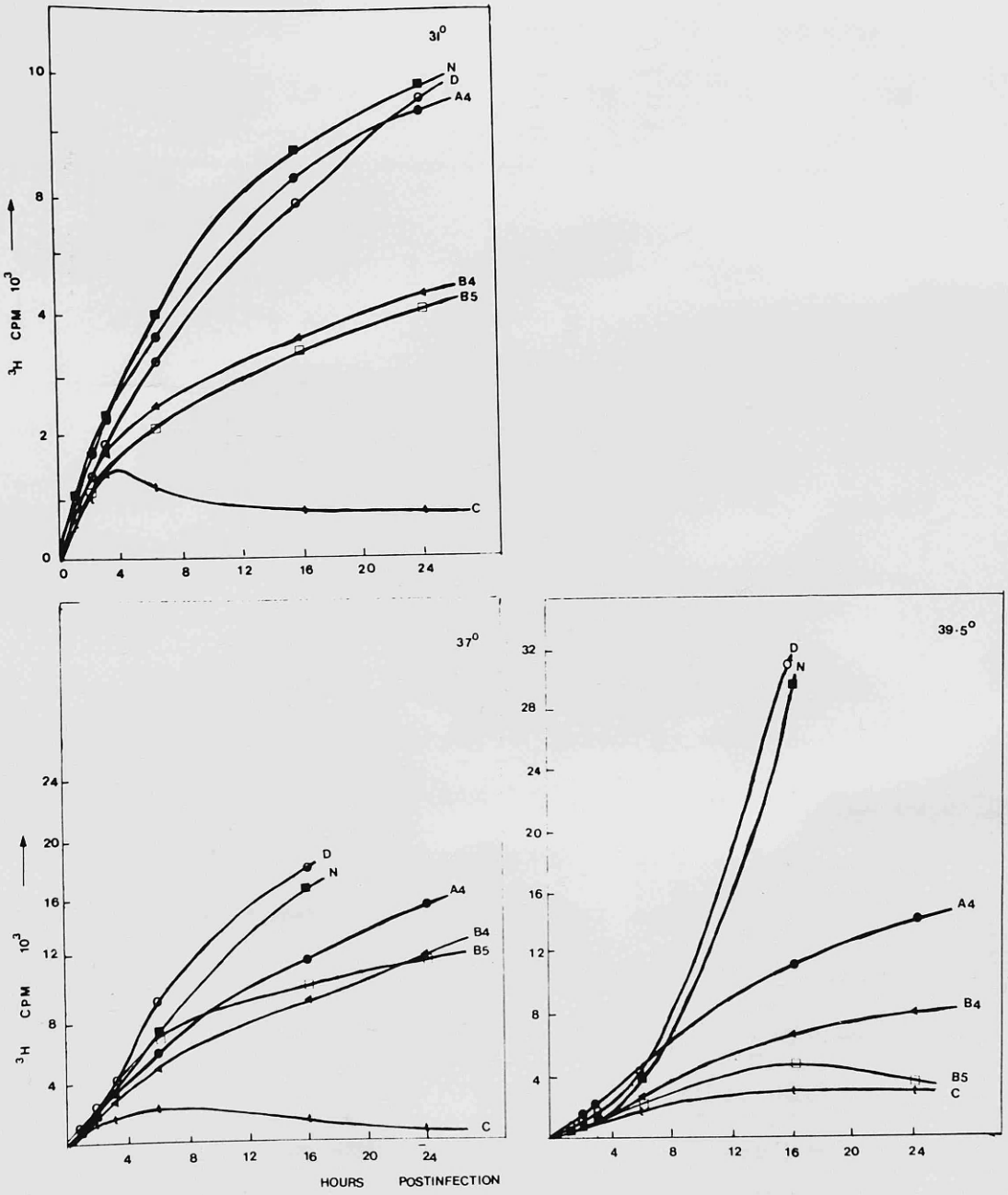
mutant D had replicated their genome more than brv mutants A_4, B_4 and B_5 at the same time, as estimated from the intensity of silver staining of nucleic acid. The maximal silver staining of RNAs extracted from cells infected with the standard brv and the brv mutant D occurred at 16 hours pi, whereas a similar peak of silver staining pattern for brv mutants A4, B4 and B5 appeared later than 24 hours pi (Figure 6.1, track 4 of panel A4 (32hr pi) and tracks 5 and 6 (48 and 72 hrs pi) in panels B4 and B5).

Viral RNA synthesis in infected cells was studied by monitoring incorporation of (5,6-^{3}H) uridine into newly synthesised RNA molecules during the transcription and replication of viral genomes. Cell monolayers in 24 well Linbro plates were infected with standard brv and the brv mutants D, A_4, B_4 and B_5 at m.o.i.s of 5-10 pfu/cell. Viral RNA synthesis was observed in the presence of 5ug/ml actinomycin D to inhibit cellular RNA synthesis. The RNA was labelled in a medium containing 0.37 MBq of (5,6-^{3}H) uridine from 1 hour pi until harvest time. At intervals of 30 min., 60 min., 90 min., and 3, 6, 16 and 24 hours after addition of radiolabelled uridine, samples were harvested in RNA solubilisation buffer (1% SDS, 8M urea, 0.5% β 2-mercaptoethanol) and assayed for TCA precipitable counts (Szilagyi and Pringle, 1979). Uninfected cell controls were treated the same way and base line values were recorded. The graphic presentation of the pattern of viral RNA synthesis at 31, 37 and 39.5^{o}C is given in Figure 6.2. It can be seen that the rate of RNA synthesis increases with rising temperature. In this assay, brv mutant D and

Figure 6.2.

Time course of RNA synthesis in MA104 cells infected with standard brv and brv mutants.

Cell monolayers (10^5 cells per well in Linbro plates) were infected at m.o.i. of 5-10 pfu/cell in the presence of 5ug/ml of actinomycin D and 0.37MBq of [5,6^{3}H] uridine. Samples were harvested at intervals of 30, 60, 90 minutes, 6, 18, and 24 hours pi as indicated on the horizontal axis. Total RNA was extracted and assayed according to Szilagyi and Pringle, 1979. The TCA precipitable counts of [5-6^{3}H] uridine representing total cell RNA were plotted on the vertical axis against the time of RNA synthesis shown on the horizontal axis in hours post infection. The figure also shows RNA synthesis in uninfected cells (curve C). The curves of RNA synthesis in virus-infected cells at each of the temperatures 31^{o}C, 37^{o}C and 39.5^{o}C are marked N, D, A_4, B_4 and B_5.


31°
37°
39·5°
N
D
A4
B4
B5
C
^{3}H CPM 10^3
HOURS POSTINFECTION

Temperature -->	31°C		37°C		39.5°C	
Hours -->	4	16	4	16	4	16
N	1.00	1.00	1.00	1.00	1.00	1.00
D	0.75	0.90	1.20	1.10	1.60	1.06
A_4	1.00	0.93	0.64	0.64	2.60	0.33
B_4	0.41	0.49	0.55	0.50	1.00	0.15
B_5	0.30	0.46	1.00	0.42	1.00	0.10

Table 6.1 Relative rates of intracellular RNA synthesis.

The RNAs synthesised in cells infected with 5-10 pfu/cell of standard brv (N) and of brv mutants D, A_4, B_4 and B_5 were assayed as TCA precipitable counts (Szilagyi and Pringle, 1979) at 4 and 16 hours pi for each virus and at the temperatures indicated. RNA synthesis of standard brv was taken as reference (equalling 1.00) and RNA synthesis of the mutants was expressed as proportion of that unit. Background counts from uninfected cells were subtracted before the ratios were calculated.

standard brv directed similar amounts of RNA synthesis, while the rest of brv mutants synthesised less RNA. Comparable RNA synthesis was observed by 4 hours pi at each of the temperatures; subsequently the differences in RNA synthesis indicated above became marked with increasing duration of infection (Figure 6.2). In Table 6.1, the values of RNA synthesised at 4 hours pi, and at 16 hours pi were compared using the rate of RNA synthesis in standard brv infected cells as a standard. At 4 hours pi the amounts of RNA produced in cells infected with brv mutants B_4 and B_5 were judged to be significantly lower at 31°C than that of the standard brv, the difference in RNA synthesis became marked by 16 hour pi, especially at the elevated temperatures (Figure 6.2). The relative rate of RNA synthesis in cells infected with brv mutants A_4, B_4 and B_5 at 16 hours pi was 3 times less at 31°C and 37°C than the corresponding values for standard brv. The difference between standard brv and mutants A_4, B_4 and B_5 RNA synthesis was most exaggerated by 5 to 10 times at 39.5°C, but the observed difference in RNA synthesis between brv mutant D and standard brv was not significant (Table 6.1). The foregoing experiments provide evidence that genome rearrangements affecting the genomic RNA segment 5 (Hundley et al., 1985) can influence the rate of overall RNA synthesis.

In order to get more detailed information on viral RNA synthesis the following experiment was performed. Confluent monolayers of MA104 cells in 50mm petri dishes were infected with the brv mutants D and A_4 in which the RNA segment 5 was rearranged into RNA bands A and E,

Figure 6.3.

RNA replication of standard brv and of the brv mutants D and A_4.

Cell monolayers were infected with standard brv and brv mutants D and A_4 and incubated for 16 hours in a phosphate free medium containing 5ug/ul actinomycin D, to which 14.8 MBq of [^{32}P] orthophosphate were added. RNA was extracted and analysed by RNA-PAGE and autoradiography. Genomic RNA segments were numbered 1 to 11 from top to bottom. Filled in arrows (➔) point to the position of rearranged RNA bands A of brv mutant D and of RNA band E of brv mutant A_4. Empty arrows (▷) indicate position of genomic RNA segment 5 in brv mutants D and A_4. C denotes extract of mock-infected cells.

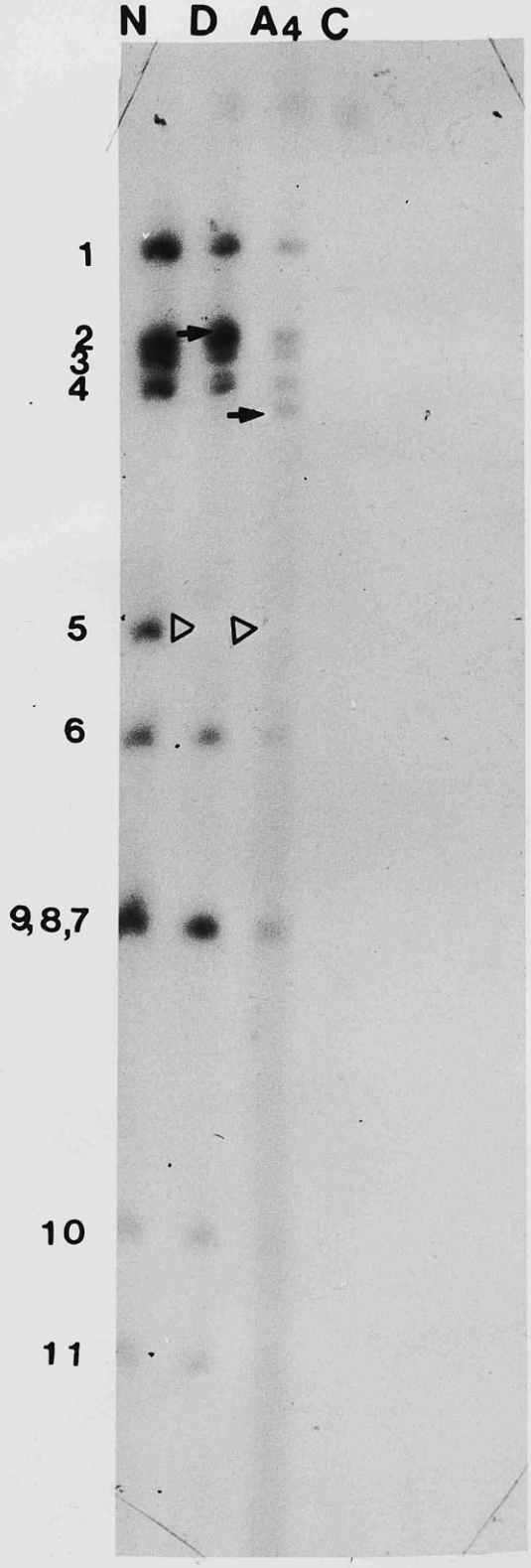

N
D
A4
C
1
2
3
4
5
6
9,8,7
10
11

Figure 6.4.

Densitometer tracings of RNAs shown in autoradiogram of Figure 6.3.

RNAs shown in the autoradiograph of Figure 6.3 were quantitated by absorption measurements on a Joyce Loebl Microdensitometer 3CS. Tracings started at the border of RNA segment 1 through to RNA segment 11 indicated on the bottom line. RNA band E was clearly resolved; RNA band A is contained in the large peak of RNA segments 2 and 3 in tracing D.

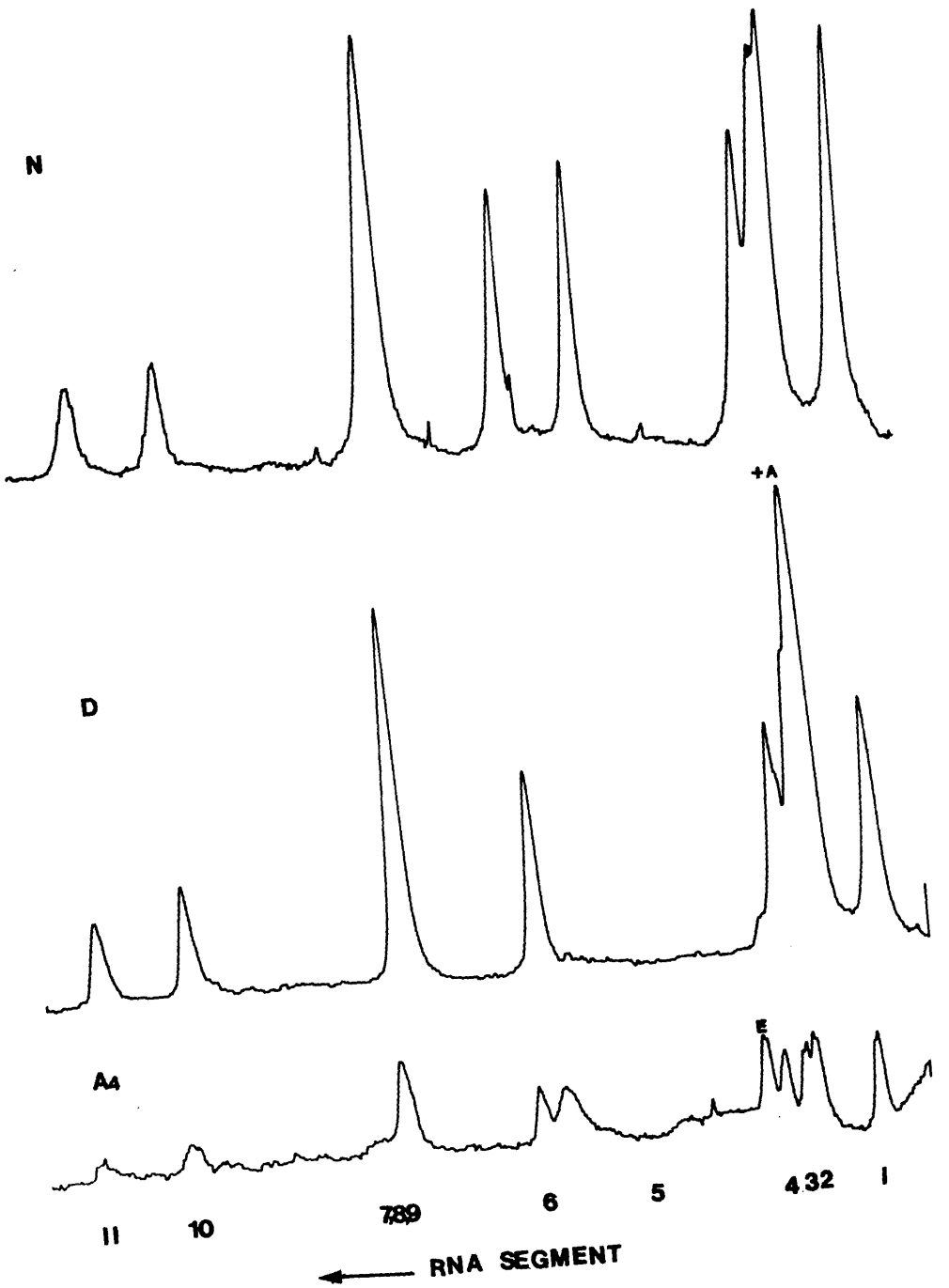

N
+A
D
E
A4
11
10
7,8,9
6
5
4
3
2
1
RNA SEGMENT

Table 6.2 Relative molar concentration of RNA segments ($x10^{-6}$) in virus infected cells.

Genomic RNA segments shown in Figure 6.3 were quantitated by computer analysis of densitometry peaks (Figure 6.4), and the percentages calculated by which each peak contributed to the total genome. The percentage of each peak of the genomes N, D and A_4 was divided by the molecular weight (in Md) of the respective RNA segment (Rixon et al., 1984) to obtain relative molar concentrations.

a The percentage shown represents the mixtures of RNA segments 2 and 3 and of segments 7, 8 and 9 respectively. The molar proportions were calculated by dividing percentage values by the sum of molecular weights.

b This peak contains RNA segments 2 and 3 and RNA band A (size 1.82×10^6 daltons).

c RNA band E (size 1.48×10^6 daltons).

Table 6.2.

		[^{32}P] RNA Segment --->							
		1	2&3[a]	4	5	6	7,8,9[a]	10	11
RNA segment size ($\times 10^6$)		2.25	3.56	1.56	1.00	0.89	2.19	0.48	0.42
Percentages	N	17.8	10.3	5.9	16.3	10.8	29.2	5.3	4.4
	D	14.3	31.1[b]	6.7	-	9.1	27.6	6.1	5.1
	A_4	14.5	26.4	6.1	9.8[c]	7.7	20.2	11.5	3.7
Relative Molar Conc. ($\times 10^{-6}$)	N	7.9	2.9	3.8	16.3	12.1	13.3	11.0	10.5
	D	6.4	5.8[b]	4.3	-	10.2	12.6	12.7	12.1
	A_4	6.4	7.4	3.9	6.6[c]	8.7	9.2	24.0	8.8

respectively, and were labelled for 16 hours in the presence of 30 MBq [^{32}P] orthophosphate per dish. The cultures were then frozen and thawed once, extracted for RNA, and the RNAs separated on a 2.8% polyacrylamide 6M urea gel, which was autoradiographed (Figure 6.3). RNA segments 1 to 11 of standard brv and the rearranged RNA bands A and E were synthesised (arrows in track D and A_4, respectively, of Figure 6.3). The autoradiograph shown in Figure 6.3 was scanned on the Joyce Loebl microdensitometer 3CS as described for silver-stained gels by Whitton et al. (1983), and densitometer tracings as shown in Figure 6.4 were obtained. They were then placed on a digitising tablet (Summagraphics ID) and each peak numbered 1-11, A and E was outlined with a stylus. The areas under peaks were calculated and their areas compared by computer programme (P. Taylor, MRC Virology Unit, written for the PDP-11 laboratory minicomputer). The percentages representing the relative amounts of each RNA segment were divided by the respective molecular weight (Rixon et al., 1984) to obtain relative molar concentrations as shown in Table 6.2. Molecular sizes of the RNA bands A and E were estimated from a graph in which the known sizes of normal segments (Rixon et al., 1984) were plotted against the distance of RNA segment migration in gel of Figure 6.3. The sizes of RNA band A and E were 1.82 and 1.48 x 10^6 daltons, respectively, (see section 2 of Results) and corresponded to 2764bp for band A and 2248bp for band E. It then worked out that the replication pattern of the rearranged bands A and E fell into a pattern very similar to that of the normal RNA

Figure 6.5.

Time course of RNA synthesis in infected cells.

Cell monolayers on 50mm petri dishes were infected with standard brv and brv mutant A_4 in the presence of 30 MBq of [^{32}P] orthophosphate and 5ug/ml actinomycin D at 5 pfu/cell. Cells were lysed and nuclei pelleted on low speed centrifugation; the supernatants containing the cell cytoplasm were extracted in phenol:chloroform and the RNA precipitated in ethanol at -70°C as described by Maniatis et al. (1982). The [^{32}P] labelled RNA segments were separated on a 2.8% polyacrylamide 6M urea slab gel and the gel dried and autoradiographed.

Tracks N: [^{32}P] labelled RNA synthesised from 0-6 hours (6N) and from 0-18 hours (18N) pi in cells infected with standard brv.

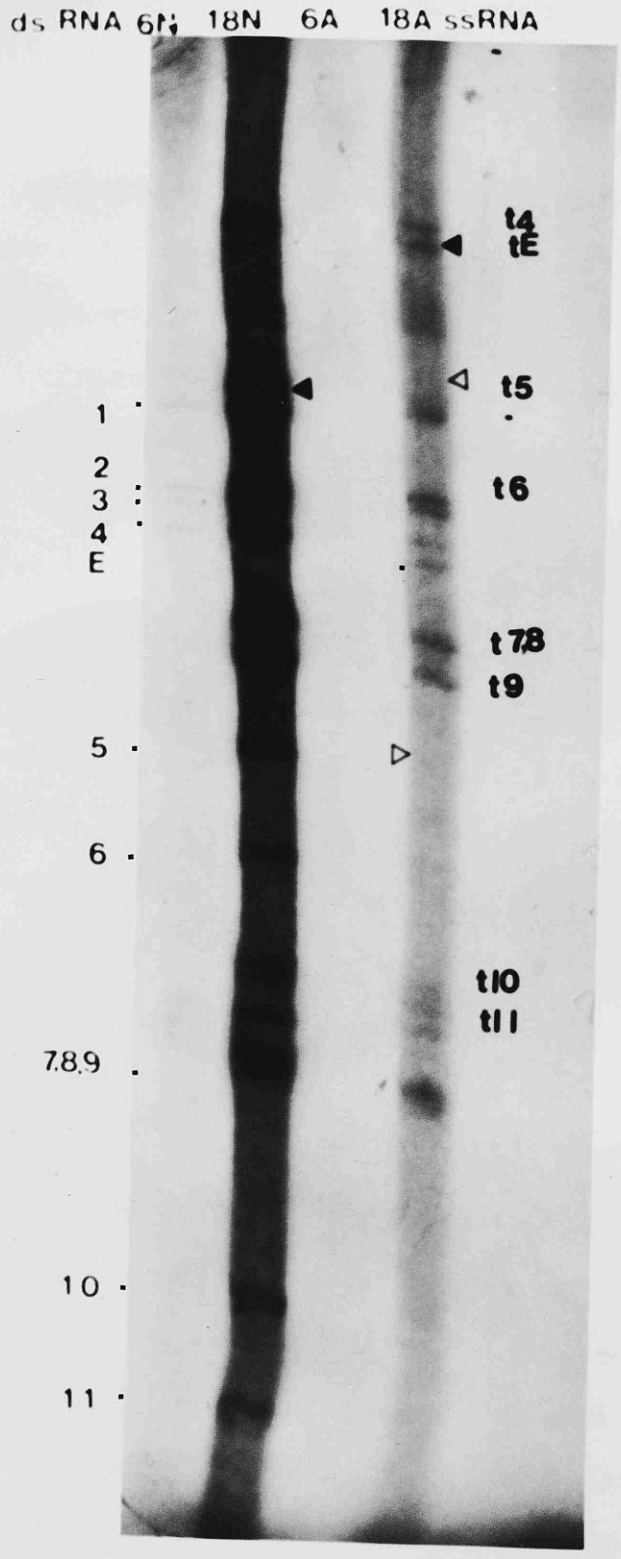
Tracks A: [^{32}P] labelled RNA synthesised from 0-6 hours (6A) and from 0 18 hours (18A) pi in cells infecte with the brv mutant A_4.

Genomic RNA segments (1-11) were numbered on the left and indicated by dots [·], while the numbers on the right (4-11) denote the ssRNA transcripts.

The filled-in arrows (▶) point to transcripts of genomic RNA segment 5 and of the rearranged RNA band E in tracks 18N and 18A, respectively.

The open arrows (▷) show the absence of RNA transcript 5 and of genomic RNA segment 5 in track 18A.

The relative positions of dsRNA segments and ssRNA trancripts are shown more clearly in Figure 5 of Hundley et al (1985) [attached].


ds RNA 6N 18N 6A 18A ssRNA
t4
tE
t5
t6
t78
t9
t10
t11
1
2
3
4
E
5
6
7.8.9
10
11

segments of corresponding size as shown in Table 6.2.

Total viral RNA was obtained from cells infected in a time course with the standard brv and the brv mutant A_4, at m.o.i. 5pfu/cell, and labelled 14.8MBq [^{32}P] orthophosphate in phosphate free Eagle's MEM containing 5ug/ml actinomycin D. The procedure described in Maniatis et al. (1982) was used to extract cytoplasmic RNAs. Cells were scraped into ice cold lysis buffer A, held at 4^{o}C for 5 minutes and vortexed. Nuclei were pelleted at low speed, (1500g for 5 minutes). The supernatants were mixed with equal volume of extraction buffer B and immediately extracted twice in phenol chloroform 1:1. RNAs were recovered after ethanol precipitation at -70^{o}C for 30 minutes. On RNA-PAGE analysis and autoradiography, the [^{32}P] labelled dsRNAs were detected by 6 hours pi in the cells which had been infected with the standard brv, and brv mutant A_4 and are shown in Figure 6.5, tracks 6N and 6A respectively. There was more of dsRNA produced in cells infected with standard brv after 6 hours than was in brv mutant A_4 infected cells at the same time.

No ssRNAs were detectable by autoradiography in the cells harvested at 6 hours pi (track N6 and A6). However, at 18 hours pi, both ssRNAs and dsRNA were present in the same RNA preparation (Figure 6.5, tracks 18N and 18A, respectively). Although the high molecular weight transcripts (ssRNAs 1-3) were hardly detectable on the autoradiogram, ssRNA transcripts derived from the genomic RNA segments 4-11 were synthesised in sufficient amounts to allow detection. The rearranged RNA band E of the brv

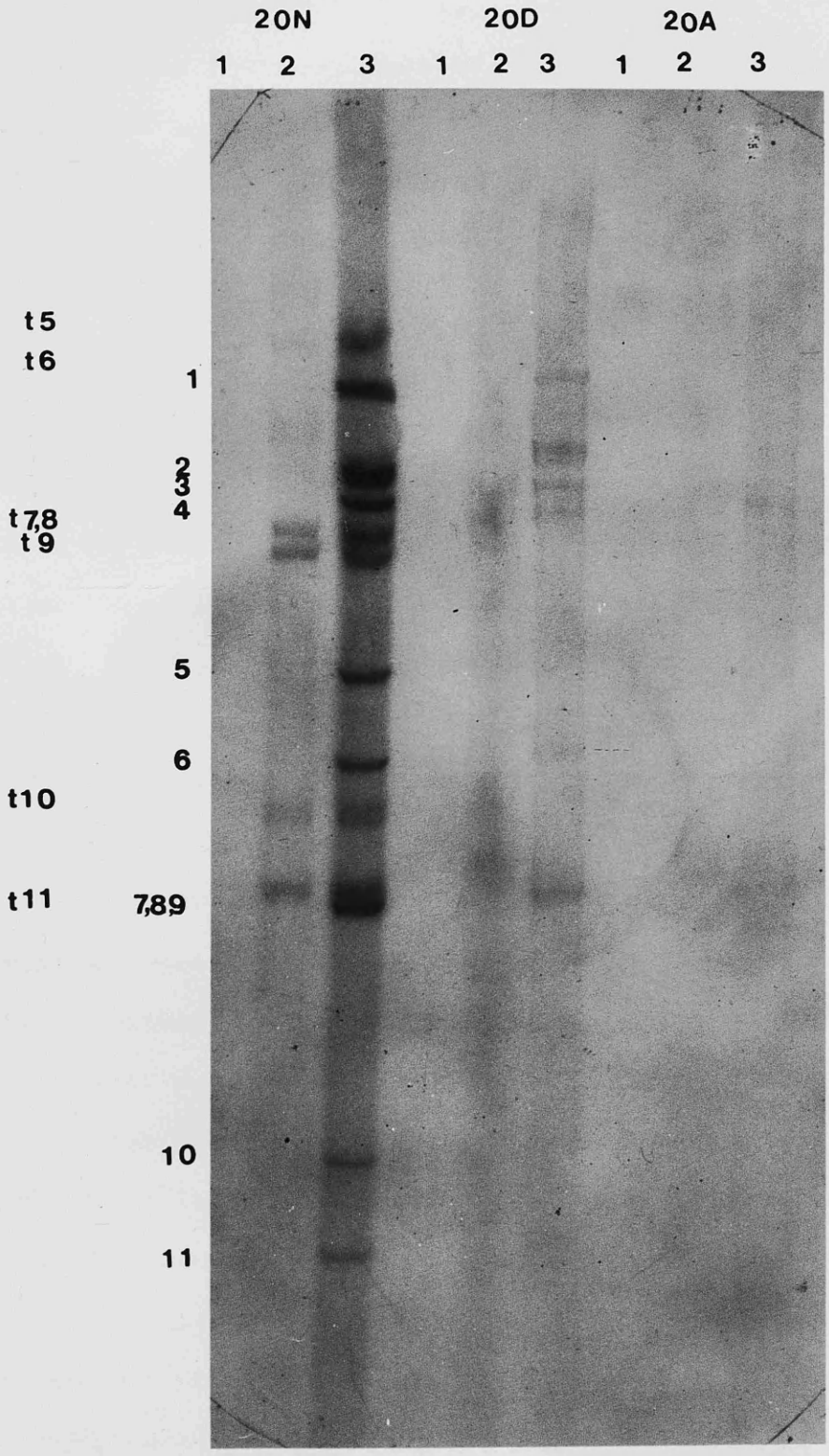
Figure 6.6.

Analysis of [^{32}P] labelled rotavirus RNA species synthesised in infected cells and separated by CF11 chromatography.

Cells were infected with 5 pfu/cell of the standard brv, mutant brv D and the brv mutant A_4 for 20 hours in the presence of 14.8 MBq [^{32}P] orthophosphate and 5ug/ml actinomycin D. The [^{32}P] labelled total RNA was extracted according to the protocol in Maniatis et al. (1982), then the RNAs were separated by CF11 cellulose chromatography (Franklin, 1966). The procedures of RNA extraction, CF11 cellulose chromatography and gel electrophoresis are detailed in the Methods section. The RNA fractions obtained at each elution step were counted for total [^{32}P]. Peak values were pooled, reprecipitated with tRNA or rRNA (final concentration 5ug) at -20^{o}C overnight in 2.5 volumes of ethanol; and the RNAs were recovered by pelleting at 11,000g on Eppendorf microfuge. Then the [^{32}P] labelled RNAs were separated on 2.8% polyacrylamide 6M urea gels according to Follett and Desselberger (1983a).

In panel 20N, 20D and 20 A: lane 1 denotes fraction which eluted in 35:65 ethanol:TSE, lane 2 fractions of RNA species which eluted in 15:85 ethanol:TSE, lane 3 fractions of RNA species eluting in TSE buffer alone.

The positions of dsRNA (genomic RNA) are numbered 1-11 on the left. RNA transcripts synthesised in-vivo can be seen in lanes 2 of panels 20N and 20D and on labelled t5-t11 on the left.


20N
20D
20A
1 2 3 1 2 3 1 2 3
t5
t6
1
2
3
4
t7,8
t9
5
6
t10
t11
7,8,9
10
11

mutant A_4 was unambiguously demonstrable in track 18A, and the ssRNA transcript E from the rearranged RNA segment E of brv A_4 could be seen in track 18A indicated with arrow. The observations indicate that brv mutant A_4 transcribed its rearranged RNA band E during infection in a similar way to the normal RNA segments.

RNA was extracted from cells which had been infected with the standard brv, brvD and brv A_4 for 20 hours in the presence of 5 ug/ml actinomycin D and 30 MBq of [^{32}P] orthophosphate per 50mm dish of MA104 cells. Molecules of ssRNA and dsRNA were then separated according to the procedure of Franklin (1966). The [^{32}P] labelled RNA was loaded on the CF11 cellulose column in 35% ethanol in TSE buffer and first eluted in 10ml of the same buffer. The buffer was changed to 15% ethanol in TSE buffer and a number of fractions were collected which contained exclusively ssRNA. In this experiment the standard brv produced a lot more ssRNA than both brvD or brv A_4. The fractions which eluted after change of buffer to TSE alone contained a mixture of dsRNAs and ssRNAs as shown in Figure 6.6, tracks 3 under panels 20N, 20D, 20A. The ssRNA which was separated from the standard brv RNA synthesised in infected cells shown in lane 2, panel 20N of Figure 6.6 was quantitated by densitometric scanning using the Joyce Loebl microdensitometer and the areas under each peak were calculated and compared by computer programme as outlined above. The results are shown in Table 6.3 and indicate that the percentages and [^{32}P] label per nucleotide of in-vivo transcription products (ssRNA) are inversely related

ssRNA	4+5	6	7,8,9	10	11
Percentage	11.5[a]	5.3	42.7[b]	12.9	27.8
Molar proportion (x 10^{-3})	3.0	3.9	12.9	17.9	42.1

Table 6.3. Relative molar concentration of genome transcripts in cells infected with standard brv.

The single stranded RNAs obtained in pool a after CF11 chromatography (Franklin 1966) of total RNA from standard brv were further separated by RNA-PAGE, and segments were visualised by autoradiography (Figure 6.6, track 2, panel 20N). The ssRNA species were then densitometrically quantitated and the peaks of RNA transcripts expressed as percent of total transcripts. Relative molar concentrations were obtained by dividing the percentage values by the number of bases in the transcripts (Rixon et al., 1984).

a Value contains ssRNA transcripts 4 and 5; molar proportion is calculated by dividing the percentage by the average of bases of transcripts 4 and 5.

b The percentage value is of transcripts of RNA segments 7, 8, 9 together. The molar proportion is calculated by dividing the percentage by the sum of bases of transcripts 7, 8 and 9.

to their molecular sizes; i.e. the smallest RNA segment 11 was transcribed most abundantly and the largest ssRNAs 1, 2 and 3 were produced in quantities not detectable even after prolonged autoradiography. The ssRNAs synthesised in cells which were infected with brv mutants D and A_4 showed insufficient intensity even after prolonged autoradiography to allow quantitation.

In order to investigate the relationship between in-vivo genome transcription and replication, cells were infected with the standard brv and brv mutants D and A_4 in a time course in the presence of 30MBq [^{32}P] orthophosphate per 50mm petri dish of cells. At 6 and 18 hours pi, the infected cells were extracted for RNA according to the procedure of Maniatis et al., 1982. Then the RNA yield was subjected to CF11 chromatography (Franklin, 1966) in 0.5ml fractions.

The fractions which eluted in the buffers containing 35% ethanol, 15% ethanol and TSE buffer alone were counted directly on the Intertechnique Liquid Scintillation Counter SL 4000 and the fractions constituting peaks were pooled. Figure 6.7 shows the pattern of RNA synthesis at 6 and 18 hours pi for the standard brv (panels N6 and N18) brv mutant D (panels D_6 and D_{18}) and brv mutant A_4 (panels A_6 and A_{18}). The fraction pools representing mainly ssRNA and dsRNAs are denoted peaks a and b, respectively. The peaks of RNA produced at 6 hours pi, are shown in a scale 10 times less than that of corresponding peaks obtained at 18 hours (Figure 6.7). Even if the fraction representing dsRNA (peaks b) contained some ssRNAs (Figure 6.3), the amount of

Figure 6.7.

Pattern of RNA synthesis in vivo.

CF11 cellulose chromatography (Franklin, 1966) was done using total RNA extracted from cells infected with standard brv and brv mutants D and A_4 in the presence of 30 MBq of [^{32}P] per 10^6 cells. The RNA samples were first adjusted to 35% ethanol in TSE and loaded onto CF11 cellulose column at 0 time. Elution with 15% ethanol in TSE was started at 2 and TSE alone was used at 3. A number of fraction (0.5 ml/fraction) for samples obtained at 6 hours pi (N6, D6, A6) and 18 hours pi (N18, D18, A18) were collected. Approximately 10ml fractions were collected at each elution step. The fractions were counted for [^{32}P] and the CPM were plotted against fraction number. Peaks a and b indicate the RNAs eluted in fractions containing ssRNAs and mainly dsRNA, respectively (Figure 6.6).

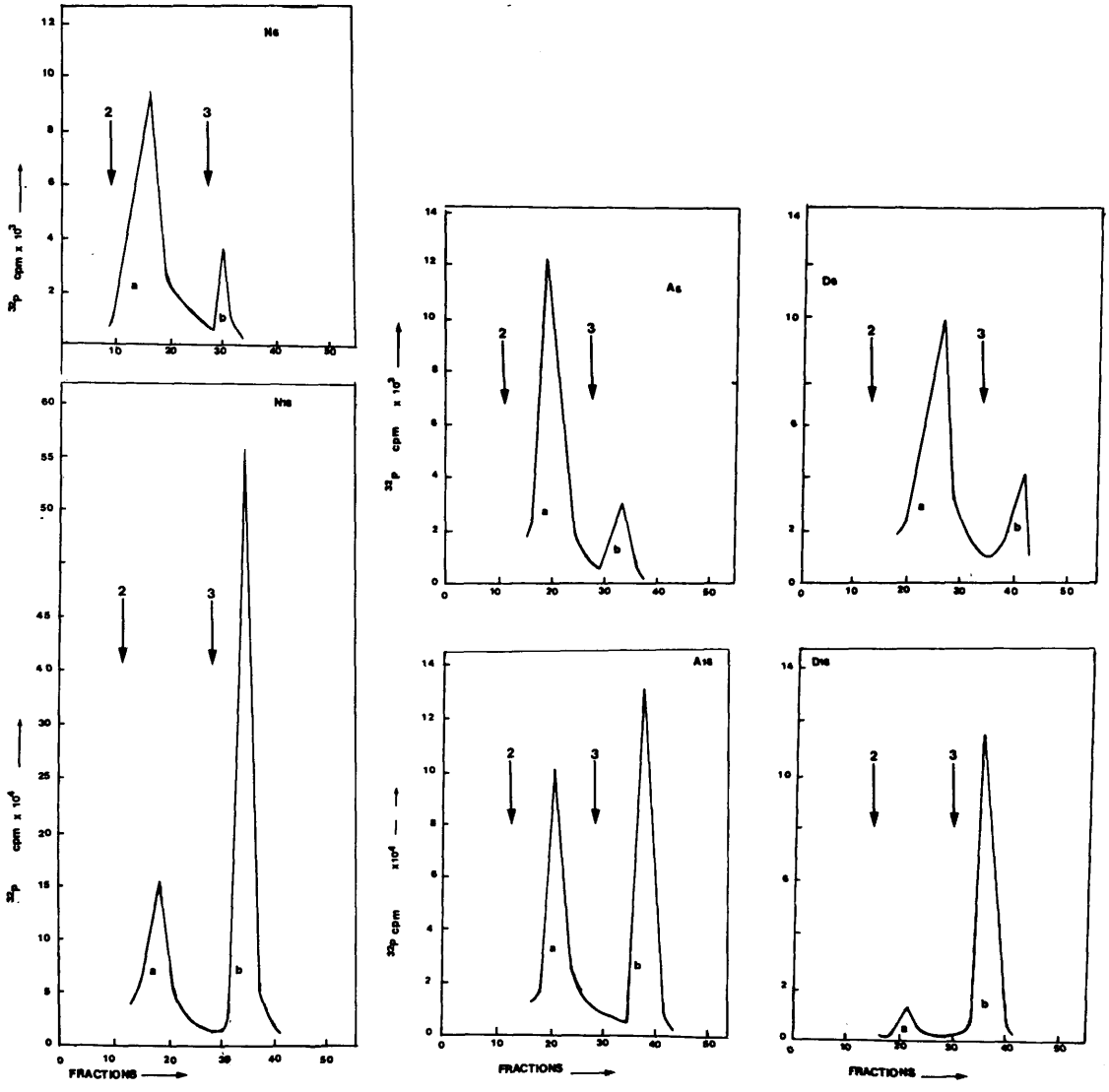

Ns
Nns
As
Ans
Ds
Dns
2
3
a
b
^{32}P cpm x 10^3
^{32}P cpm x 10^4
FRACTIONS

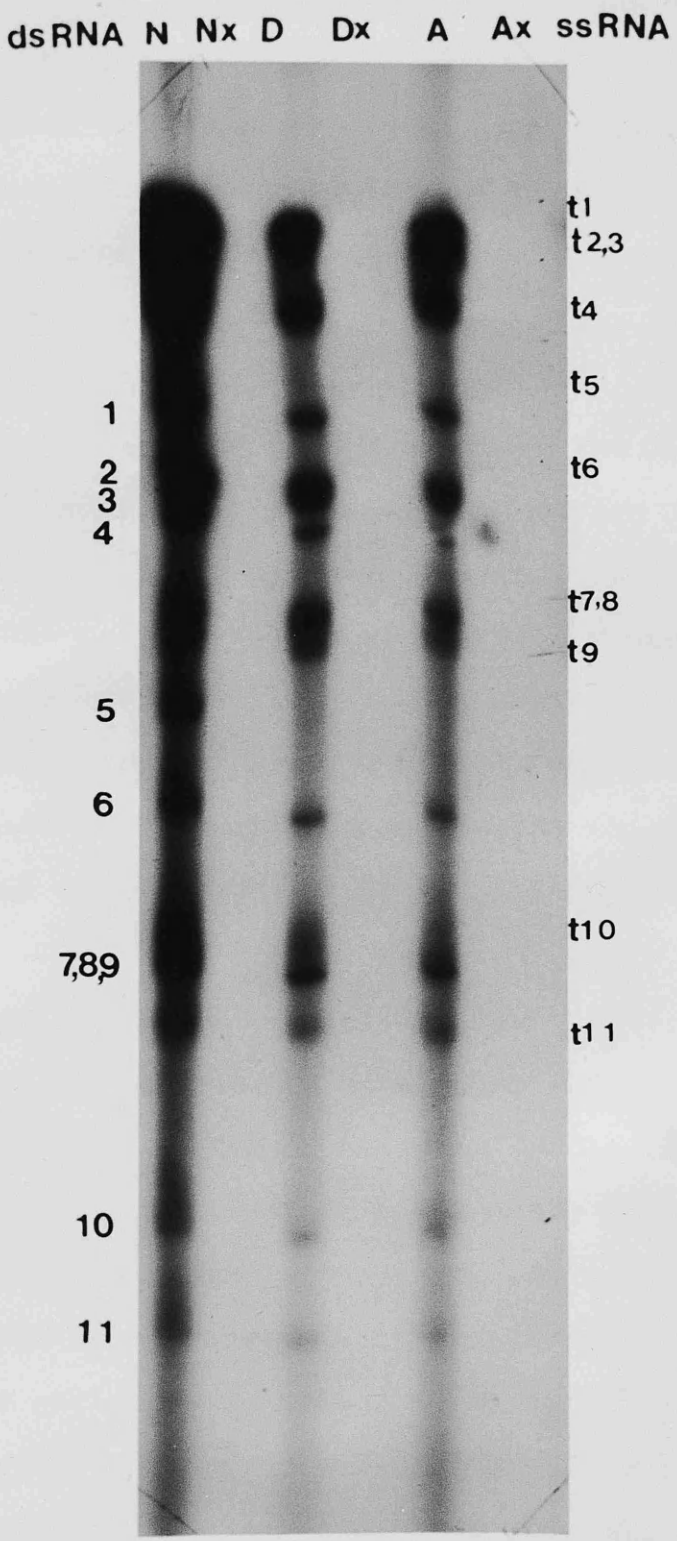
Figure 7.1.

Effect of cycloheximide on viral RNA synthesis.

The autoradiograph shows [^{32}P]-labelled RNA extracted from cells which were incubated for 16 hours after infection in the presence and absence of 20ug/ml cycloheximide. The cells were infected at m.o.i. of 5 pfu/cell with standard brv and brv mutants D and A_4 and labelled in phosphate free Eagle's MEM to which 14.8 MBq/ml of [^{32}P] orthophosphate had been added. The total RNA extracted from the cell cytoplasm (Maniatis et al., 1982) was separated on 2.8% polyacrylamide 6M urea slab gel (Follett and Desselberger, 1983a).

Tracks N, D and A show the [^{32}P] labelled RNA synthesised in the absence of cycloheximide in cells infected with the standard brv and brv mutants D and A_4, respectively. Tracks N^X, D^X and A^X were loaded with cellular [^{32}P] labelled RNA synthesised after infection with standard brv and brv mutants D and A_4, respectively, in the presence of 20ug/ml cycloheximide.

Genomic dsRNAs are numbered on the left from 1 to 11.


dsRNA
N
Nx
D
Dx
A
Ax
ssRNA
1
2
3
4
5
6
7,8,9
10
11
t1
t2,3
t4
t5
t6
t7,8
t9
t10
t11

ssRNAs synthesised at 6 hour pi (peaks a) were in excess of the dsRNA in ratios for brv D 4:1; brvA_4 5:1 and standard brv 10:1 as determined by comparison of the areas under peaks. Higher absolute [^{32}P] counts were recorded for ss and dsRNA fractions at 18 hours pi than for fractions obtained at 6 hours pi, and the ssRNA and dsRNA species were present in proportions ranging from 1:4 for standard brv; 1:9 for brv D; and 2:3 for brv A_4 (Figure 6.7). The fact that the peaks b of all panels of Figure 6.7 were mixtures of dsRNAs and ssRNAs (Figure 6.3) meant that the apparent transition from predominant ssRNA to dsRNA during late infection (18 hours pi) was slightly smaller than represented by the relative sizes of peaks. However, failure to resolve pure species of dsRNA in TSE alone does not obscure the observation that at 6 hours pi, the brv D, brvA_4 and standard brv synthesised more ssRNA than the mixture of ds and ssRNAs represented in peak b in each case.

7. Effect of cycloheximide on viral RNA synthesis.

Confluent cell monolayers on 50mm petri dishes starved of phosphate overnight were then infected for 16 hours in the presence of 5ug/ml actinomycin D and of 30 MBq [^{32}P]-orthophosphate with and without 20ug/ml cycloheximide. The infected cells were extracted for total RNA synthesised over the 16 hour period as described by Maniatis et al., 1982. Infection with brv D, brv A_4 and standard brv which was allowed to proceed in the presence of 20ug/ml cycloheximide did not produce either genomic ssRNA or dsRNA that could be detected, not even after prolonged exposure as

shown in Figure 7.1, lanes N^X, D^X, A^X. Control samples which were treated similarly but had no cycloheximide added, showed synthesis of both dsRNA and ssRNA, (Figure 7.1, lanes N, D and A_4). Since cycloheximide was used to inhibit protein synthesis in infected cells, it might be concluded that some viral protein synthesis was required for genome replication of both the standard brv and brv mutants.

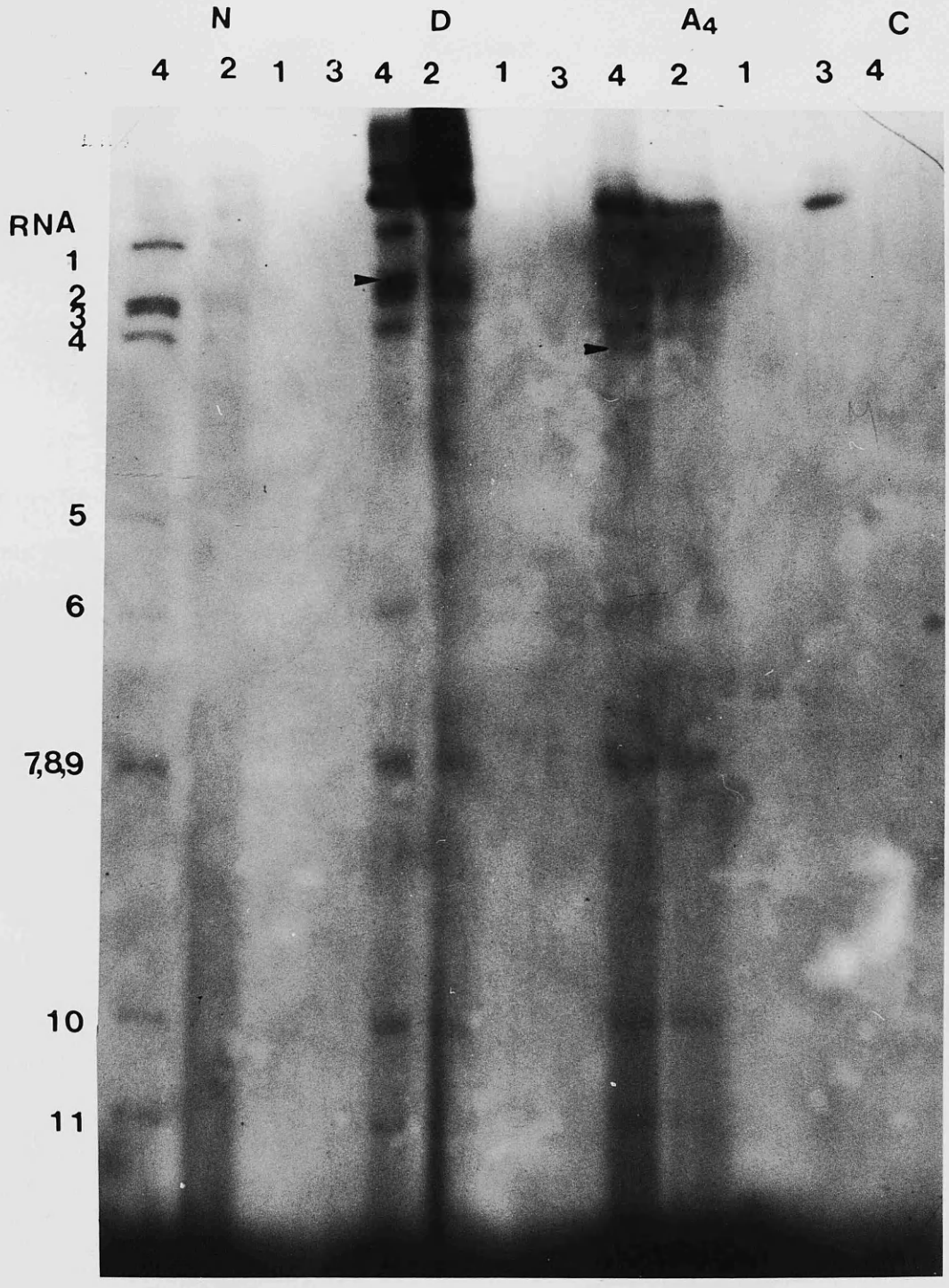
RNA synthesis of standard brv and of mutants D and A_4 was followed in a time course under conditions where protein synthesis was blocked with 20ug/ml cycloheximide. Parallel controls were set up without cycloheximide. Cells were infected with 5 pfu/cell of standard brv and the brv mutants D and A_4. The RNAs were extracted at different times pi and analysed by RNA-PAGE and autoradiography. The infected cell cultures which were [^{32}P]-labelled from 0 to 8 hours pi in the presence of 20ug/ml cycloheximide did not contain detectable amounts of dsRNA or ssRNA (Figure 7.2, tracks 1 under panels N, D and A_4). In those samples which were [^{32}P] labelled without cycloheximide from 0-8 hours pi, both ssRNA and dsRNAs were present (Figure 7.2, tracks 4). To demonstrate the effect of absence of protein synthesis on viral genome transcription and replication , infected cell cultures were pulse labelled with [^{32}P] from time 0-6 hours pi without cycloheximide. Then cycloheximide was added to the labelling medium (final concentration 20ug/ml), and the infection maintained for a further two hours. Total RNA extracted from these samples was analysed on gels (Figure 7.2, tracks 2). It was found that under these conditions RNA was produced in distinctly smaller quantities than when

Figure 7.2.

Time course of rotavirus RNA synthesis in-vivo in the presence and absence of cycloheximide.

The autoradiogram shows [^{32}P] labelled RNA that was extracted from cells infected with standard brv and brv mutants D and A_4 and labelled with 30 MBq of [^{32}P] orthophosphate per 50mm petri dish of MA104 cells in the presence and absence of 20ug/ul cycloheximide. The [^{32}P] labelled RNA was separated on 2.8% polyacrylamide 6M urea slab gels as described by Follett and Desselberger (1983a).

Panels N, D and A_4 show profiles of RNA synthesised in cells infected with standard brv and brv mutants D and A_4. Tracks 4: [^{32}P] labelled RNA synthesised from 0 to 8 hours pi without cycloheximide; Tracks 2:[^{32}P] labelled RNA synthesised in the absence of cycloheximide from 0-6 hours pi; then virus infection and label were maintained in the presence of cycloheximide until 8 hours pi; Track 3: [^{32}P] labelled cell extract from 0-8 hours pi in the presence of cycloheximide; Tracks 1: [^{32}P] labelled cells in the presence of cycloheximide from 0-6 hours pi. Change medium to [^{32}P] without cycloheximide for a further 2 hours. Included for comparison were extracts from uninfected cells, panel C, which were treated in the same way as infected cells, tracks 1. Genomic RNAs were numbered 1-11 from top to bottom. Arrows (►) point to the rearranged RNA bands A and E in panel D and A_4, respectively.


N
D
A4
C
4 2 1 3 4 2 1 3 4 2 1 3 4
RNA
1
2
3
4
5
6
7,8,9
10
11

infected cell cultures were continuously labelled for 8 hours without cycloheximide. Uninfected cells were not found to synthesise any appreciable amounts of RNA in the presence of actinomycin D whether or not the labelling medium contained cycloheximide (Figure 7.2). Two bands of nucleic acid migrating slower than the genomic dsRNA segment 1 were observed in virus infected cell cultures and could be cellular RNA bands.

8.1 In-vitro transcription of rotavirus genomes.

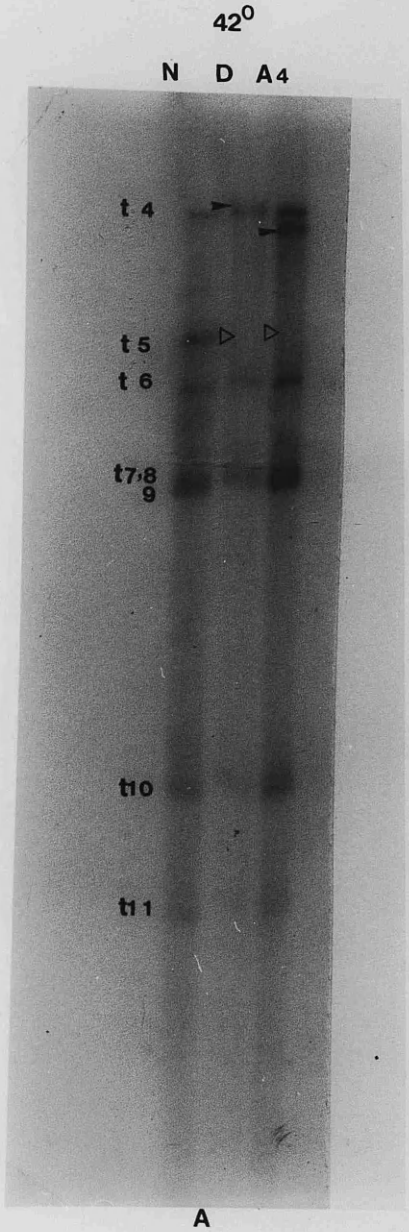
In-vitro transcription assays were employed to demonstrate whether or not the brv mutants carrying rearranged RNA bands D, E, G and H were capable of transcribing their abnormal genomes as it was found already described for the brv mutant D (Hundley et al., 1985). Standard brv and the brv mutants D, A_4, B_4 and B_5 were incubated in the in-vitro transcription mixture (Flores et al., 1982a) for 6 hours as described in Methods. The resulting RNA was extracted and analysed by RNA-PAGE and autoradiography. Groups of transcripts indicated in Figure 8.1 as t4-t11 were resolved representing the transcripts of genomic RNA segments 4-11. The rearranged RNA bands A, E, F, G and H were similarly transcribed as shown by arrows in Figure 8.1, panels A and B. There was no detectable ssRNA transcript from RNA segment 5 in the transcription mixtures of the brv mutants (open arrows, Figure 8.1). High molecular sized transcripts from the genomic RNA segments 1-3 were present in amounts that could hardly be detected after prolonged autoradiography.

Figure 8.1.

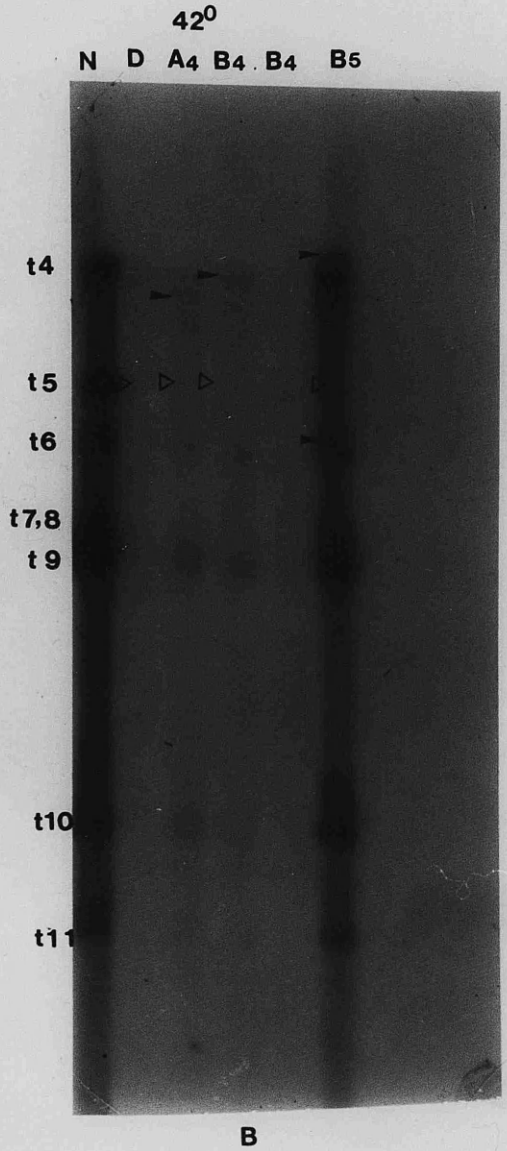
In-vitro transcription of rotavirus genomes.

The [^{32}P]-orthophosphate labelled RNA transcripts were synthesised in-vitro from the genomes of the standard brv and brv mutants D, A_4, B_4 and B_5 in a reaction which was catalysed by the virion-associated RNA polymerase. To the reaction mixture (total volume 400ul) virions amounting to 1.5ug of protein was added. The conditions of the in-vitro transcription assay are described in Methods and were according to Flores et al. (1982a) and Hundley et al. (1985). The autoradiograms Panels A and B show RNA transcripts synthesised at 42^{o}C. Viruses used in the in-vitro transcription assay are indicated on top of each track. In this experiment the brv mutant B_4 did not synthesise detectable amounts of transcripts (track B_4) in panel B. Open arrows (▷) indicate the missing transcript 5 in all brv mutants which do not have the normal genomic RNA segment 5. Filled arrows (►) indicate the RNA transcripts from rearranged segments A, E, G and H in tracks D, A_4, and B_5, respectively, at 42^{o}C.

Transcripts are denoted on the left and numbered in order of increasing size from t11 to t4.


42⁰
N D A4
t 4
t 5
t 6
t7,8
9
t10
t11

A


42⁰
N D A4 B4 B4 B5
t4
t5
t6
t7,8
t 9
t10
t11

B

Figure 8.2.

Pattern of in-vitro transcription at 42°C.

In-vitro transcription reaction mixtures were set up as described in Figure 8.1. The reaction was followed for 5 hours by taking aliquots of 20ul every hour. Ten ul of sample were spotted on Whatman filter disc in duplicate and dried. [^{32}P] orthophosphate incorporated during the transcription of genomes of the brv mutants D, A_4, B_4 and B_5 was assayed as TCA precipitable material and counted in PPO-toluene scintillation medium, as described by Bollum (1968) and Szilagyi and Pringle (1979). Counts incorporated (cpmx10^{-3}) and were plotted against the time (in hours) of incubation at 42°C. The rates for transcription for the brv mutants are compared with that of standard brv (N) under the same conditions.

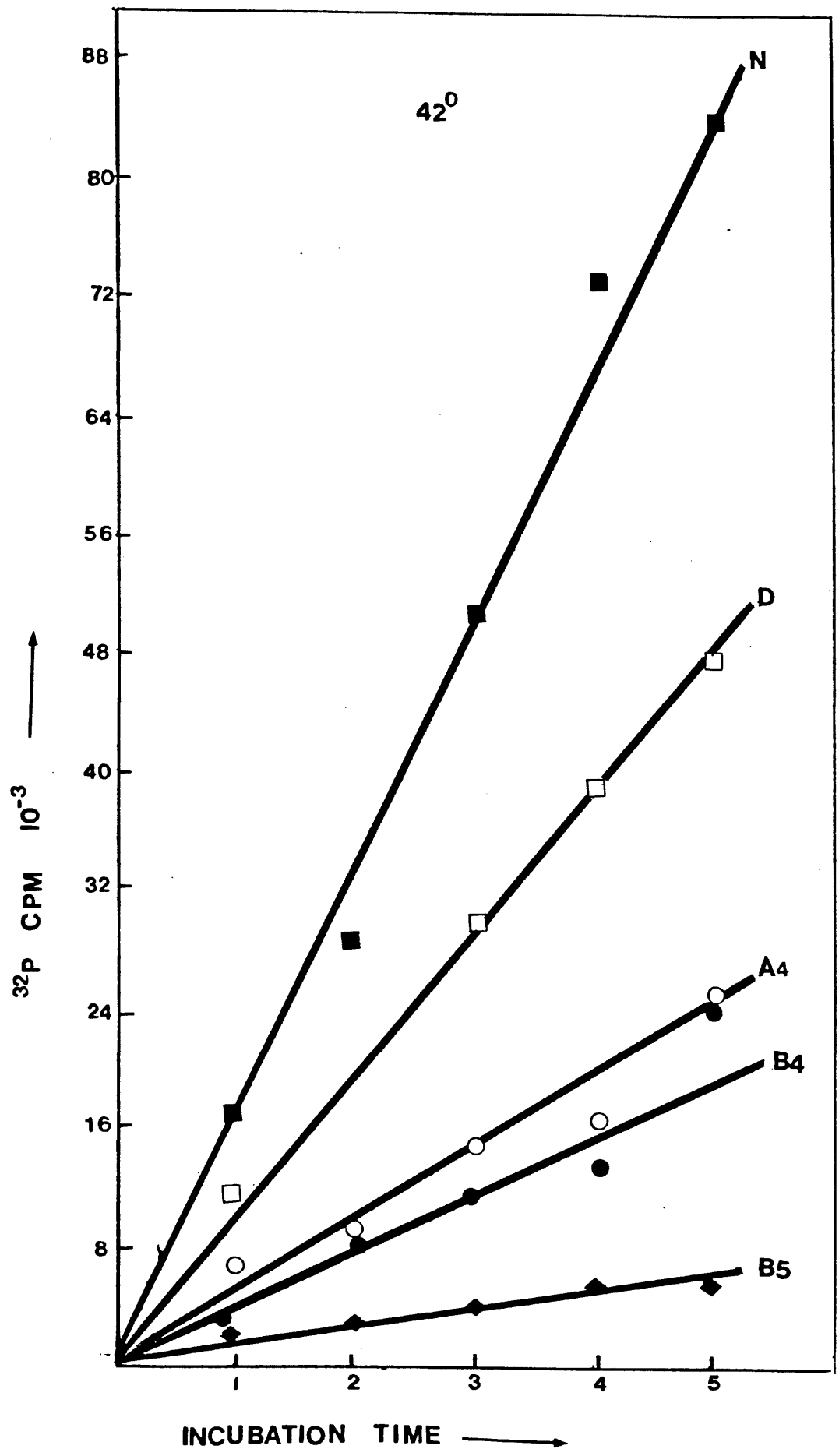
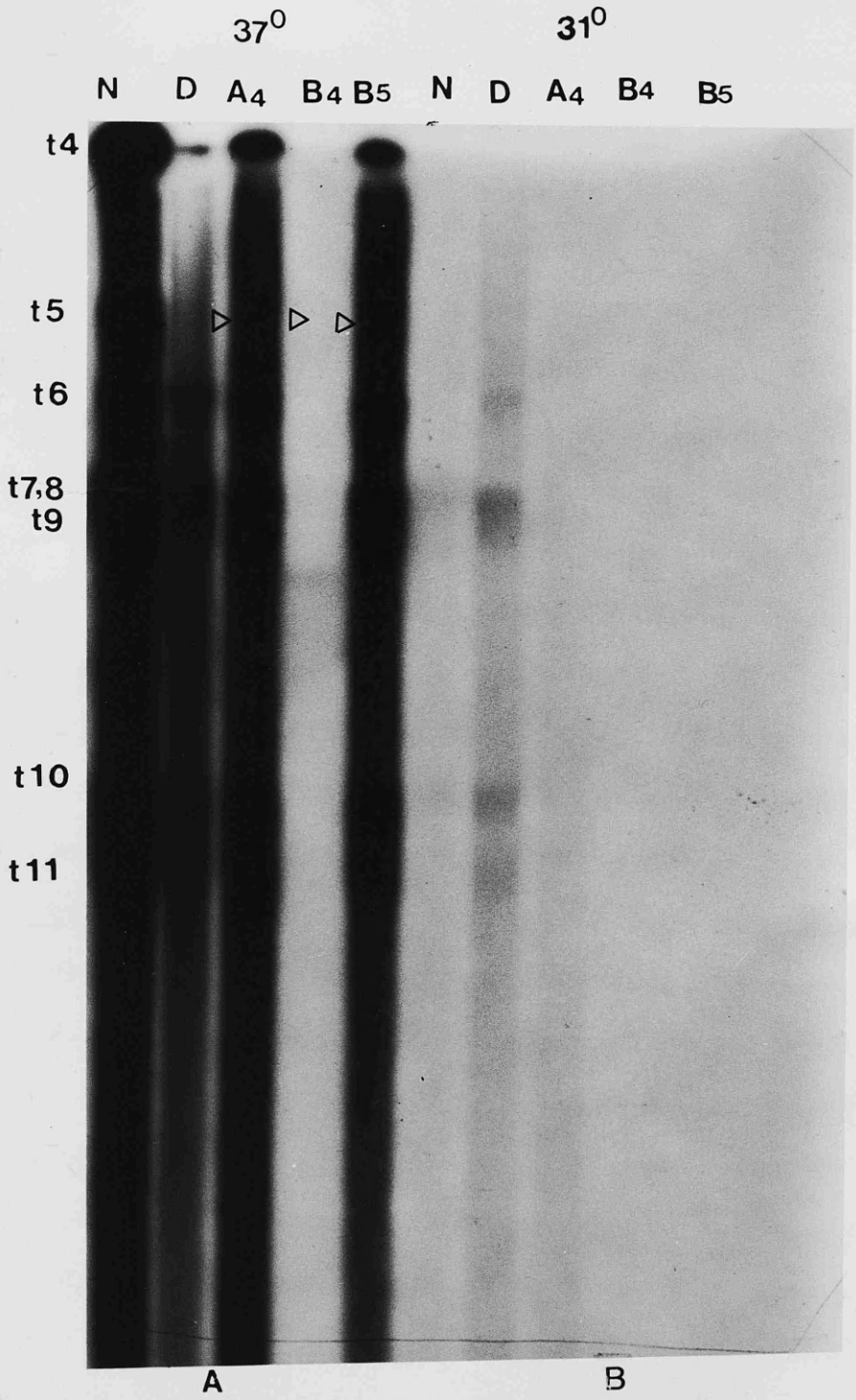

42°
N
D
A4
B4
B5
88
80
72
64
56
48
40
32
24
16
8
1
2
3
4
5
32P CPM 10-3
INCUBATION TIME

Figure 8.3.

Effect of temperature on in-vitro transcription.

The autoradiogram shows [^{32}P] labelled ssRNA transcripts of brv mutants D, A_4, B_4 and B_5 produced at 37^{o}C panel A and 31^{o}C panel B. Included for reference was standard brv (track N). In-vitro transcription was carried out as described in Methods (Flores et al., 1982a; Hundley et al., 1985). See Figure 8.1.

Transcripts t11 to t4 were numbered from bottom to top in order of increasing size of the transcribing genomic segment. Open arrows (▷) point to the missing t5 in the transcripts of brv mutants.


37°
31°
N
D
A4
B4
B5
N
D
A4
B4
B5
t4
t5
t6
t7,8
t9
t10
t11
A
B

Kinetics of genome transcription in-vitro were followed under reaction conditions previously optimised at 42°C (Cohen et al., 1979; Flores et al., 1982a). The amount of virus contained in approximately 1.5ug protein as estimated by the Lowry method was put into each of the in-vitro transcription reactions and incubated for 5 hours at 42°C.

RNA polymerase activity of standard brv and of each of the brv mutants D, A_4, B_4 and B_5 was measured as TCA precipitable [^{32}P] counts (Bollum, 1968; Szilagyi and Pringle 1979). A comparison of the RNA polymerase activities is shown in Figure 8.2: it can be seen that the pattern of transcription was similar being linear for 5 hours of incubation. However, the rates of RNA synthesis were different. The brv mutants synthesised RNA at 3-10 times lower rates than standard brv but it was observed that the concentration of virus particles recovered from partially purified brv mutants was also lower by 30-90 fold. When in-vitro transcription of brv genomes was set up at temperatures 31°C and 37°C, for 5 hours, there was transcription as shown in Figure 8.3, panel A and B. It was noted that standard brv as well as the brv mutants synthesised ssRNAs in-vitro but the amount of transcription was much lower at 31°C than at 37°C. From the autoradiographs shown in Figure 8.3, panels A and B, a similar number of ssRNA bands/segments was visible at 37°C and 31°C, respectively, as demonstrated at 42°C (Figure 8.1) indicating that at suboptimal temperatures the brv genomes were transcribed fully.

To evaluate the transcription efficiency of the individual RNA segments, a densitometer tracing produced

Virus	RNA segment 2,3,4[a]	5	6	7,8,9[a]	10	11
N	10.2 (0.13)	18.5 (12.2)	12.6 (9.3)	37.1 (11.2)	12.4 (16.2)	9.2 (13.9)
D	21.7[b] (0.21)	-	17.8 (13.2)	42.3 (12.8)	13.7 (17.9)	5.2 (7.9)
A_4	11.5 (0.14)	6.8[c] (3.1)	11.9 (8.8)	45.2 (13.6)	20.1 (26.3)	5.0 (7.6)

Table 8.1. Relative frequency of genome transcription in-vitro.

Standard brv and mutants D and A_4 were in-vitro transcribed to produce ssRNAs (Figure 8.1). The ssRNAs were densitometrically scanned, and the areas under peak compared: the percentages under each peak are shown to represent frequency of genomic segment transcription in-vitro. Relative molar concentrations were calculated by dividing the percentage by the number of nucleotides of the transcripts (Rixon et al. 1984), and are given in parenthesis ($\times 10^{-3}$).

(a) The relative [^{32}P] incorporated per nucleotide is calculated from by dividing the percentage by the sum of the bases of ssRNA transcripts 7, 8 and 9, and of 2, 3 and 4 (Rixon et al., 1984).

(b) Relative molar concentration was calculated by dividing the percentage by the sum of bases of ssRNA transcripts of 2, 3, 4 and A (band A containing 2764 bp).

(c) This value represents ssRNA transcript of band E (2248bp).

from the autoradiograph shown in Figure 8.1, panel A, were placed on the Summagraphics 1D Digitising tablet and each peak was outlined with a stylus. The areas were calculated and compared by computer programme "DENS" (P. Taylor, MRC Virology Unit). A comparison of RNA transcripts of standard brv and the mutants D and A_4 by percentage of each transcript being made and relative amounts of [^{32}P] incorporated per nucleotide of transcripts are given in Table 8.1. It was evident that transcription of the genome segments 4 to 11 on the whole was inversely proportional to size of transcribing segment. Under the conditions of in-vitro transcription (Flores et al., 1982a), the transcript of RNA segment 10 was observed more frequently than that of the smallest genomic RNA segment 11; the genomic RNA segment 6 of the standard brv was transcribed far less than the next larger RNA segment 5. Transcripts of the genomic RNA segment 1, 2 and 3 were barely visible on the autoradiographs as to be included in this quantitative evaluation.

9 Viral protein synthesis in infected cells.

Confluent cell monolayers on 50mm petri dishes were infected at a m.o.i. of 5 p.f.u./cell with standard brv and the brv mutants D, A_4, B_4 and B_5. Proteins were pulse-labelled for 2 hours at 14 hours pi in hypertonic Eagle's medium containing 7.4MBq/ml of [^{35}S] methionine. The cell lysates were analysed by SDS-PAGE and autoradiography: twelve virus specific proteins were identified and named VP1-VP12 as shown in Figure 9.1. None

Figure 9.1.

Protein synthesis in MA104 cells infected with standard brv and brv mutants D, A_4, B_4 and B_5.

Cell monolayers were infected at m.o.i. of 5 pfu/cell, incubated for 14 hours and then pulse labelled with 7.4 MBq/Ml [^{35}S]-methionine for 2 hours in 2ml of hypertonic Eagle's medium. Cells were washed, disintegrated in dissociation buffer and the proteins separated on a 12% polyacrylamide slab gel which was autoradiographed.
Track N: profile of proteins synthesised in cells infected with standard brv; tracks D, A_4, B_4 and B_5: profiles of proteins synthesised in cells infected with the brv mutants D, A_4, B_4 and B_5, respectively. Track C contains proteins from uninfected [^{35}S] methionine labelled cells.
Track SP shows the reference ^{14}C protein size markers, Amersham, CFA 626 (after prolonged exposure, SP^x) and molecular weights (in Kd) are indicated on the left.
Filled arrow (→) points to protein VP5A only found in cells infected with the brv mutant A_4; open arrows (▷) point to the position of VP5 missing in all brv mutants but seen in track N.

Viral proteins (VP) were numbered using the nomenclature of McCrae and Faulkner-Valle, 1981 and are indicated to the right. VP7pr denotes non-glycosylated precursor of VP7 (McCrae and Faulkner-Valle, 1981).

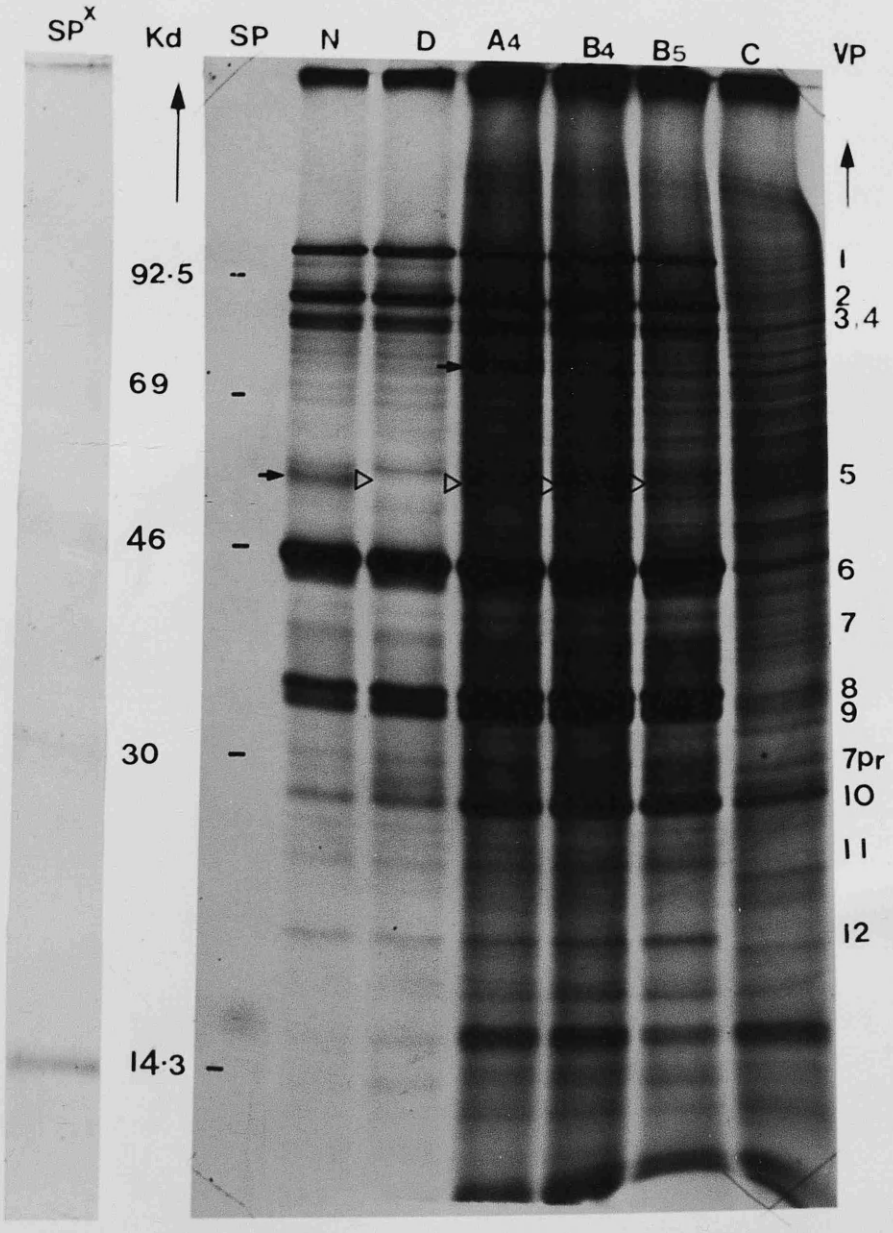

SP^x
Kd
SP
N
D
A4
B4
B5
C
VP
92·5
69
46
30
14·3
1
2
3,4
5
6
7
8
9
7pr
10
11
12

Table 9.1.

Comparisons of molecular weight determined for brv proteins produced in infected cells.

Viral proteins VP1-VP12 shown in Figure 9 were assessed for size in Kilodaltons (Kd) using the method of Shapiro et al., 1967. The distance each of the VP1-12 migrated in SDS-polyacrylamide gel was measured and the corresponding molecular weight read off from a reference curve relating $\log_{10}$ molecular weight of ^{14}C proteins (track SP, Figure 9.1) and their distances under identical conditions of gel electrophoresis. The resulting molecular weight determinations were compared with the published molecular weights for brv proteins (Source indicated in the table).

Table 9.1.

Nomenclature according to ref h	ref i	Protein size according to Matsuno et al., 1979	Thouless 1979	Carpio et al., 1981	Sabara et al., 1982	This Thesis	Predicted from RNA sequence
VP1	I_1	130.0	135.5	123.0	92.0	112.0	
VP2	I_2	115.0	95.1	84.0	86.0	93.3	
VP3	I_3	97.0	85.9	79.0	83.7	85.1	
VP4				78.0	74.6	83.1	88[a]
VP5	O_1	68.0	53.2	64.0	-	57.5	
VP6	I_4	41.0	42.7	48.0	48.8	45.0	41.8[b]
VP7	O_2	34.0	38.2	39.0	41.9	38.0	35->38.3[c]
VP8	NS_1	28.0	32.9	34.0	37.8	34.7	33.0[d]
VP9	NS_2	24.5	31.2	-	36.3	32.4	31.3[e]
VP10	O_3	17.6	26.4	28.0	29.3	26.3	28.0[f]
VP11	O_4	-	24.6	-	16.1	22.4	21.7[g]
VP12	NS_3	13.5		16.0	14.8	18.6	20.5[f]

a Lopez et al., 1985, partial sequence of VP3 of SA11
b Cohen et al., 1984
c Ellermann et al., 1983
d Dyall-Smith et al., 1983b
e Ward et al., 1984a
f Baybutt and McCrae, 1984
g Ward et al., 1985
h McCrae and Faulkner-Valle, 1981
i Thouless, 1979

* The molecular weight estimates in this thesis (Table 9.1) fit very well the molecular weights predicted from the nucleotide sequences.

of the brv mutants D, A_4, B_4 and B_5 synthesised VP5, mutants D, B_4, B_5 did not synthesise a protein that could be considered a product of their respective rearranged RNA bands A, F and G. The brv mutant A_4, which carries rearranged RNA band E migrating below the normal RNA segment 4 (Figure 2.1), synthesised a protein designated VP5A (arrow-marked in track 3, Figure 9.1). The molecular weight of the abnormal VP5A was 74.6Kd, a size between that of VP4, 83.1Kd and VP5, 57.5Kd.

Molecular weights of VP1-12 shown in Table 9.1 were estimated according to Shapiro et al. (1967): a reference curve relating $\log_{10}$ molecular weight of ^{14}C protein size markers (Figure 9.1, track SP) to the distance of migration in the gel was constructed. Although the estimated molecular weights of VP1-12 compare well with the published values (Table 9.1), there were differences in the molecular weight values of individual viral proteins depending on the gel system used. In the absence of a unified system of naming rotavirus proteins, the nomenclature proposed by Mason et al., 1980 and used by various authors (McCrae and Faulkner-Valle, 1981, Arias et al., 1982, Estes et al., 1982, Hundley et al., 1985) was employed throughout the thesis.

10 Morphology and structural proteins.

When standard brv and the brv mutant A_4 were centrifuged to equilibrium in caesium chloride gradients, three visible bands of virus were observed (Figure 10.1). The estimated density of each of the fractions p, d, and s, was 1.34, 1.36 and 1.39 g/ml, respectively. The virus particles banding

Figure 10.1.

Purification of rotavirus particles in caesium chloride gradients.

The diagram represents the relative banding positions of rotavirions after isopycnic ultracentrifugation, 115,000g for 16 hours on AH650 rotor in a CsCl gradient (density of initial suspension 1.4g/ml). Positions p, d and s correspond to banding positions for empty, double capsid and the single capsid rotavirus particles. The densities of fractions p, d and s were determined from their refractive index according to the formula of Brunner and Vinograd (1965) and were found to be 1.34, 1.37 and 1.39 g/ml, respectively.

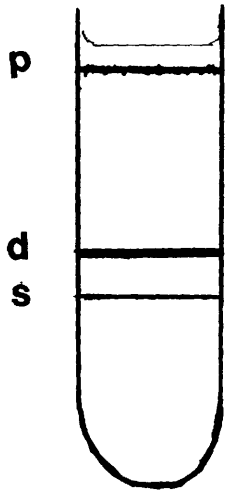
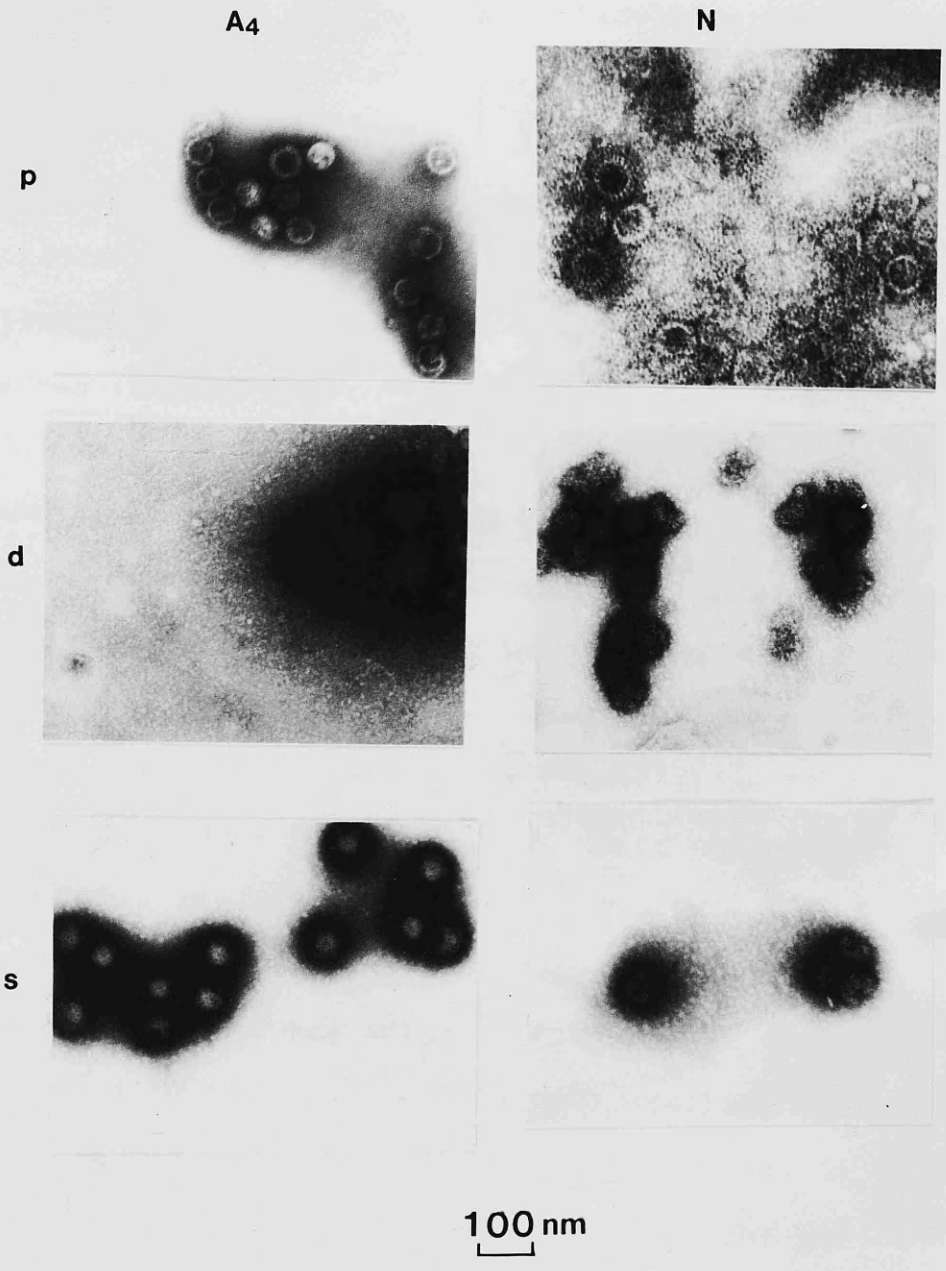

p
d
s

Figure 10.2.

Morphology of virus particles derived from the standard brv and mutant A_4.

The micrographs show rotavirus particles photographed under an electron microscope JEOL 100B at an accelerating beam of 80KV and at instrument magnification of 78,000. The fractions p, d and s (of density 1.34, 1.37 and 1.39, respectively) were collected from virus bands formed during isopycnic centrifugation of virus preparations N and A_4 at 115,000g for 16 hours (Figure 10.1). Virus particles were pelleted by centrifugation after overnight dialysis against PBS at 4°C, and resuspended in a small volume of PBS. Aliquots of 5ul of the virus concentrates were applied to Formvar coated-copper grid, blotted dry and contrasted with 1% phosphotungstic acid at pH 7.0 according the Follett and Desselberger (1983a).


A4
N
p
d
s
100 nm

at the density in CsCl 1.34 (band p) were empty particles. Virions collected from band d (1.36 g/ml) were the double capsid virus particles whereas virus particles banding at the density 1.39 (band s) represented single capsid particles (Figure 10.2). Virus particles of brv mutant A_4 were found to be similar in shape and size to those of the standard brv (Figure 10.2). Sizes of the virus particles of equivalent density was estimated at instrumental magnification of 78,000 and no major differences between virus particles of A_4 compared with those of the standard brv were observed (Figure 10.2).

Of the genomically rearranged brv mutants, only brv mutant A_4 synthesised an extra protein (VP5A) in infected cells (Figure 9.1). In an attempt to define the structural status of the normal protein product of RNA segment 5 (VP5) and of the mutant protein VP5A, cells infected with brv mutant A_4 and standard brv were labelled with 7.4MBq/ml of [^{35}S] methionine in 30ml of Eagle's MEM until complete CPE developed. The infected cell cultures were frozen and thawed once, clarified of cell debris by low speed centrifugation and the virus particles were banded by equilibrium centrifugation in a CsCl gradient (Novo and Esparza, 1981). The purified virus particles of the two viruses shown in Figure 10.2 were analysed by SDS-PAGE. For standard brv and the mutant A_4 double capsid (d), single capsid (s) and virus core (p) proteins were compared in tracks 1 and 2; 3 and 4; 6 and 5 respectively, of Figure 10.3. In no case was VP5A found in purified virions of the brv mutant A_4.

The protein band migrating close to the position of VP5 as seen in infected cells (arrow track N) appeared in virus preparations of both brv A_4 and standard brv (Figure 10.3, double dots [:]). This protein band was virus-specific judged from its absence from uninfected cell controls (track C, Figure 9.1) but could not be regarded as representing an authentic VP5 since the same protein band was detectable in brv mutant A_4 which lacks coding capacity of RNA segment 5. The protein band (arrowed in track 6 of Figure 10.3) shows a protein of size similar to the VP5 and might be a contaminating cellular material easy to pick up in core fraction (p) of the standard brv. Virus particles of standard brv purified in bands d and s did not contain VP5 (Figure 10.3, tracks 1 and 3).

Both standard brv and brv mutant A_4 consist of the same number of structural proteins VP1, VP2, VP3/4, VP6, and VP7. The VP6 is the major structural core protein (Table 10.1). Under the conditions of SDS-PAGE, the double capsid particles produced a protein band larger than VP1 (in Figure 9.4, tracks 1 and 2) and a protein band marked VP8C was observed to migrate in front of VP8 in infected cells (tracks N and A_4, Figure 10.3). The result can be interpreted as showing that VP8C was derived from VP4 by trypsin cleavage to result in the VP5C and VP8C proteins in analogy to the VP3 cleavage found in the SA11 virions (Lopez et al., 1985).

Relative concentrations of viral structural proteins of the standard brv and brv mutant A_4 were obtained by densitometry of the autoradiograph shown for Figure 10.3.

The areas under each peak were measured and compared by computer. Relative molar proportions of the structural proteins were calculated and are shown in Table 10.1. It was observed that the outer capsid consisted of proteins VP4 and VP7 which were present in the double capsid virus particles of both the standard brv and brv mutant A_4. The amount of outer capsid proteins diminished 4-fold in the single capsid virus particles of standard brv (track 3, Figure 10.3; and Table 10.1). The protein profile of single capsid particles of brv mutant A_4 was too light for similar densitometric analyses but clearly showed specific viral proteins (Figure 10.3, tracks 4 and 4^x). Tentative cleavage product of VP4 marked with double dots (:) VP5C and VP8C were detectable in protein profiles of virus cores (p) and double capsid (d) particles of both standard brv and brv mutant A_4.

Single capsid virions consisted of the structural proteins VP1, VP2, VP3 and VP6 of which VP6 was the major constituent (30-60%) of total viral protein. The main difference in the proteins of the double capsid virions was found in the amount of VP2 and less of VP6 (Figure 10.3) which were underrepresented in the brv mutant A_4 (Table 10.1). Of the viral glycoproteins, VP7 was found to be abundant in the double capsid virions of standard brv and brv mutant A_4 in amounts exceeding 30% of total protein whereas the VP10 contributed 0.5%. Table 10.1 illustrates that assembly of rotavirus structural proteins did not occur in equimolar proportions.

Figure 10.3.

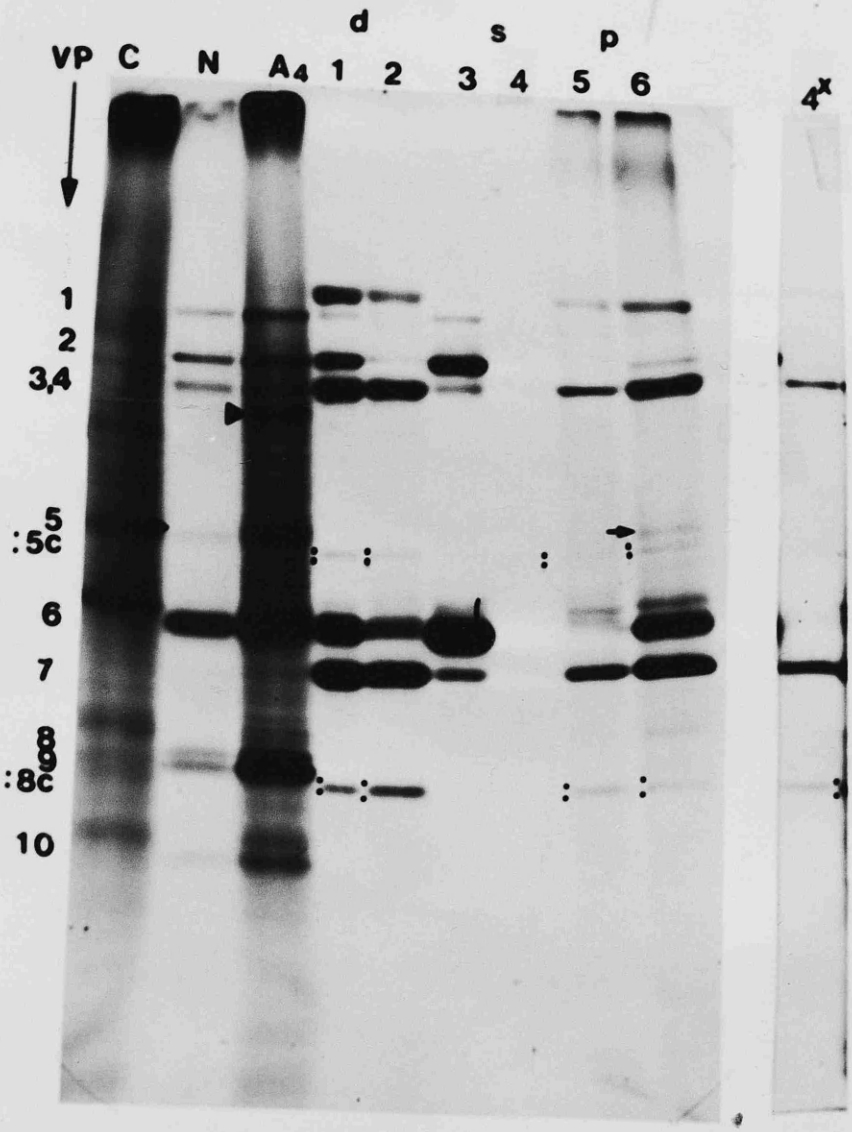
Structural proteins of standard brv and of brv mutant A_4.

Roller cultures of $2x10^8$ MA104 cells were infected with standard brv and brv mutant A_4, labelled with 1.48 MBq/ml of [^{35}S] methionine in 30ml of Eagle's MEM from 1 hour pi onwards and kept until complete CPE was reached. Viruses were purified by ultracentrifugation in 1.4g/ml CsCl solution and three bands - p, d and s - containing empty particles, double capsid and single capsid virus particles were obtained (Figures 10.1 and 10.2). Virus preparations were boiled in protein dissociation buffer and the proteins separated by SDS-PAGE.

Tracks 1 and 2: proteins of the double capsid particles of standard brv and of mutant brv A_4, respectively; Tracks 3 and 4: proteins of single capsid virus particles of standard brv and brv mutant A_4; Tracks 6 and 5: proteins of empty particles of standard brv and brv mutant A_4. Triangles (▶) denote the VP5 and VP5A seen in [^{35}S] methionine labelled cells infected with standard brv (track N) and brv mutant A_4 (in track A_4).

Double dots (:) indicate trypsin cleavage products of VP4 (Clark et al., 1981; Lopez et al., 1985).

Included for comparison is the lysate of [^{35}S] methionine labelled mock-infected cells (track C). Track 4^x denotes a longer and darker exposure of track 4.


d
s
p
VP
C
N
A4
1
2
3
4
5
6
4x
1
2
3,4
5
:5c
6
7
8
9
:8c
10

Virion	Viral proteins ---->							
	1	2	3/4	5C	6	7	8C	10
N^d	0.06	1.17	1.85	0.38	7.20	8.36	1.79	0.18
A^d	0.20	0.14	2.79	0.27	5.00	10.3	3.15	0.22
N^s	0.26	2.00	0.44	0.0	13.80	2.69	0.19	0.20

Table 10.1. Relative molar concentrations of rotavirus structural proteins.

Structural proteins shown in Figure 10.3, tracks 1, 2 and 3 were scanned densitometrically and the percentage of each structural protein was calculated. Relative molar concentrations (x 10^4) were obtained by dividing the percentage of each viral protein by the molecular weight (in Kd) given in Table 9.1, column "This Thesis". 5C and 8C denote trypsin-cleavage products of VP4 which were observed in the purified virions but not in cells infected with standard brv or brv mutant A_4.

N^d, A^d denote the double capsid particles of standard brv and brv mutant A_4 respectively.

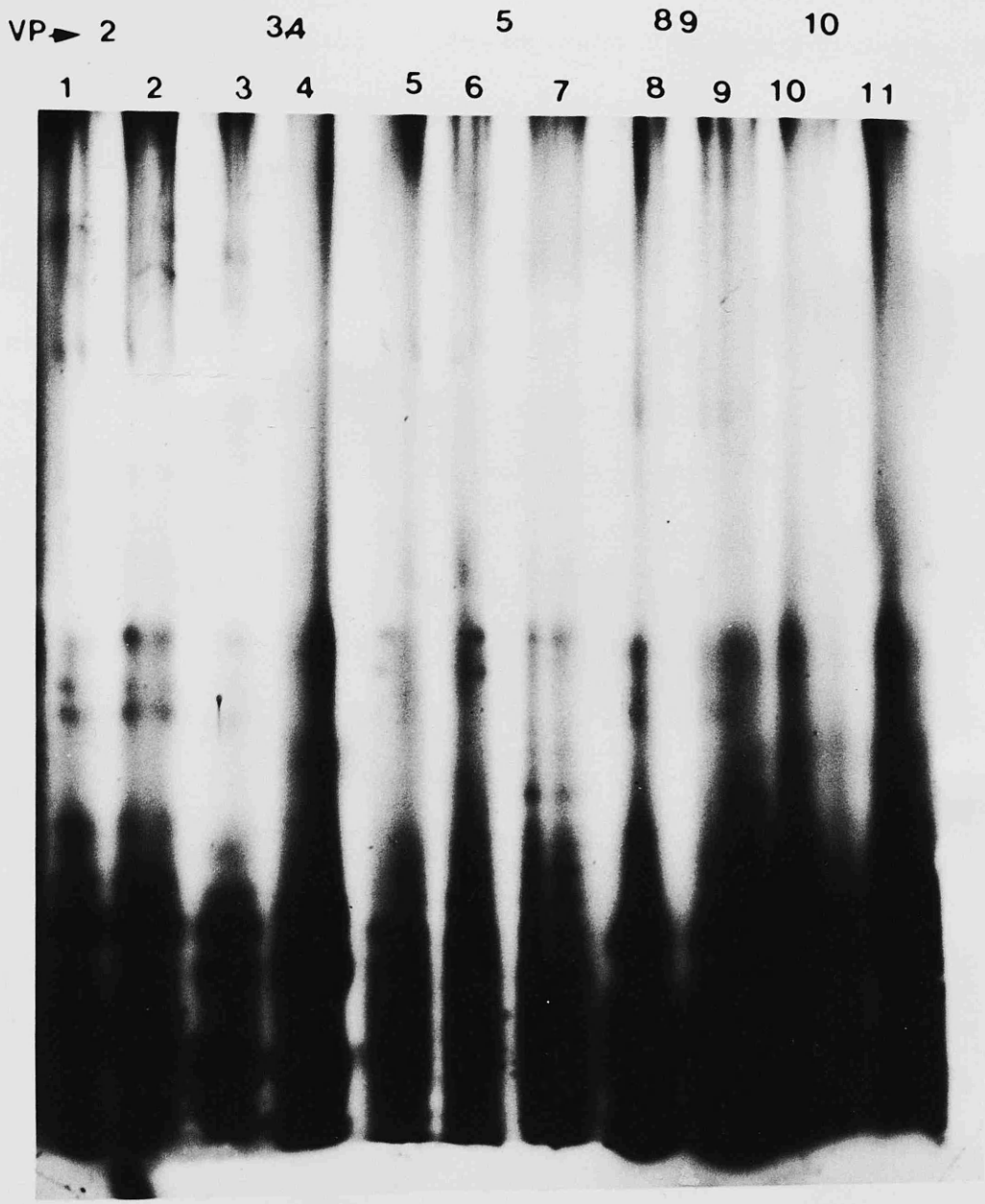
N^s denotes the single capsid particles of standard brv.

Figure 11.1.

One dimensional peptide maps of rotavirus-specific proteins.

Viral proteins synthesised in infected cells were separated on a 12% polyacrylamide gel and selected proteins of standard brv and brv mutant A_4 were sliced out, loaded into the slots of a 15% polyacrylamide gel and digested with 20ug/slot of Staphylococcus aureus V8-protease in sample buffer. The peptides generated by partial proteolysis were separated by electrophoresis (Cleveland et al. 1977). The viral proteins used are indicated on top (VP2-VP10).
Tracks 1, 3, 5, 8 and 10 contain [^{35}S]-methionine labelled peptides of VP2, VP3/4, VP5, VP6 and VP10, respectively, of standard brv.
Tracks 2, 4, 7, 9 and 11 contain [^{35}S]-methionine labelled peptides of VP2, VP3/4, VP5A, VP6 and VP10 of brv mutant A_4.
Track 6 contained peptides produced from cellular protein comigrating in the position of the normal VP5 in the protein profile of brv mutant A_4.

A diagram indicating the position of peptide bands as visualised on the original autoradiograph is attached in the pocket.


VP
2
3A
5
8 9
10
1
2
3
4
5
6
7
8
9
10
11

11 Comparative peptide mapping of viral proteins.

Among the genomically rearranged brvs, only the brv mutant A_4 was found to produce a novel virus-specific protein, VP5A, during infection. Therefore, viral proteins synthesised in cells infected with the brv mutant A_4 were selected for comparative one-dimensional peptide mapping - and compared with the corresponding proteins of standard brv. Gel pieces containing the protein were cut out of a preparative 12% gel, loaded into slots of a 15% polyacrylamide gel and digested in situ with 20ug of Staphylococcus aureus V8-protease per slot. The peptides were separated by electrophoresis overnight at room temperature as described (Cleveland et al., 1977). The maps (Figure 11.1) obtained after autoradiography of the [^{35}S] methionine labelled tryptic peptides showed close similarities for the VP1, VP2, VP3, VP6 and VP8/9 of standard brv and brv mutant A_4. The peptide map for the mutant VP5A was similar to that of VP5 (Figure 11.1, tracks 7 and 5). There were apparent differences observable in the large peptides of VP5A which were not found in VP5. However, these differences could not be ascertained because both VP5 and VP5A repeatedly produced smeary maps under the conditions used for peptide mapping.

In order to determine a relationship between the normal VP5 and the mutant VP5A, two-dimensional peptide mapping with its improved resolution and sensitivity was employed (as described in Methods, according to MacDonald 1980). The VP6 proteins of standard brv and brv mutant A_4 were included for reference purposes. The tryptic peptide

Figure 11.2.

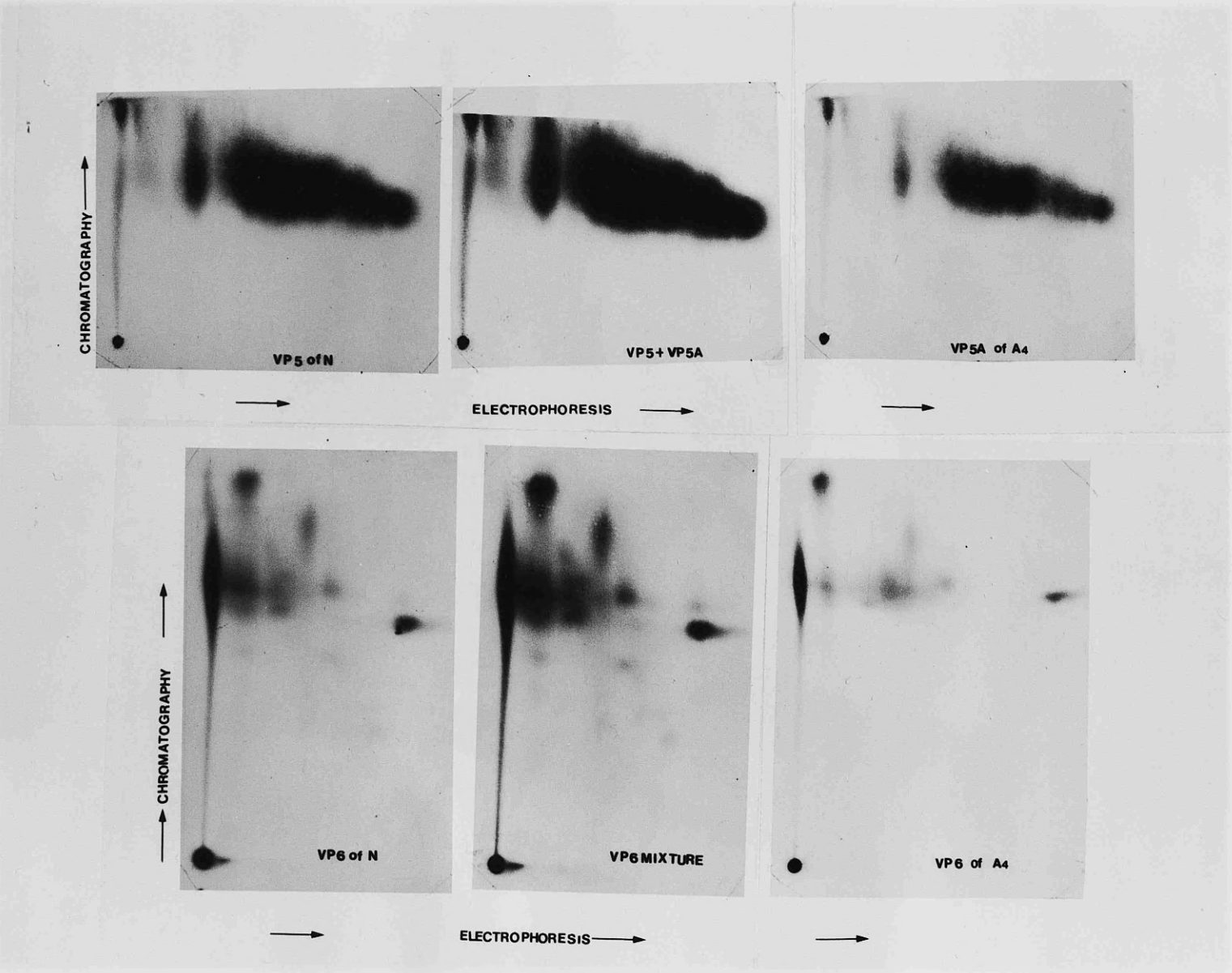
Two-dimensional peptide mapping of VP5 and VP5A and VP6

The viral proteins VP5 of standard brv and VP5A of brv mutant A_4 were cut out from a preparative gel of [^{35}S] methionine labelled proteins produced in infected cells. The proteins VP5 and VP5A were digested with trypsin; the tryptic peptides were spotted on TLC sheets, electrophoretically separated in first dimension at pH 3.5 followed by ascending chromatography in a second dimension as described in Methods after MacDonald, 1980.

Panel A: peptides of VP5 and VP5A with the mixture of the two shown in the middle;

Panel B: peptide maps of VP6 of brv A_4 and standard brv with the mixture of the two shown in the middle (included in the assay for comparison and reference).

Electrophoresis in the first dimension was from left to right (indicated) at 550V for 45 minutes. Chromatography was from bottom to top, direction shown.


CHROMATOGRAPHY
VP5 of N
VP5+ VP5A
VP5A of A4
ELECTROPHORESIS
CHROMATOGRAPHY
VP6 of N
VP6 MIXTURE
VP6 of A4
ELECTROPHORESIS

maps of VP5 and VP5A were very similar (Figure 11.2, panel A) suggesting strongly that the mutant protein VP5A was related to VP5 in sharing common amino acid sequences. Figure 11.2 panel B shows similar maps of VP6 proteins of standard brv and brv mutant A_4 which appear to be very closely related. This observation, taken together with the similarities found in the RNase T1-resistant oligonucleotide maps of RNA segments 5 and E (see Results 3.2) provides evidence that RNA segment E was rearranged from the normal RNA segment 5.

12 Effect of multiplicity of infection on protein synthesis.

After infection of cells with the brv mutants A_4, B_4 and B_5 cellular protein synthesis was less suppressed than by standard brv or mutant D (see Figure 9.1 and Hundley et al., 1985). For this reason, protein synthesis was investigated at different m.o.i.s. Cell monolayers on 50mm petri dishes were infected for 16 hours at 40 and 1 pfu/cell with standard brv and brv mutants D, A_4, B_4 and B_5. The proteins were pulse labelled in 2ml of Eagle's MEM containing 7.4 MBq/ml of [^{35}S] methionine for 2 hours at 14 hours pi and then separated by SDS-PAGE. At both m.o.i.s, all viral proteins were synthesised (Figure 12.1). Although the pulse labelling was performed under hypertonic conditions which blocks cellular RNA synthesis (Nuss et al., 1975; Racaniello and Palese 1979) cellular background was more marked in the protein profiles of the brv mutants A_4, B_4 and B_5 (Figure 12.1). The brv mutant D inhibited host cell protein synthesis to an extent almost comparable with that of

Figure 12.1.

Effect of multiplicity of infection on protein synthesis.

Confluent cell monolayers were infected at 40 pfu/cell with standard brv and the mutants D, A_4, B_4 and B_5 (tracks 1-5, respectively). Infection at 1 pfu/cell was used for standard brv and the mutants D, A_4 and B_4 (tracks 6-9, respectively). Pulse labelling of proteins was carried out at 16 hours pi for 2 hours using 2ml of Eagle's MEM containing 7.4 MBq/ml [^{35}S] methionine. The proteins were separated on 12% polyacrylamide gels. Track C were the [^{35}S] labelled uninfected cells controls. Viral proteins were numbered VP1-12 from top to bottom.

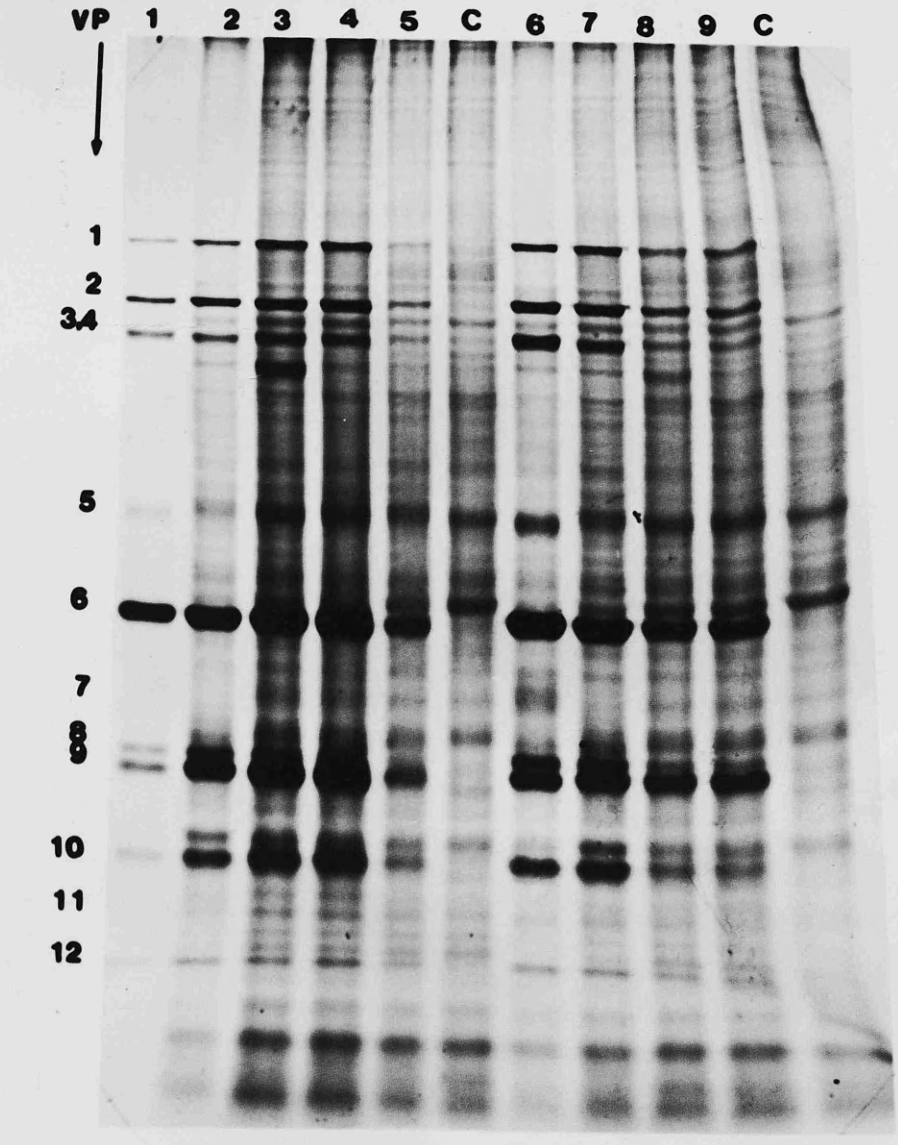

VP
1
2
3
4
5
C
6
7
8
9
C
1
2
3,4
5
6
7
8
9
10
11
12

Table 12.1. Relative molar concentrations of intracellular viral proteins VP1-VP12.

The figures given in moles indicate relative proportions of VP1-VP12 for the standard brv (N) and the brv mutants D, A_4, B_4 and B_5. The viral proteins were synthesised intracellularly at m.o.i. 5 shown in Figure 9.1 and 1 and 40 pfu/cell as shown in Figure 10.1. Percentages of viral proteins were calculated from densitometer tracings of the autoradiographs shown in Figures. 9.1 and 10.1. These percentages represented relative amounts assuming a comparable methionine content per viral protein. Relative molar concentration (x 10^4) were obtained by dividing the percentage by the respective molecular weight shown in Table 9.1, column "This Thesis".

The values obtained after infecton at moi of 5 and those obtained after infection at mois of 1 and 40 were from different experiments.

Table 12.1.

Virus	m.o.i.	Viral protein (VP) ---> 1	2	3+4	5	6	7	8	9	10	11	12
N	5	1.0	1.2	0.4	1.0	11.1	0.2	1.5	0.7	0.3	0.2	0.2
	40	0.5	1.2	1.2	2.1	10.3	0.7	1.3	2.0	4.8	0.3	1.3
	1	0.6	1.1	1,2	2.0	10.4	0.8	0.8	1.7	4.7	0.3	1.6
D	5	1.3	1.3	0.2	-	12.6	0.5	2.0	0.7	0.2	0.3	0.3
	40	0.6	1.2	0.8		10.7	0.2	1.5	2.3	4.6	0.3	1.7
	1	0.8	1.5	1.2		9.2	0.3	1.0	2.2	4.4	0.4	1.9
					5A							
A_4	5	0.9	1.0	0.2	1.1	8.8	0.4	2.9	2.0	0.7	0.3	0.3
	40	0.5	0.8	0.5	0.7	6.2	0.6	0.8	9.2	2.9	0.3	1.6
	1	0.6	1.0	0.5	0.9	6.7	0.4	0.3	7.3	2.8	0.3	2.8
B_4	5	1.4	1.3	0.4	-	8.8	0.3	2.0	2.9	0.6	0.5	0.5
	40	0.5	0.9	0.5		7.4	0.3	0.1	7.6	3.5	0.5	1.0
	1	0.7	0.9	0.6		7.7	0.5	0.3	7.6	2.5	0.6	4.8
B_5	5	1.0	1.6	0.4	-	10.8	0.4	2.9	1.2	1.5	0.6	0.6
	40	0.2	1.9	0.7		7.0	0.3	3.6		3.3	0.7	1.3

* However, at low moi mutant D does not switch off the host cell response very efficiently either.

standard brv (tracks 2 and 7, Figure 12.1). This result and that shown in Figure 9.1, suggest that the brv mutants A_4, B_4 and B_5 have a less inhibitory effect on host cell protein synthesis than standard brv and brv mutant D.* During infection at high m.o.i., the nonstructural protein VP9 was produced in amounts at least twice that of standard brv in the cells which were infected with the brv mutants A_4, B_4 and B_5 (Figure 12.1). This observation may suggest a possible interplay of VP9 and host cell metabolism since it occurs in increased amounts in the brv mutant infected cells in which host protein synthesis is inefficiently shut off.

Microdensitometer scanning technique (Whitton et al., 1983) was used to analyse the viral proteins synthesised in cells infected at different m.o.i.s (Figure 12.1). Applying the computer programme DENS (P. Taylor, MRC Virology, written for PDP11 mini computer) the area under each densitometer peak was calculated and expressed as percentage of of total viral protein in each infected cell lysate. It was found that at 16 hours pi, the brv mutants D, A_4, B_4 and B_5 synthesised proteins in proportions similar to those produced by standard brv N_L (Table 12.1). when compared within the same experiment.

The abnormal VP5A of brv mutant A_4 was detected in molar proportions compatible with its intermediate molecular weight between VP5 and VP4. The relative molar proportion of individual viral proteins in standard brv and brv mutants D, A_4, B_4 and B_5 varied slightly with changes of m.o.i. (Table 12.1). However, it was found that the relative molar proportions of proteins synthesised by each of the viruses

Figure 13.1.

Time course of protein synthesis in infected cells.

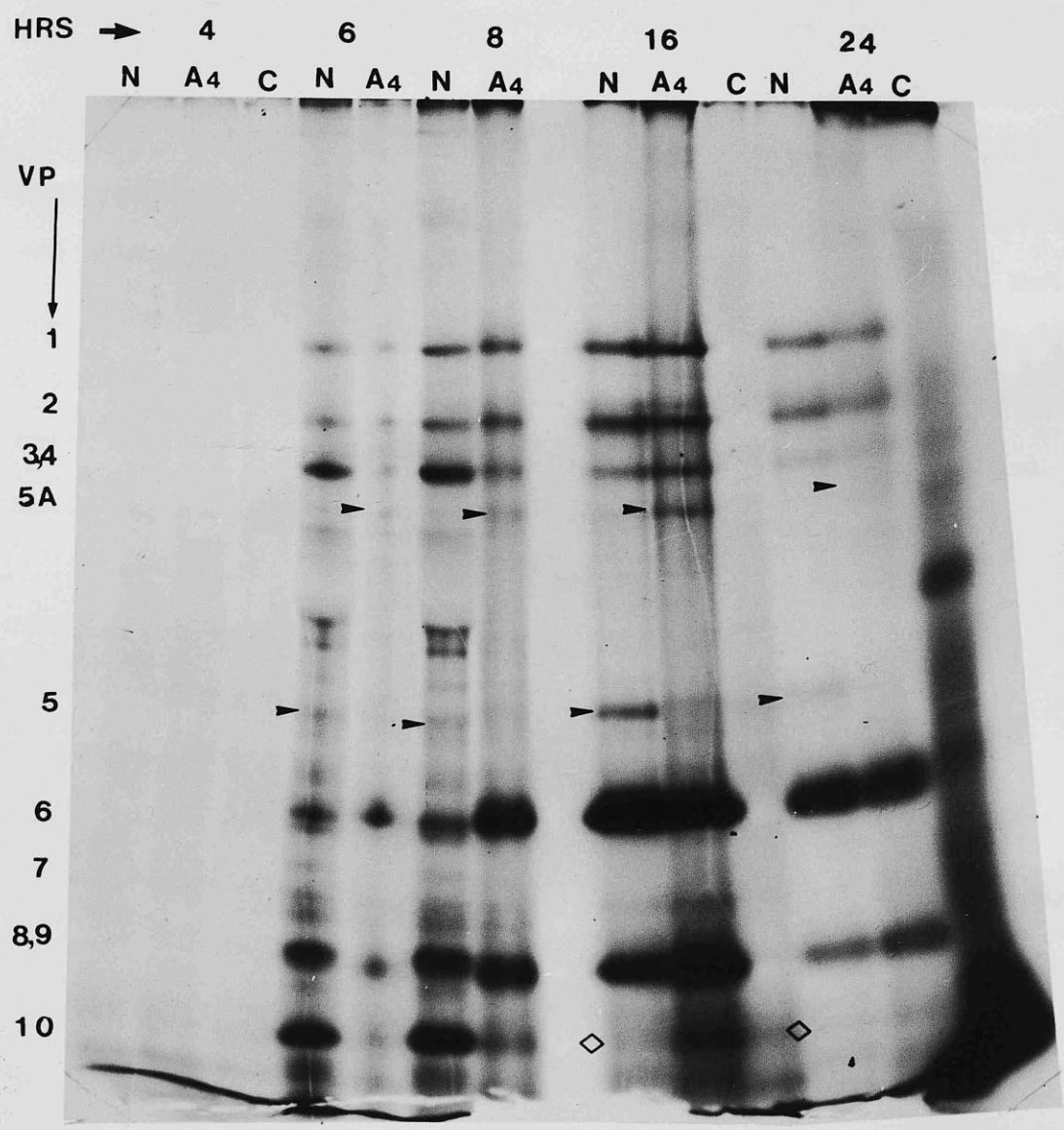
MA104 cells were infected for 4, 6, 8, 16 and 24 hours (indicated on top of gel) with standard brv and brv mutant A_4. The proteins were pulse labelled with 7.4 MBq/ml of [^{35}S] methionine for 2 hours under the conditions of hypertonic shock (described in Methods).

Tracks N: [^{35}S] methionine labelled proteins produced in cells infected with the standard brv; tracks A_4: [^{35}S]-methionine labelled proteins produced in cells infected with brv mutant A_4; tracks C:[^{35}S]-methionine labelled proteins produced in uninfected cells.

Arrows (►) point to VP5A of brv A_4 (tracks A_4) and VP5 of standard brv (tracks N).

Open diamond (◇) denotes diminishing VP10 in cells infected with standard brv for 16 and 24 hours (tracks 16N, 24N).

The position of viral proteins VP1-VP10 is indicated on the left.


HRS
4
6
8
16
24
N
A4
C
N
A4
N
A4
N
A4
C
N
A4
C
VP
1
2
3,4
5A
5
6
7
8,9
10

were unequal indicating that the translation of viral mRNA was not equimolar.

13 Time course of protein synthesis in infected cells.

Intracellular viral proteins were pulse labelled with [^{35}S] methionine at 4, 6, 8, 16 and 24 hours pi (Figure 13.1). Virus-specific proteins were found to be synthesised in full complement of VP1-VP12 at 6 hour pi in cells infected with the standard brv and with the brv mutant A_4. The mutant protein VP5A was detected at the same time, 6 hours pi, as the normal VP5. The observation suggests that there was no sequential protein synthesis in either the genomically rearranged brv mutant A_4 or the standard brv.

Identifiable viral proteins VP1-VP10 excluding VP7 and VP12 which were present in amounts unsuitable for densitometry, were evaluated using densitometry computer programme "DENS" (P. Taylor) to calculate and compare percentages of individual viral proteins (Table 13.1). It was observed that both standard brv and brv mutant A_4 produced viral proteins in amounts depending on duration of infection. Between 8 and 16 hours pi, VP6 of the standard brv increased three fold in contrast to the VP6 of the brv mutant A_4 which only showed a 20 % increase. During the same period, the glycoprotein (VP10) synthesised in cells infected with the standard brv showed a marked decline of $\geq$ 30 fold at 16 hours pi (Figure 13.1); while the VP10 produced in brv mutant A_4 infected cells declined 2 fold between 8 and 16 hours pi.

During another time course of infection to investigate

Infected cell preparation	Viral protein (VP) ---> 1	2	3/4	5	6	7	8/9	10
8N	7.2	6.7	17.5	2.2	16.4	0	19.1	30.2
16N	6.2	11.6	13.5	3.3	53.9	0	21.3	0.67
8A	8.9	9.4	5.3	2.4[a]	40.9	0	24.8	8.1
16A	7.5	6.4	3.7	3.1[a]	48.3	0	26.2	4.9

Table 13.1. Intracellular viral proteins at different times post infection.

The values denote percentage of each viral protein VP1-VP10 represented at the time of assay 8 and 16 hours pi for the virus standard (N) and the brv mutant A_4 (A). The percentages were calculated from areas of densitometer tracings of a gel shown in Figure 10.2.

[a] Percentage of VP5A

Figure 13.2.

Post-translation modification of [^{35}S]methionine-labelled viral proteins VP1-VP10 marked on the left were studied by pulse-chase procedure described in Methods.

Tracks 1 and 2 show pulse labelled proteins synthesised at 8 hrs pi in cells infected with standard brv and brv mutant A4 respectively.

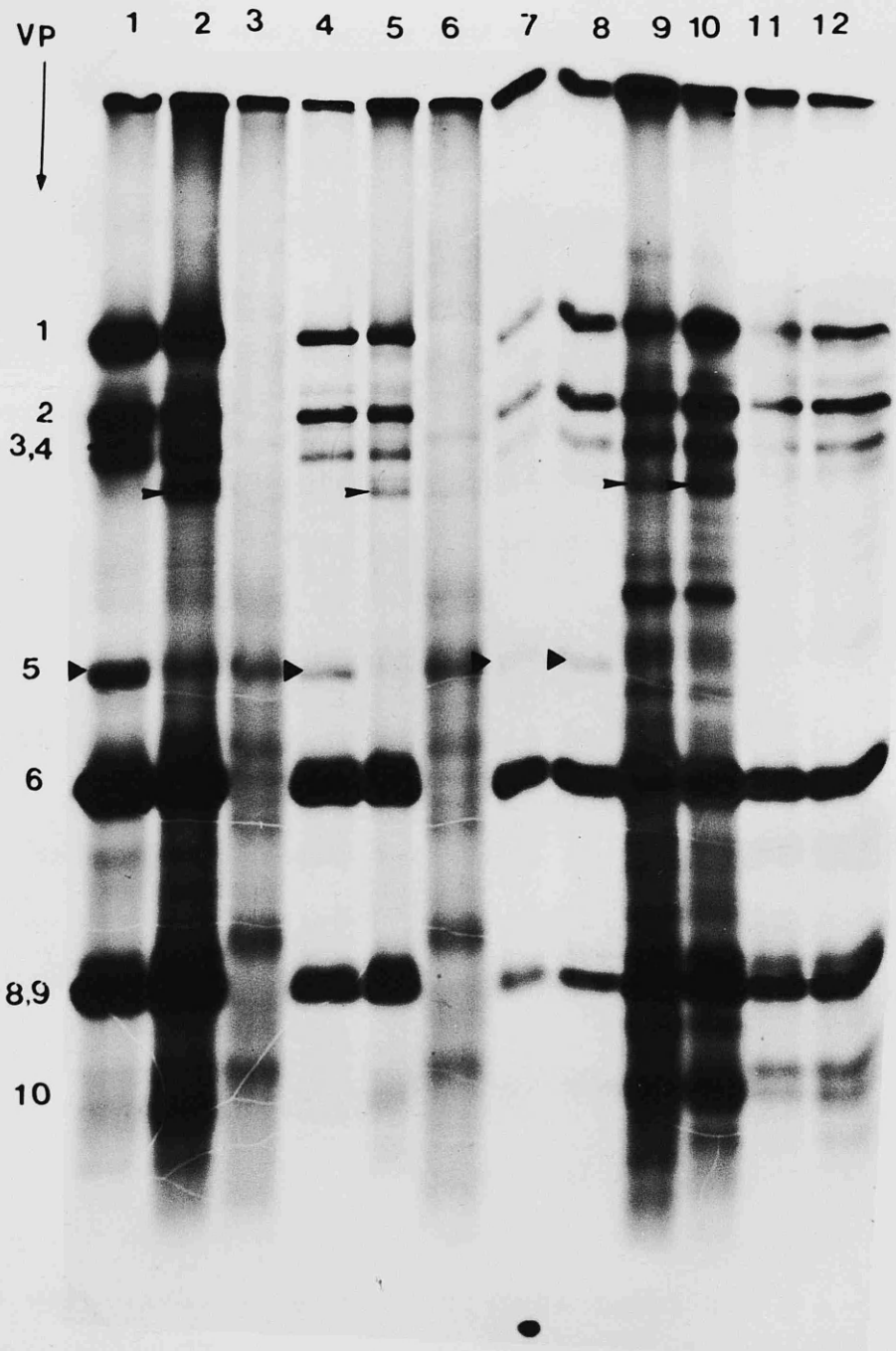
Tracks 4 and 7 show pulse labelled proteins from cells infected with standard brv at 16 hrs pi.

Tracks 5, 9 and 12 show pulse labelled proteins of standard brv, brv mutant A4 and mutant D respectively.

Tracks 8, 10 and 12 show proteins of standard brv, brv mutant A4 and brv mutant D after a 2 hr chase in 300 times excess unlabelled methionine.

Tracks 3 and 6 show protein labelling in uninfected cells at 8 and 16 hrs respectively.

Arrow [►] points to VP5A; triangle [▶] denotes VP5.


VP
1
2
3
4
5
6
7
8
9
10
11
12
1
2
3,4
5
6
8,9
10

stability of proteins, the brv mutant A_4 and standard brv were compared and protein synthesis was monitored by SDS-PAGE (Figure 13.2). The viral proteins were pulse labelled for 2 hours in hypertonic Eagle's medium containing 7.4MBq/ml of [^{35}S] methionine and then chased in a medium lacking [^{35}S] methionine but containing a 300-fold excess of unlabelled methionine. The viral proteins VP5 and VP5A remained unchanged after a chase period of 2 hours (tracks 7, 8 and 9, 10 respectively in Figure 13.2) suggesting that these proteins were not post-translationally modified. Like the rest of viral proteins shown VP1-VP10, the glycoproteins VP2, VP6, VP7 and VP10 were unchanged in their mobility on the gel before and after the chase period (Figure 13.2).

14. Effect of tunicamycin on protein synthesis in infected cells.

Posttranslational modification/processing of intracellular viral proteins was investigated using tunicamycin, an inhibitor of N- linked glycosylation (Heifetz et al., 1979; Dunphy et al., 1985). Cell monolayers were infected with standard brv and brv mutants D and A_4 in either presence or absence of tunicamycin at a concentration of 5ug/ml from 1 hour postadsorption. The proteins were labelled with [^{35}S] methionine for 2 hours at 14 hours and then separated on 12% polyacrylamide-SDS gels. The proteins produced in cells that were infected in the absence of tunicamycin showed normal profiles and there was no observable mobility change in the glycoproteins VP2, VP6,

Figure 14.1.

Effect of tunicamycin on protein synthesis in infected cells.

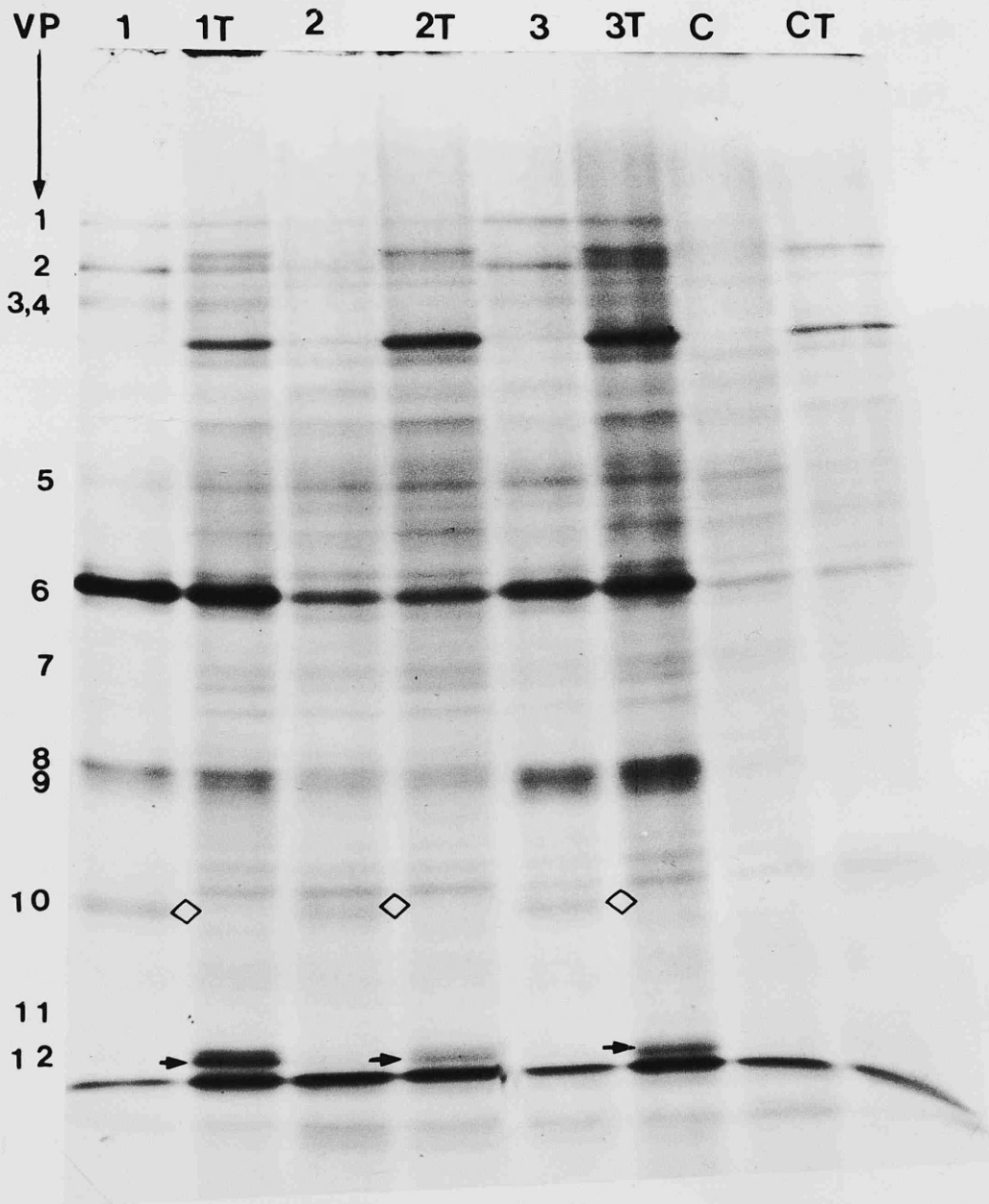
Confluent cell monolayers were infected with 1 pfu/cell of standard brv and brv mutants D and A_4. The proteins synthesised were pulse labelled with 7.4 MBq/ml of [^{35}S] methionine in 2ml of methionine free Eagle's MEM for 2 hours at 16 hours pi, and then separated on 12% polyacrylamide SDS slab gel (Elliott et al., 1984).

Tracks 1, 2, 3 show [^{35}S] methionine labelled proteins found in cells infected with standard brv and brv mutants D and A_4, respectively, in the absence of tunicamycin.

Tracks 1T, 2T and 3T show [^{35}S] methionine labelled proteins produced in the presence of 5ug/ml tunicamycin in cells infected with standard brv and brv mutants D and A_4, respectively.

C and CT denote the proteins of uninfected cells, [^{35}S] methionine labelled in the absence (C) and presence (CT) of 5ug/ml tunicamycin.

Closed arrow (➔) points to accumulation of VP12 in tunicamycin treated infected cells, open diamond (◇) to VP10 diminishing under tunicamycin treatment.


VP
1
1T
2
2T
3
3T
C
CT
1
2
3,4
5
6
7
8
9
10
11
12

Virus	Concentration (ug/m) of tunicamycin		
	0	1	5
N	$4x10^5$	$1x10^4$	$4x10^4$
D	$8x10^5$	$4x10^4$	$8x10^4$
A_4	$7x10^4$	$3x10^3$	$1x10^3$

Table 14.1.

Effect of tunicamycin on rotavirus infectivity.

Cell cultures were infected at 1 pfu/cell with standard brv and the brv mutants D and A_4. The infection was maintained in the absence (0) and presence of tunicamycin 1 and 5ug/ul until CPE was complete. Virus yields (pfu/ml) were determined by plaquing the viruses at 37°C on MA104 cell monolayers under 0.5% agar overlay and 5ug/ul pancreatin in Eagle's MEM lacking phenol red (Methods).

VP7 and VP10, (Figure 14.1, tracks 1, 2 and 3). In contrast, the extracts of cells which were infected and labelled in the presence of 5ug/ml tunicamycin contained changes in mobility of VP10 (indicated with open diamond in Figure 14.1) and the concurrent accumulation of VP12 (arrowed in tracks 1T, 2T and 3T, Figure 14.1). The observable effect of tunicamycin on viral protein synthesis was uniform for standard brv as for brv mutants D and A_4 and demonstrated that VP10 was derived from VP12 by N-linked glycosylation. Furthermore, this experiment provided evidence that VP2 and VP6 were not glycosylated in N-glucosidic linkage since both proteins were insensitive to tunicamycin. With tunicamycin treatment of infected cells, the yield of virus was reduced by 1 log unit compared with the virus produced in absence of tunicamycin (Table 14.1) whether or not the virus was a brv mutant or standard brv. The major structural glycoprotein VP7 was produced in such small amounts during infection that it was neither easily detected nor could the non glycosylated forms of the viral protein be identified. Under tunicamycin treatment, two prominent cellular proteins, one above VP2 and the other comigrating with VP5A, were induced (Figure 14.1, tracks 1T, 2T, 3T, CT).

15 Viral glycoproteins of genomically rearranged brv mutants.

Synthesis of glycoproteins was studied in cells which were infected with standard brv and the brv mutants D, A_4, B_4 and B_5 in an attempt to find out whether or not there

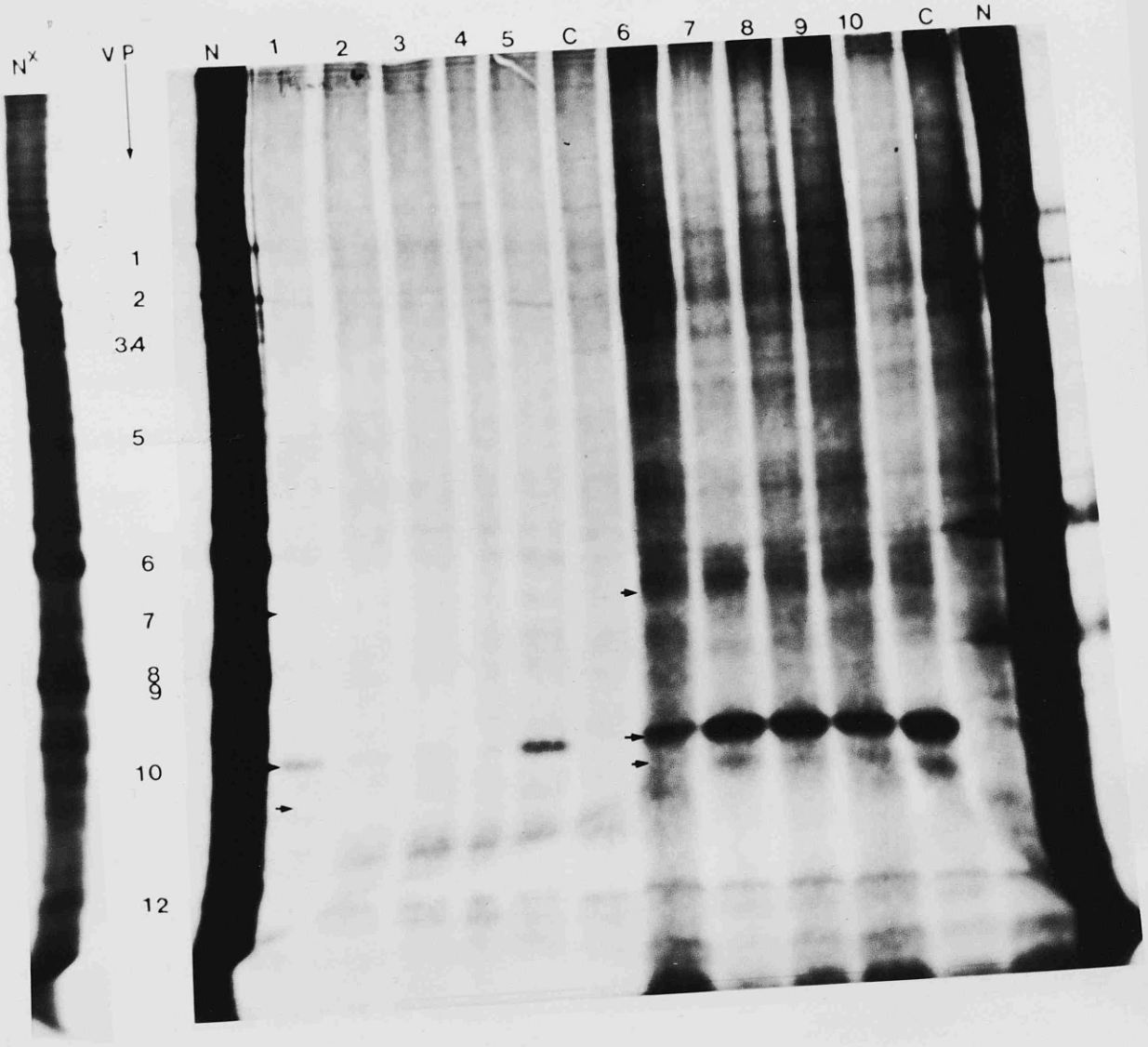
Figure 15.1.

Virus-specific glycoprotein synthesis in infected cells.

MA104 cells were infected in duplicate with standard brv and brv mutants D, A_4, B_4 and B_5. The infected cells were then labelled in parallel with either tritiated mannose (tracks 1-5) or tritiated glucosamine (tracks 6-10) and the proteins separated on a 12% polyacrylamide gel and autoradiographed as indicated in Methods.

Track N shows [^{35}S] methionine labelled proteins of cells infected with standard brv. Tracks C: lysates of uninfected cells labelled with either mannose (between tracks 5 and 6) or glucosamine (between tracks 10 and N).

Reference viral proteins were designated VP1-VP12 to the right of the gel. Closed arrows point to incorporation of [^{3}H] mannose (tracks 1-5) and of [^{3}H] glucosamine (tracks 6-10) in the viral proteins VP7, VP10 and VP10C.
Autoradiography was for 4 weeks at -70^{o}C.
N^{x} denotes a lighter exposure of track N.


N^x
VP
N
1
2
3
4
5
C
6
7
8
9
10
C
N
1
2
3.4
5
6
7
8
9
10
12

were changes associated with genome rearrangements. Infected cells were labelled continuously with either (^{3}H) mannose (Figure 15.1, tracks 1-5) or (^{3}H) glucosamine (Figure 15.1, tracks 6-10). (^{3}H) Mannose was detected in al VP2, VP6, VP7, VP10 and VP10C. But the VP7, VP10 and VP10C, incorporated (^{3}H) mannose was very accentuated in the glycoproteins produced in cells which were infected with the standard brv and brv mutant B5 (tracks 1 and 5 in Figure 15.1). Irregular sugar labelling was consistently seen with the VP7, VP10 and VP10C of some of the brv mutants. In a parallel experiment, (^{3}H) glucosamine was used to label glycoproteins. Without exception (^{3}H) glucosamine was incorporated in the glycoproteins VP7, VP10, VP10C of the brv mutants and standard brv (Figure 15.1, tracks 6-10). The viral proteins VP2 and VP6 showed a fuzzy labelling pattern with (^{3}H) glucosamine but it was clear from the labelling with [^{3}H] mannose that the proteins contained the sugar moieties.

To follow the pattern of glycoprotein synthesis during infection, a time course was performed using the standard brv and the brv mutant D. For 6, 16 and 24 hours, infected cells were labelled with (^{14}C) mannose and cell lysates analysed by SDS-PAGE and autoradiography (Figure 15.2). At 6 hours pi, clear labelling of glycoprotein VP10 of brv mutant D was discernable. Later in infection, (^{14}C) mannose was found to be incorporated specifically in VP2 and VP6 of both standard brv and the brv mutant D. There were clear differences (once reproduced) in the mannose contents of VP7 and VP10 (arrow in Figure 15.2, track 4) being reduced or absent in the brv

Figure 15.2.

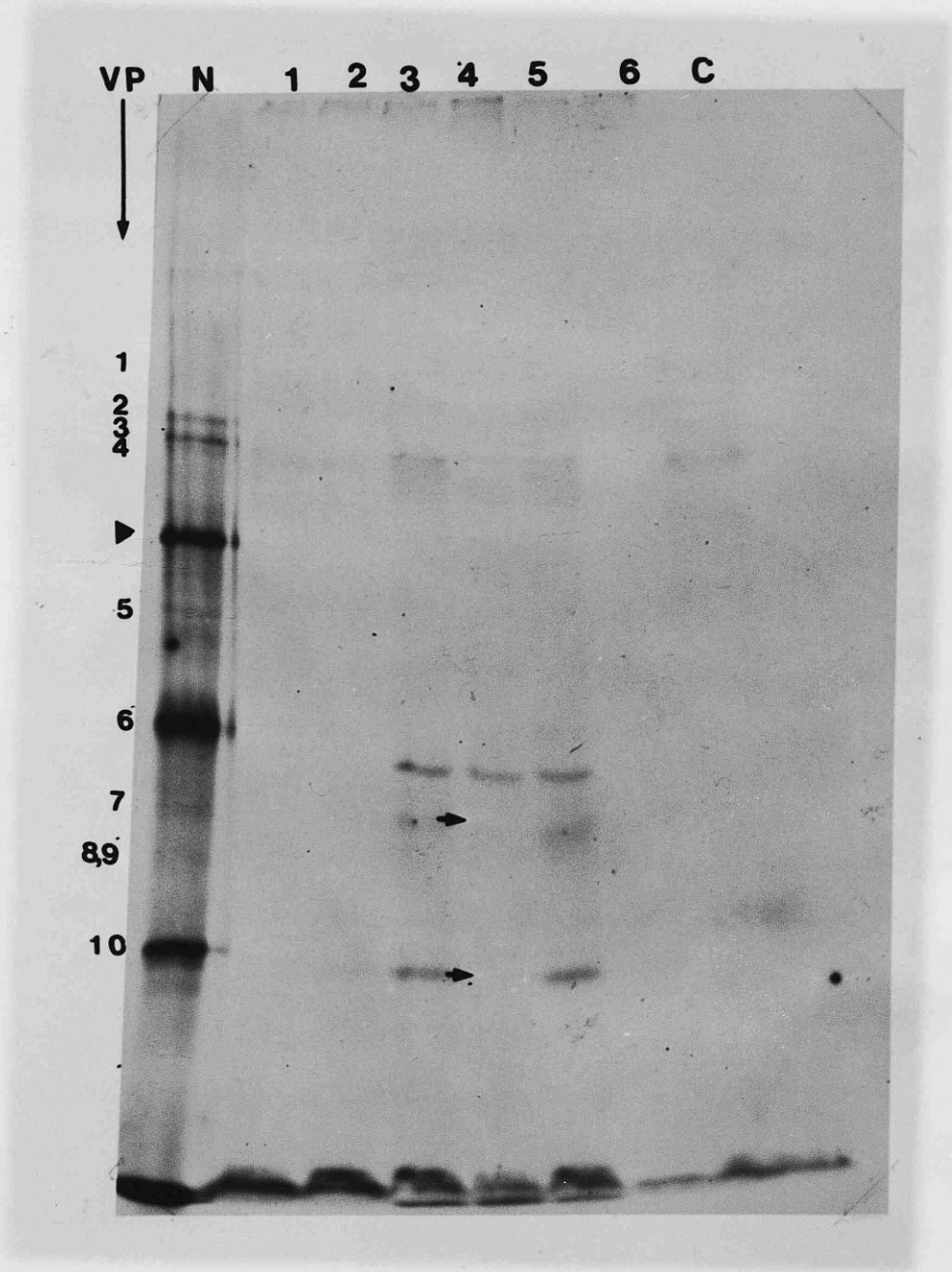
Time course of glycoproteins synthesis.

An autoradiogram showing proteins which were synthesised in cells infected with standard brv and brv mutant D for 6 hours (tracks 1 and 2, respectively); 16 hours pi (tracks 3 and 4) and 24 hours pi (tracks 5 and 6). The cells were labelled from 1 hour pi until harvest time with 0.37 MBq of [^{14}C] mannose for each 50mm petri dish of cells. Track C was uninfected cells labelled with [^{14}C] mannose under the same conditions for 16 hours.

Track N shows [^{35}S] methionine labelled proteins of cells infected with standard brv.

Closed arrows (⟵) point to diminished VP7 and VP10 in cells infected with brv mutant D (track 4).

Closed triangle (▶) indicates unusually heavy cellular protein found in [^{35}S]-methionine labelled infected cells (track N).


VP
N
1
2
3
4
5
6
C
1
2
3
4
5
6
7
8,9
10

mutant when as compared with the corresponding proteins of standard brv (Figure 15.2, track 3). The absence of (^{14}C) mannose labelling in 24 hour samples of brv D infected cells was largely due to loss of material because of more advanced CPE at this time point.

The foregoing findings suggest that genome rearrangements were associated with abnormalities in the compositions of the viral glycoproteins VP7 and VP10.

16 Antigenic relationship among the brv mutants.

Radioimmunoprecipitation (IP) tests were employed in order to compare the antigenicity of standard brv and of brv mutants D, A_4, B_4 and B_5. Using polyclonal antisera raised against standard brv and brv mutant D, it was shown that there was no serological difference in the IP reactions of major antigens, between the brv mutants and standard brv (Figures 16.1 and 16.2, tracks 1-5).

The role of glycosylation for antigenicity was examined by using antigens obtained from virus which had been labelled in the presence of tunicamycin. On IP with the antisera against standard brv and against brv mutant D, major antigens common to both namely VP1, 2, 3, 4, 6, VP7 and 10 were immunoprecipitated. The non glycosylated forms of VP7 and VP10 (arrow-marked in Figures 16.1 and 16.2) were not reactive in this test. The antigens derived from cells which were infected with the standard brv, the brv mutants D, A_4, B_4 and B_5 were labelled with [^{35}S] methionine in the presence and absence of tunicamycin, and IP assays with antiserum against brv mutant A_4 were carried out. The IP

Figure 16.1.

Antigenic relatedness among the genomically rearranged brv mutants investigated by radioimmunoprecipitation (RIP).

Antigens were prepared in cells infected with standard brv and the brv mutants D, A_4, B_4 and B_5 and labelled with 7.4 MBq/ml of [^{35}S]-methionine. They were each reacted with hyperimmune polyclonal polyvalent antiserum raised against the standard brv. RIP was achieved with staphylococcus protein A (immunoprecipitin) using the procedure described by Palfreyman et al. (1984) as detailed in Methods. The immunoprecipitates were boiled in dissociation buffer and the proteins separated on 12% polyacrylamide SDS-gel as described by Elliott et al., 1984 and Hundley et al., 1985. Tracks 1 to 5: polypeptides immunoprecipitated from antigens prepared in the absence of tunicamycin of standard brv and the brv mutants D, A_4, B_4 and B_5 in order. Tracks 1T to 5T: IP reactions of corresponding antigens which were obtained after treatment of infected cells with tunicamycin. Tracks N, NT: [^{35}S]-methionine labelled proteins of cells infected with standard brv without (N) or in thepresence of tunicamycin (NT). Closed arrows (►) show IP reaction with antigen vpr7 (smaller than the glycosylated VP7). Open diamonds (◇) indicate the absence of IP reaction in the area of VP7 and VP10 for the tunicamycin treated antigens. Uninfected cells treated (CT) and untreated (C) with tunicamycin were included for comparison. Viral proteins VP1-VP10 were marked on the left.

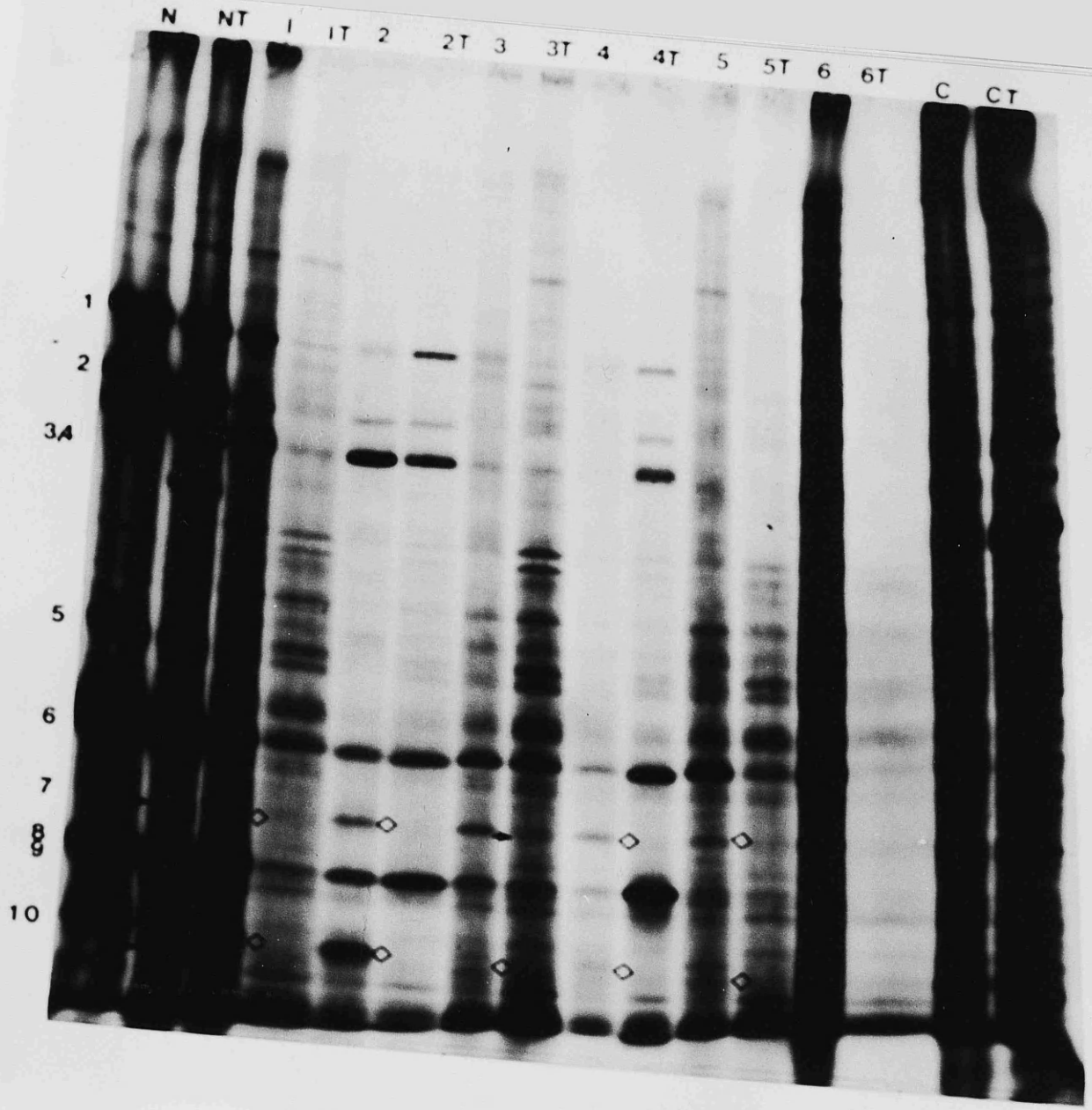

N
NT
1
1T
2
2T
3
3T
4
4T
5
5T
6
6T
C
CT
1
2
3A
5
6
7
8
9
10

Figure 16.2.

Radioimmunoprecipitation using the antigens described in Figure 16.1 and the polyconal antiserum raised against brv mutant D.

The procedure was as described in Legend to Figure 16.1 except that an antiserum directed against brv mutant D was used.

Tracks 1 to 5: RIP proteins using antigens N, D, A_4, B_4 and B_5, respectively.

Tracks 1T to 5T denote corresponding antigens which were prepared in the presence of tunicamycin.

Tracks 6, 6T contain uninfected cells labelled without and with tunicamycin, respectively.

Tracks D and DT show [^{35}S]-methionine labelled proteins from cells infected with brv mutant D without and with tunicamycin respectively.

Tracks C and CT: uninfected cell lysates after corresponding treatment.

Arrows (►) show absence of IP reactions in the antigens treated with tunicamycin.

Viral proteins VP1-VP10 were numbered on the right.

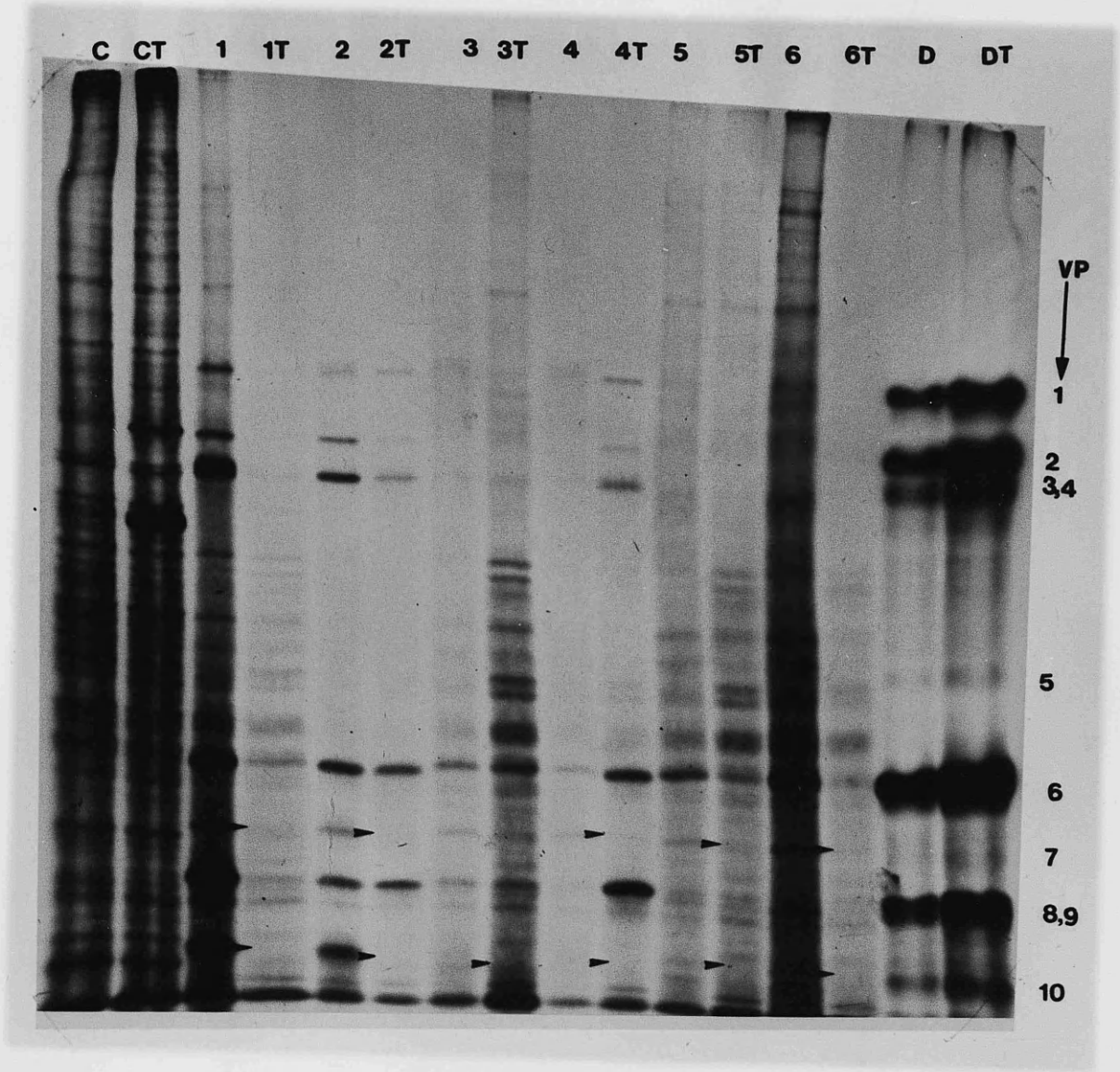

C
CT
1
1T
2
2T
3
3T
4
4T
5
5T
6
6T
D
DT
VP
1
2
3,4
5
6
7
8,9
10

Figure 16.3.

Radioimmuno-precipitation assay using a polyclonal antiserum raised against brv mutant A_4.

Tracks 1 to 5 contain IP reactions using [^{35}S]-methionine labelled antigens prepared in the absence of tunicamycin in cells infected with standard brv and brv mutants D, A_4, B_4 and B_5 respectively; tracks 1T to 5T show the IP reactions in which the corresponding tunicamycin-treated [^{35}S]-methionine labelled antigens were used.

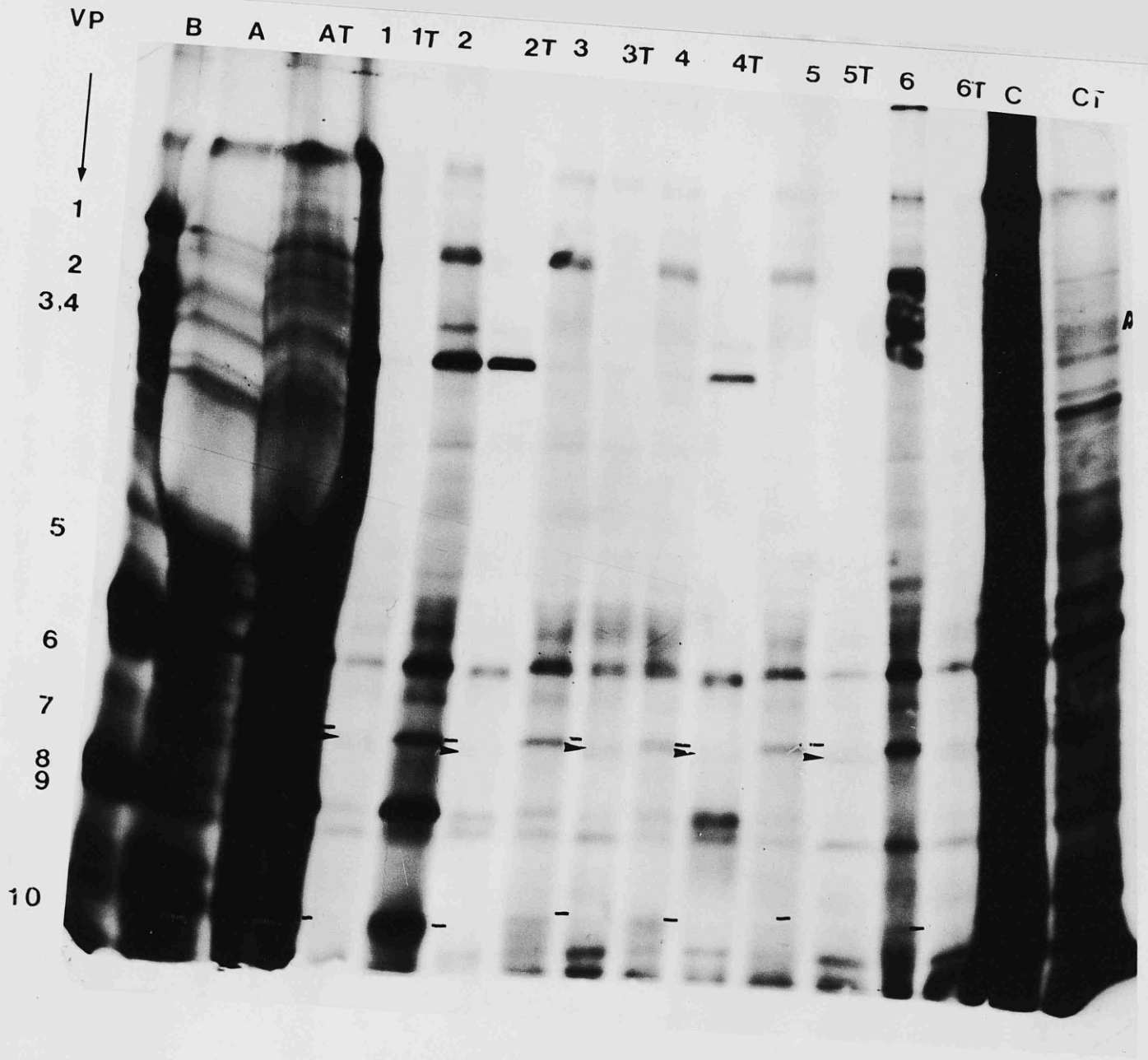
Tracks 6 and 6T contain uninfected cell antigens labelled with [^{35}S] methionine in the absence and presence of tunicamycin, respectively.

Tracks C and CT contain [^{35}S]-methionine labelled proteins of uninfected cell extracts without and with tunicamycin treatment respectively.

Track B: [^{35}S]-methionine labelled intracellular proteins produced in cells infected with brv mutant A_4. Antigens of brv mutant A_4 prepared from cells infected without and with tunicamycin treatment were shown in tracks A and AT, respectively.

Lines (—) show absence of IP reaction in the VP7 and VP10.

Closed arrow (►) indicates an IP reaction corresponding to VP7 in all tunicamycin treated antigen preparations.


VP
B
A
AT
1
1T
2
2T
3
3T
4
4T
5
5T
6
6T
C
CT
1
2
3,4
5
6
7
8
9
10
A

* This conclusion is based on the reactivity of a single antiserum and needs confirmation.

reactions showed that no differences were observed between the brv mutants and standard brv (Figure 16.3). In contrast to the IP reactions in which antisera directed against standard brv and mutant D were used, the IP reactions with antiserum directed against mutant A_4 showed that the glycosylated VP7 was immunologically recognised (Figures 16.3, tracks 1-5). In addition, the anti A_4 serum immunoprecipitated the non-glycosylated and possibly partially glycosylated forms of VP7 (arrow-marked in Figure 16.3, tracks 1T-5T) without difference between standard brv and brv mutant antigens. This IP reaction was absent when the antisera against brv D and standard brv were used (Figures 16.1 and 16.2).

The results presented above showed that anti N and anti D polyclonal sera recognised both the antigen VP7 and the non-glycosylated VP7 of brv mutant A_4 (Figure 16.1 and Figure 16.2; tracks 3 and 3T). But these antisera were unreactive with homologous non-glycosylated form of VP7. It was also observed that anti A_4 contained antibodies which reacted with both forms of VP7 antigens synthesised in cells infected with all brv mutants as well as the standard brv. This is an indication of a subtle antigenic change involving site(s) on the major serotype specifying antigen (VP7) of brv mutant A_4.* Considering that brv mutant A_4 had a diminutive defect in the mannose content of glycoprotein, VP7 (shown in Figure 11.2) these observations suggest that glycosylation of VP7 had an influence on antigenic determinants expressed on the viral protein.

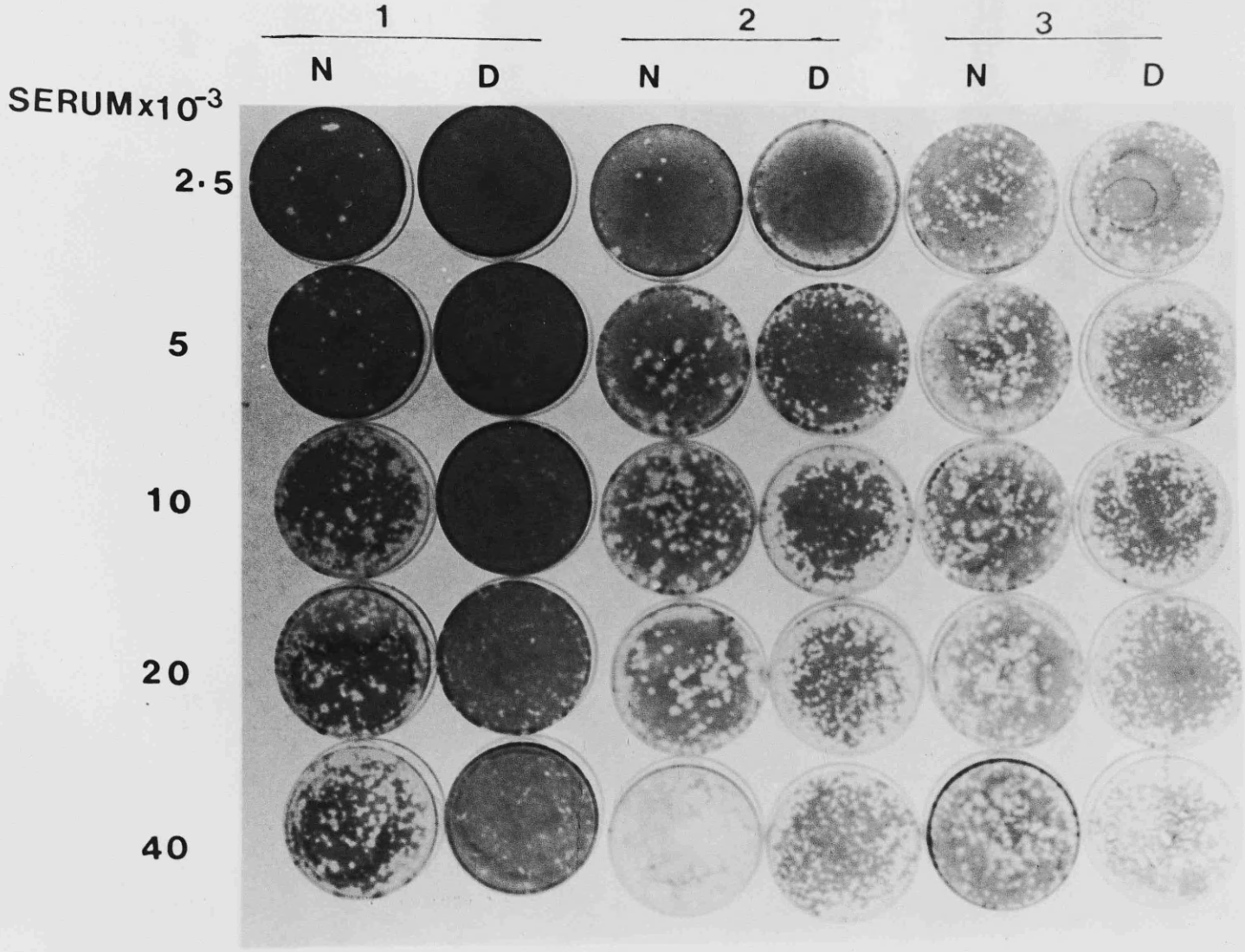
The VP5 and VP5A proteins were not immunoprecipitated in

Figure 17.1.

Serological relationship among the brv mutants.

Plaque reduction neutralisation assays to determine serotype of the brv mutants D and A_4 using antisera raised against brv D, brv A_4 and the standard brv were set up as described in Methods. Shown are plaques produced when the brv mutant D (D) and standard brv (N) were plated after treatment with antisera against brv N (Panel 1) mutant D, (Panel 2), and mutant A_4 (Panel 3). The antiserum dilutions 2,500 to 40,000 (indicated on the left) were reacted with a constant dose of approximately 180 pfu of standard brv or brv mutant D contained in an equal volume of PBS. Neutralisation titre was determined at the 60% plaque reduction.

The plaques formed by the brv mutant A4 in homologous plaque reduction neutralisation assay (titre 7,000) were microscopic and were not included in this figure.


1
2
3
N
D
N
D
N
D
SERUM x10-3
2·5
5
10
20
40

any of the tests and neither were the nonstructural proteins VP8, VP9 and VP12.

17 Serotypic relationships among the brv mutants.

Plaque reduction neutralisation assays were performed as described in Methods according to the procedure of Wyatt et al. (1982, 1983a) in order to investigate serotypic relationships between brv mutants D and A_4 and the standard brv. In Figure 17.1, panels 1 and 2, rabbit hyperimmune serum raised against both the standard brv and brv mutant D were observed to neutralise standard brv and brv mutants D and A_4 to similar extent. The titres (reciprocal of antiserum dilution causing 60% reduction in plaque count) calculated from Figure 17.1 for antiN and anti D were 8 x 10^3 and 4 x 10^3, respectively; they were not judged to be significantly different.

The anti A_4 (polyclonal polyvalent antiserum raised against the brv mutant A_4) gave a homologous titre of 7 x 10^3. As seen in Figure 17.1, panel 3, the neutralising antibody titres against both the standard brv and the brv mutant D were less than 2.5 x 10^3. In another titration - the neutralising titres were found to be 1500 against standard brv and 1200 against brv mutant D.

18 Reassortment of rearranged bands of genomic rotavirus RNA.

Gene reassortment was found to contribute to emergence of "new" virus strains of influenza in nature (Desselberger et al., 1978). In a well studied system of influenza

virus, gene segment reassortment has been used to construct single gene reassortants which had potential uses as live attenuated vaccines (Maassab et al., 1981, Murphy et al., 1984). While for rotaviruses gene reassortment was initially used to rescue non-cultivatable hrvs (Greenberg et al., 1981) it soon became a powerful method of mapping functions of the RNA segments (Greenberg et al., 1982; Kalica et al., 1983; Hoshino et al., 1985b and Offit and Blavat 1986). Most recently, from mixed infections of human and simian rotaviruses in-vitro , rotavirus reassortants were obtained carrying single gene substitutions coding for hrv serotypes 1, 2 and 4 specificity and were considered as candidate strains for a live attenuated vaccine to be used in man (Midthun et al., 1985).

With the above mentioned implications and potential uses of gene-reassortment it was of special interest to find out whether or not the rearranged bands of genomic RNAs were able to reassort. From mixed infections of cells with human rotaviruses possessing rearranged genomes and with standard brv, reassortants were obtained in which rearranged bands of the hrv genome replaced certain normal RNA segments of the brv genome both structurally and functionally (Allen and Desselberger, 1985).

The RNA segment reassortment experiments were done in an attempt to find a genetic equivalent of the rearranged RNA band A found in the brv mutant D. Another important question to be answered was whether or not a successful reassortment into the genome of brv mutant D would alter

growth parameters (e.g. plaque size). Also under consideration was to see if there was a chance for the rearranged band A to revert to its previous position of normal RNA segment 5 under the conditions of segment reassortment.

The results shown in Figure 18.1 were obtained following the protocol described by Garbarg-Chenon et al., 1984. Monolayers of secondary Rhesus monkey kidney (RMK) cells in roller tubes were mixedly infected with the brv mutant D (at a m.o.i. of 10^{-3} pfu/cell) and with approximately 10^4 $TCID_{50}$ of tissue culture adapted, but non-plaquing human rotavirus (hrv A59). After complete cpe was reached, the yield was plaque titrated and plaques picked and amplified in MA104 cells under liquid overlay. Three rounds of plaque-to plaque purification, followed by RNA extraction and analysis by RNA-PAGE were performed to obtain the RNAs of brv/hrv A59 reassortants shown in Figure 18.1. Out of 36 plaques that were picked at the beginning of the experiment, five were reassortants. Two plaque isolates were reassortants carrying an hrv RNA segment 5 while the rearranged RNA segment A was lost (Reassortants B320-2 and B317-1; Figure 18.1, tracks 2 and 5). The other three reassortants carried RNA segment 8 from hrv A59 but retained the rearranged RNA band A (Figure 18.1, tracks 1, 3 and 4; reassortants designated B320-1, B320-4 and B320-6 respectively). The genomes carrying the reassorted RNA segments were stably maintained.

There were two possibilities as to the source of the RNA segment 5 seen in the reassortants B320-2 and B317-1 (Figure

Figure 18.1.

Gene reassortment between brv D and hrv A59.

The brv mutant D which lacks the genomic RNA segment 5 and has a rearranged RNA segment A was reassorted with the non-plaquing, tissue culture adapted human rotavirus hrv A59. The procedure as described by Garbarg-Chenon (1984) was employed. RNA extracted from twice plaque purified reassortants (virus isolates) was analysed by RNA-PAGE followed by silver staining according to Follett and Desselberger (1983a).

Tracks N and D show the genome of standard brv and of brv mutant D, respectively.

Tracks 1 to 5 show the RNAprofiles of reassortants designated B320-1, B320-2, B320-4, B320-6 and B317-1, respectively. Reassortants in tracks 2 and 5 possess RNA segment 5 derived from hrv A59 (closed arrows) and have lost the RNA band A (open arrows). Reassortants in tracks 1, 3 and 4 have derived RNA segment 8 from hrv A59.

RNA segments (1-11) are indicated to the right.

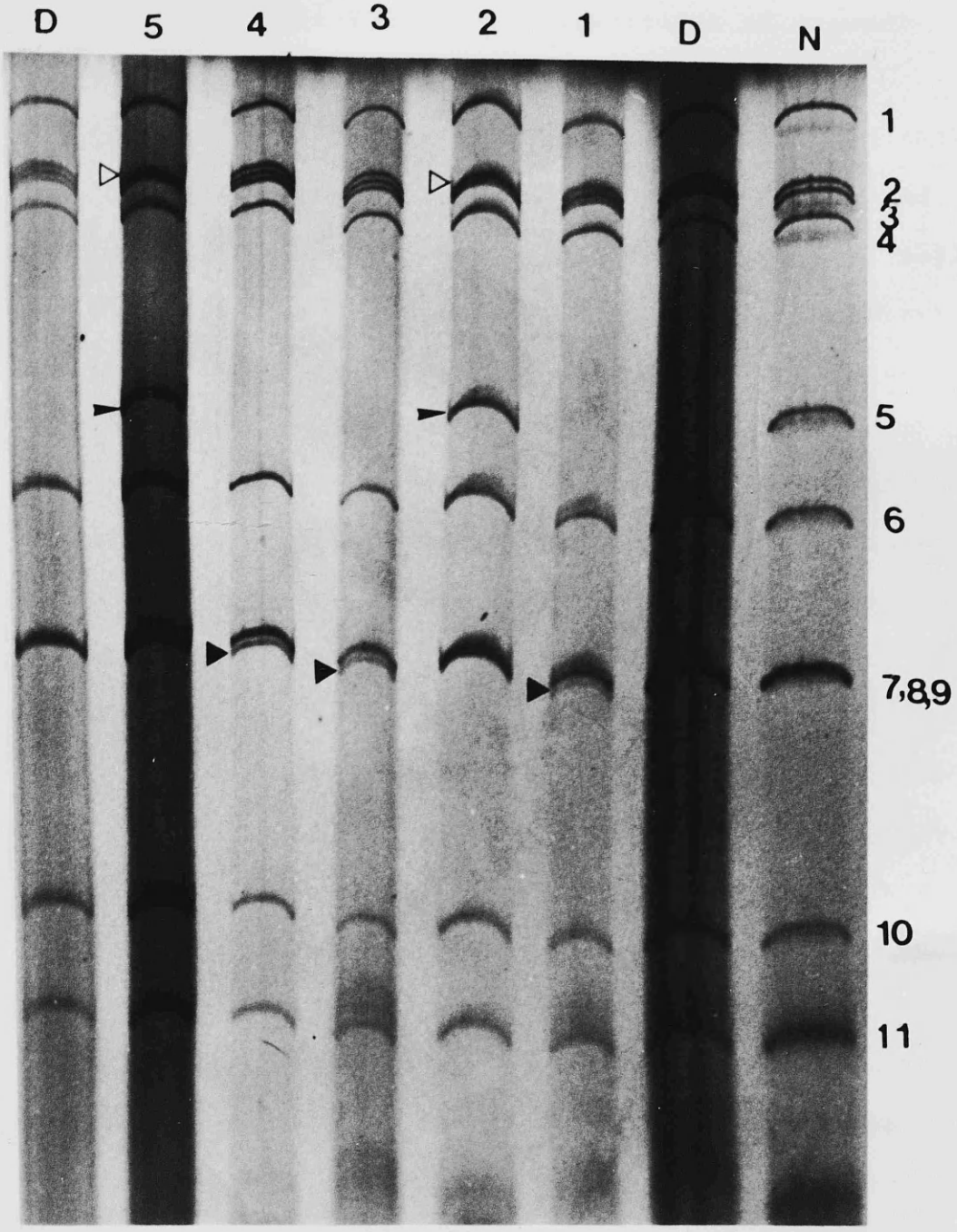

D
5
4
3
2
1
D
N
1
2
3
4
5
6
7,8,9
10
11

Figure 18.2.

Confirmation of the origin of the RNA segment 5 seen in the brvD/hrv A59 reassortants.

Standard brv, brv mutants D and A_4, hrv A59 and the reassortant viruses B317-1 of B320-2 were grown to complete CPE. Virus was pelletted through 30% sucrose cushion at 115,000g for 90 minutes and RNA extracted (Follett and Desselberger, 1983a). The RNAs samples were divided in portions to be separated on 2.8% polyacrylamide 6M urea slab gel as follows:

Tracks N, D, A_4 and S: Reference RNA profiles of standard brv, brv mutants D and A_4, and hrv A59, respectively; tracks 2 and 5: RNA profile of reassortants B320-2 and B317-1, respectively.

Track 2 + S: Mixture of RNAs of reassortant B320-2 and hrv A59.

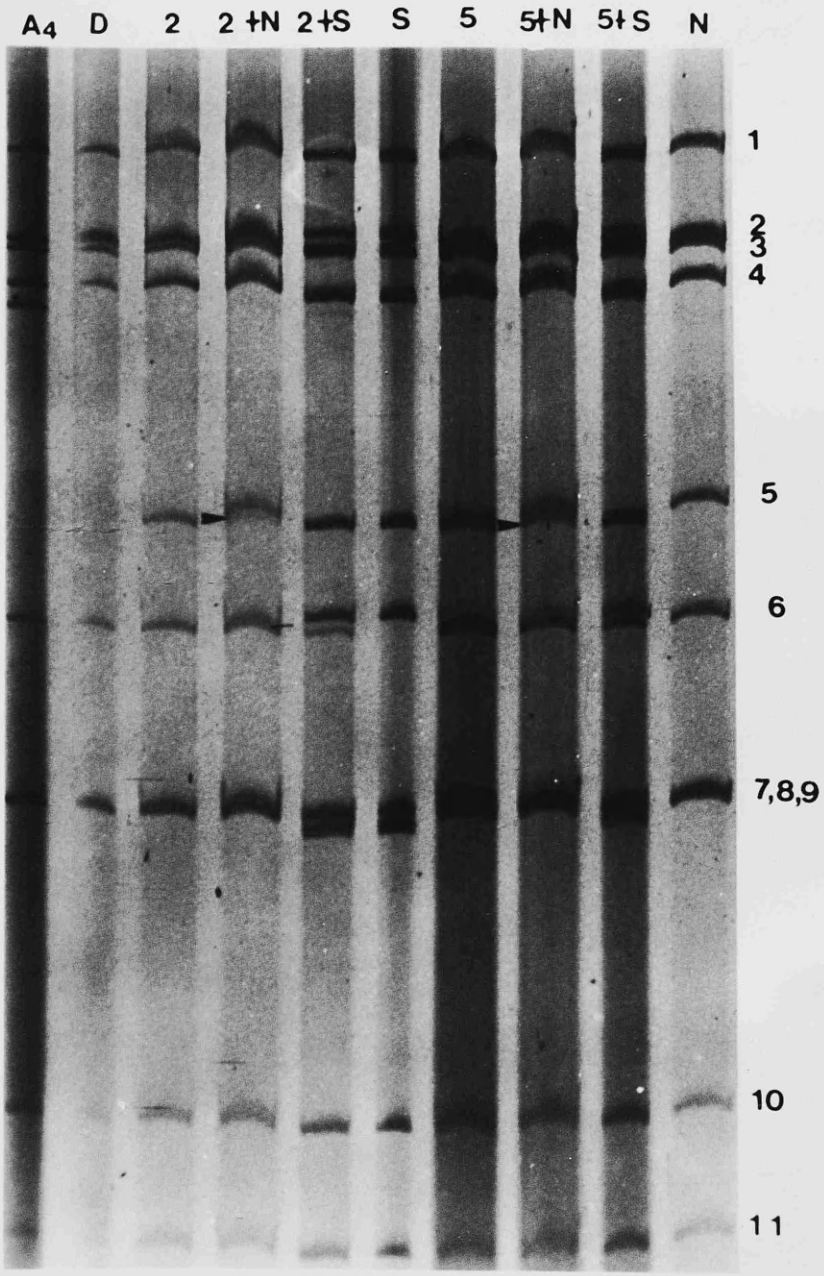
Track 5 + S: Mixture of RNAs of reassortant B317-1 and hrv A59.

Track N + 2: Mixture of RNA of standard brv and reassortant B320-2.

Track N + S: RNA mixtures of standard brv and hrv A59.

Genomic RNA segments are numbered 1-11.

Arrow (►) points to the RNA segment 5 of hrv A59 which has reassorted into the brv D genome.


A4
D
2
2+N
2+S
S
5
5+N
5+S
N
1
2
3
4
5
6
7,8,9
10
11

18.1, panel A, tracks 2 and 5). Besides the possibility that hrv A59 which has a RNA segment 5 migrating faster than that of standard brv donated the RNA segment, it could be argued that the rearranged RNA band A reverted to the normal position of RNA segment 5 of standard brv. To decide between these possibilities, RNAs of standard brv and of each of the reassortant RNAs B320-2 and B317-1 were mixed and co-electrophoresed). It turned out that the reassorted new RNAs segment found in the reassortants B320-2, B317-1 (Figure 18.2, track 2 and 5) respectively, did not comigrate with RNA segment 5 of standard brv (Figure 18.2, tracks 2+N and 5+N) but it comigrated with RNA segment 5 of hrv A59 (Figure 18.2 tracks 2+S and 5+S). Thus the hrv A59 was designated as the donor of the genomic segment 5 into the brv mutant D genome, and the hrv A59 segment 5 was genetically equivalent to the rearranged band A since the two were found to be exchanged. To confirm that the RNA segment 8 found in the reassortants B320-1, B320-4 and B320-6 (shown in Figure 18.1, tracks 1, 3 and 4) was donated by the hrv A59, mixtures of RNAs of the reassortant and hrv A59 were coelectrophoresed together with single RNAs (Figure 18.3). It was evident that the RNA segment 8 seen in the reassortants B320-1, B320-4 and B320-6 and marked (·) (Figure 18.3, tracks 1, 3 and 4) comigrated with the RNA segment 8 of hrv A59 (track S) as indicated in tracks 1+S, 3+S and 4+S. This observation was further confirmed using RNA mixtures of reassortants and the brv A_4 RNA: RNA segment 8 was not derived from the parent brv D which has the same migration pattern of segments 7, 8 and 9 as brv

Figure 18.3.

Confirmation of the origin of RNA segment 8 found in brv A/hrv 59A reassortants.

The RNAs of brv mutants D and A_4, of hrv A59, and of the reassortants were extracted as described in Legend to Figure 18.2. Individual and mixed RNAs were electrophoresed on 2.8% polyacrylamide 6M urea gels and silver stained.

The following track designations were used: S, RNA of hrv A59; 1, reassortant B320-1; 3, reassortant B320-4; 4, reassortant B320-6; D, RNA of brv D; A_4, brv mutant A_4 (included as reference for RNA segment 7, 8, 9).
1+S, reassortant B320-1 + hrv A59; 3+S, B320-4 + hrv A59; 4+S, B320-6 + hrv A59; 1+A_4, B320-1 + brv A_4; 3+A_4, B320-4 + brv A_4; 4+A_4, B320-6 + brv A_4.

The dots indicate the reassorted RNA segment 8 which comigrates with the segment 8 of hrv A59 in track S.

The genomic RNA segments were numbered 1-11 on the right.

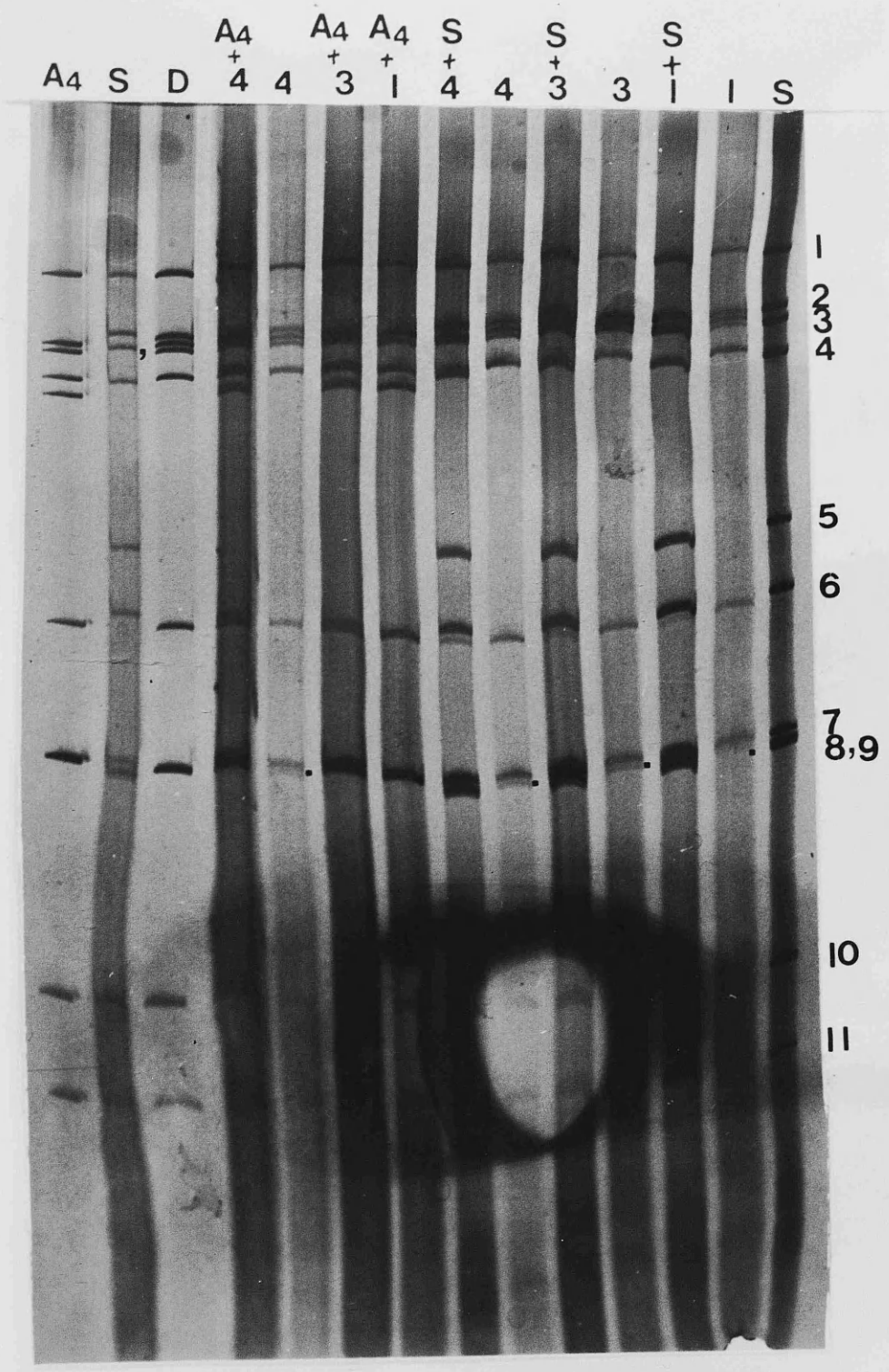

A4
S
D
A4 + 4
4
A4 + 3
A4 + I
S + 4
4
S + 3
3
S + I
I
S
1
2
3
4
5
6
7
8,9
10
11

Figure 18.4.

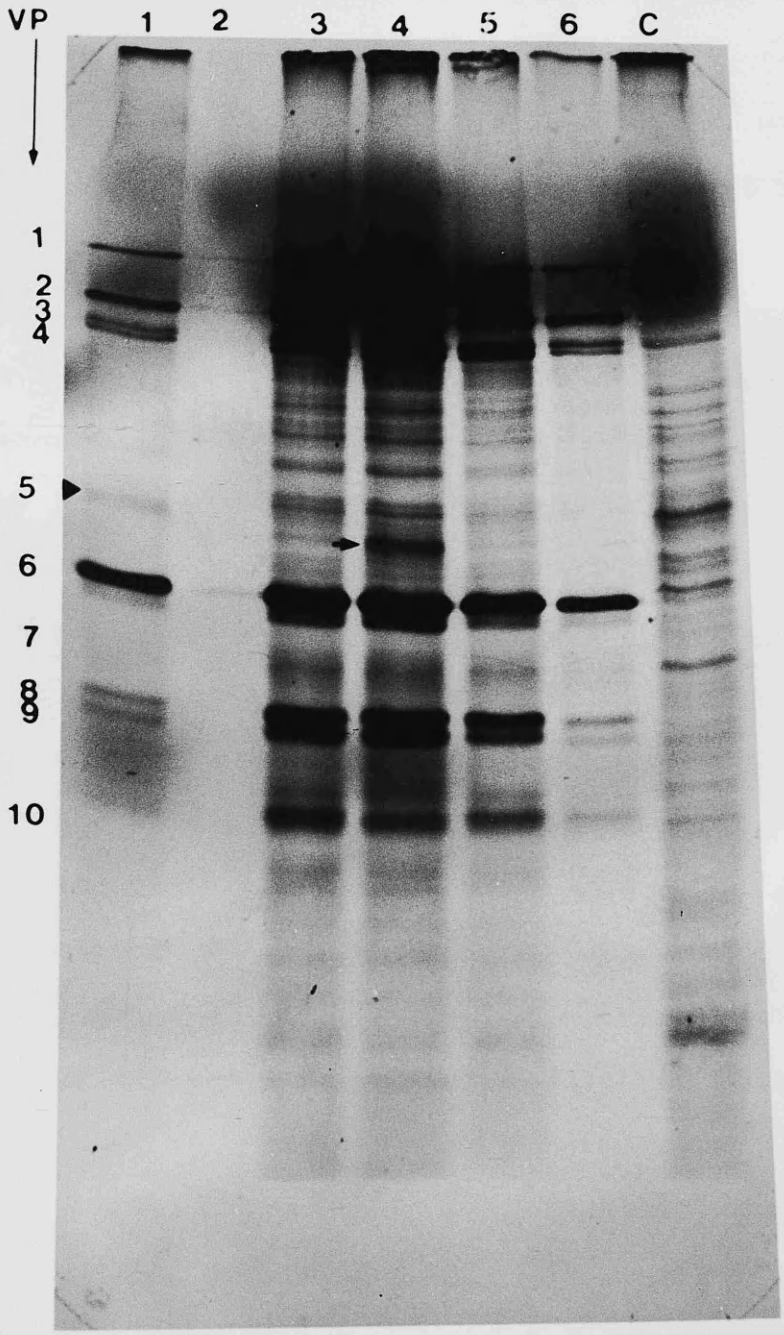
Proteins synthesised in cells infected with brv/hrv A59 reassortants.

Monolayers of MA104 cells were infected with twice plaque purified brv/hrv A59 reassortants, standard brv and brv mutant D. Intracellular proteins were pulse labelled for 2 hours at 16 hours pi with 7.4 MBq/ml [^{35}S] methionine in hypertonic Eagle's medium (total volume 2ml) and solubilized. Cell lysates were separated on a 12% polyacrylamide SDS gel which was autoradiographed.
Tracks 1 and 2: [^{35}S] methionine labelled proteins produced in cells which were infected with standard brv and brv mutant D, respectively.

Tracks 3 to 6: [^{35}S] methionine labelled proteins which were produced in cells infected with the reassortants B320-1, B320-2, B320-4 and B320-6, respectively.

Track C: Uninfected cell control labelled under the same conditions.

Arrow (→) points to the VP5 synthesised in the reassortant B320-2 carrying RNA segment 5 hrv A59.


VP
1
2
3
4
5
6
C
1
2
3
4
5
6
7
8
9
10

mutant A_4 (Figures 2.1 and 18.3).

The reassortants described above were used to infect cells, and virus-specific proteins synthesised during this infection were analysed by SDS-PAGE. The protein profiles of the reassortant viruses were compared with the protein profile of standard brv (Figure 18.4). The reassortant B320-2 (track 4, Figure 18.4) produced a VP5 which was smaller than the normal VP5 from the standard brv and appeared to be coded for by RNA of the human rotavirus A59. The rest of viral proteins produced by the reassortants were of the same sizes as those of the standard brv. Although the reassortants B320-1, B320-4 and B320-6 had acquired RNA segment 8 from hrv A59, there was no change in the gel mobility of the VP8 or VP9 in each of the reassortant protein profiles (Figure 18.4, tracks 3, 5 and 6). A control track of hrv A59 is not shown in Figure 18.4. Growth of the A59 virus on stationary cell cultures was poor and therefore no labelling attempt has been made.

Plaque reduction neutralisation assays were performed to test whether or not the RNA segment reassorted into the genome of brv mutant D had introduced a change in serotype. The reassortants were reacted with antiserum raised in rabbits against the brv mutant D (Figure 18.5). Plaque reduction tests with A59 virus could not be performed because an antiserum was not available and although hrv A59 was tissue-culture adapted, was non-plaquing. Neutralising antibody titres taken at 60% plaque reduction using the criteria set by Greenberg et al. (1981) and Hoshino et al. (1985a) were between 5×10^3 and 9×10^3 as compared with the homologous antibody titre of 4×10^3.

Thus, plaque reduction assays revealed no significant differences between the brv/hrv A59 reassortants and the

Figure 18.5.

Serotypic relationships among brvD/hrv reassortants.

Doses of 20-90 pfu of the reassortants B320-1, B320-2, B320-4 and B320-6, brv D and standard brv were reacted at 4°C with equal volume of antiserum directed against brv mutant D (in dilutions 500 to 10.000). The mixture was plated on 50mm petri dishes of MA104 cell monolayers under 0.5% agar overlay containing 5ug/ml pancreatin. Plaques were counted after 7 days of incubation at 37°C. For all viruses the antiserum titre causing 60% reduction in plaque counts was found to be between 5,000 and 8,000.

parental virus, brv D, to suggest a change in serotype. The fact that the reassortants B320-1, B320-4 and B320-6 which had acquired RNA segment 8 from hrv A59 reacted with the antiserum D the same way as those reassortants which had their RNA segment 5 donated by the hrv A59 (Figure 18.5), indicated that the reassorted RNA segment 8 did not code for serotype specifying antigens.

The brv mutant D and the reassortants B320-1, B320-2, B320-4 and B320-6 formed plaques of size of 2-3mm (Figure 18.5) which were comparable in size with those of the standard brv. The observation suggests that plaque size was not dependent on exchange of normal RNA segments 5 or 8. It is known that the capacity of plaque formation is coded for by RNA segment 4 (Kalica et al., 1983).

DISCUSSION

A Genomic heterogeneity among naturally occurring human rotaviruses.

The human rotaviruses (hrvs) were obtained from E.M. positive stool samples of children who presented with acute gastroenteritis at St. Thomas Hospital, London, East Birmingham Hospital, Birmingham, and Ruchill Hospital, Glasgow, between 1975 and 1983. The hrv genomes were extracted and electrophoretically separated on gels (RNA-PAGE) which were silver stained (Herring et al., 1982; Follett and Desselberger, 1983a; Follett et al., 1984). Among the 100 hrv samples, thirteen different electropherotypes (electrophoretic mobility pattern of genomic RNA on gel, Rodger et al., 1981; Schnagl et al., 1981; Estes et al., 1984) were observed as shown in Figures 1.1 and 1.2. The usage of "long" (L) and "short" (S) electropherotypes was derived from the relative migration of the genomic segments 10 and 11 (Rodger et al., 1981; Schnagl et al., 1981; Follett et al., 1984). Within the two main groups L and S there were numerous distinguishable variants here denoted by a small letter subscript (Follett et al., 1984). Three hrv electropherotypes Ld, S and Sa (Figure 1.1) were similar to those reported by Follett et al. (1984) during the gastroenteritis outbreaks of 1981/1982 and 1982/1983, but the rest differed. The finding of thirteen hrv electropherotypes circulating in the human population of Great Britain over a period of eight years indicates genome diversity which had been described to be as

extensive (with up to nineteen different electropherotypes recorded) in previous epidemiological studies (Kalica et al., 1978; Rodger et al., 1979; Espejo et al., 1980a; Rodger et al., 1981; Schnagl et al., 1981; Street et al., 1982; Follett and Desselberger, 1983a; Pereira et al., 1983; Follett et al., 1984; Dimitrov et al., 1984). Eighty percent of the hrvs obtained over the eight year period were of the long electropherotypes. Many authors (Espejo et al., 1979, 1980a; Rodger et al., 1981; Schnagl et al., 1982; Kutsuzawa et al., 1982b; Dimitrov et al., 1984; Follett et al., 1984; Konno et al., 1984) also observed a predominance of hrvs of the long electropherotypes. In this context it is of interest that the hrvs of the short electropherotypes are more difficult to adapt to growth in tissue cultures and grow to lower titres (Kutsuzawa et al., 1982a; Taniguchi et al., 1982; Garbarg-Chenon et al., 1984; Hoshino et al., 1985a); this possibly suggests that their replication in the human host is less successful than that of hrvs of the long electropherotypes. During the 8 year period of monitoring rotavirus infections, rotaviruses with atypical electropherotypes, so-called pararotaviruses and defined as non group A (Pedley et al., 1983, 1986) and similar to those described by Hung et al. (1983, 1984) and Snodgrass et al. (1984a) were found.

In one case (faecal sample 5485 shown in Figure 1.3) a genome profile consisting of apparently 12 RNA segments was observed with an extra genomic RNA segment migrating slower than the normal RNA segment 2. The extra RNA segment was considered to reflect a natural mixed infection as described

previously (Lourenco et al., 1981; Spencer et al., 1983; Nicolas et al., 1984). However, when propagating a simian SA11 rotavirus preparation with an apparent doubling of RNA segment 4 in the profile at limiting dilutions, Pereira et al. (1984) were able to separate clones showing a slower and faster moving RNA segment 4, called 4S and 4F respectively, in otherwise identical RNA profiles and demonstrated that what appeared as extra genomic segment 4 in the uncloned SA11 preparation was a stable genomic segment 4 of another SA11 genome comprising a different subpopulation. Further passage of the SA11 clone 4S and RNA electropherotyping (even using 12.5% polyacrylamide gels; Sabara et al., 1982a) did not show any more splitting in the RNA segment 4. Although this observation can be taken to explain the RNA profile found in sample 5485, it could not be confirmed for lack of a tissue culture adapted virus. As the virus 5485 was collected in one diarrhoea episode, its quantity was insufficient for Northern blotting to test the sequence relatedness (Street et al., 1982; Pedley et al., 1984) of the additional RNA band. The explanation of the existence of the 12th genomic segment of 5485 by a reassortment event that could have occurred during mixed infection (Sabara et al., 1982a; Garbarg-Chenon et al., 1984; Allen and Desselberger, 1985; Gombold and Ramig, 1986) was untenable as packaging of more than 11 RNA segments was never observed.

Rotavirus infections in otherwise healthy children are acute and virus shedding is normally limited to one week after the onset of diarrhoea. However, children with

This observation gave rise to the experiments in which brv was serially passaged at high moi (Hundley et al, 1985) and from which finally the brv mutants with genome rearrangements analysed in this thesis were obtained.

*

severe combined immunodeficiency (SCID) were found to become chronically infected with rotaviruses (Saulsbury et al., 1980; Booth et al., 1982; Chrystie et al., 1982; Pedley et al., 1984; Dolan et al., 1985 and Eiden et al., 1985). Rotaviruses obtained from two children with SCID over periods of 7 weeks and $8^1/_2$ months, respectively, were found to have highly abnormal RNA profiles (Pedley et al., 1984). The genomes consisted of more than 11 segments and in one case (U.H.) the normal segment 11 was lost from the profile. The loss of the normal segment 11 has been described for viruses obtained from immunodeficient children (Dolan et al., 1985; Eiden et al., 1985) as well as in normal neonates (Besselaar et al., 1986).

The abnormal genomes of the hrv generated during chronic infection of children with SCID (Pedley et al., 1984) consisted of several dsRNAs distributed between the normal RNA segments 1-7 of the hrv genome profile, and were shown by Northern blot hybridisation using [^{32}P]-labelled segment-specific cDNA probes to be derived from normal genomic segments migrating faster on the gel. Thus they did not show a RNA pattern typical for DI RNAs (Holland et al., 1980, 1982) but seemed to have arisen by concatemer formation (Pedley et al, 1984). The question of whether or not mosaic structures (Fields and Winter, 1982) had contributed to the formation of the abnormal bands (Pedley et al., 1984) remains unanswered.

B Evidence for rearrangements of genomic RNA segments of brv.

Initially the hrvs possessing rearranged genomes could not be propagated in cell cultures. In an attempt to reproduce in tissue culture events leading to the emergence of the rearranged rotavirus genomes, the cultivatable brv (UK Compton strain) was serially passaged at high m.o.i. and brv mutants lacking the normal RNA segment 5 but containing abnormally positioned RNA bands A-H were obtained (Hundley et al., 1985 and Figure 2.1). And although the conditions of the virus growth were likely to generate DI particles (von Magnus, 1954; Huang and Baltimore, 1977; Holland et al., 1980; 1982), the genomically rearranged brv mutants were different in that their replication was independent of the helper function of standard brv. When plaque purified stocks of brv mutants were raised in tissue culture, the abnormal RNA segments A-H were maintained; in no case were RNAs observed which were smaller than the smallest rotavirus RNA segment 11 (Hundley et al., 1985). Evidence in favour of RNA segment rearrangements was obtained from isolated RNA segment A: although the RNA was contaminated with some RNA of segment 2, the oligonucleotide (ON) map was similar to that of the normal RNA segment 5 (Hundley et al., 1985). Using the ON mapping technique (Follett and Desselberger, 1983b; Hundley et al., 1985) the RNAs of band E of brv mutant A_4 and of normal RNA segment 5 were compared. Close similarity of the large (unique) ONs was observed, indicating that RNA band E shared nucleotide sequences with the genomic RNA segment 5 of standard brv (Figure 3.2).

The ON maps showed one clear difference of a large ON missing in the T_1 map of RNA band E, and the absence of such a unique large ON might have resulted from deletions occurring during sequence rearrangements. Cross hybridisation experiments to Northern blots using [^{32}P]-labelled cDNA probes specific for RNA segment 5 and for RNA bands A and E (prepared by random priming with calf thymus DNA fragments according to Street et al. (1982) and Allen and Desselberger (1985)) revealed that probes made from bands A and E as templates only hybridised to the homologous sequences and to RNA 5 but to no other segments (U. Desselberger, unpublished experiments). This finding makes it unlikely that mosaic structures contribute to the concatemer formation of bands A and E (U. Desselberger, personal communication).

The mechanism leading to the formation of genome rearrangements is not clear at present: for its elucidation molecular cloning and sequencing of the rearranged bands are in progress (U. Desselberger, personal communication).

C Growth characteristics of brv mutants carrying rearranged genomes.

The effect of temperature on virus infectivity was tested at 31°C, 37°C and 39.5°C (Table 4.1). The brv mutants D, A_4, B_4 and B_5 grew and produced infectious virus titres that responded to differences in temperature during incubation. By applying the criteria of Greenberg et al. (1981) for determining temperature sensitivity (ts), it was found that none of brv mutants had a difference in virus titre between temperatures of $\geq$ 2.7 $\log_{10}$ units.

It remains to determined whether or not the differences in the titre of the mutants at different temperatures amounting to up to 30-fold above control level can be mapped to a mutation of one or several genes.

Since neither the brv mutants nor the standard brv was temperature sensitive (according to this reference), it was concluded that the loss of genomic RNA segment 5 and the emergence of RNA bands A-G in the genomes of brv mutants were not associated with ts lesions.*

Specific infectivities (i.e. the virus particle counts (VPC):infectivity ratio) were determined at 37°C for the brv mutants D, A_4, B_4 and B_5 and compared with that of standard brv: no significant differences were found as all VPC/pfu ratios were between 30-100, representing a $\log_{10}$ difference of 0.5 units but were higher than the log difference 0.3 corresponding to the VPC/pfu value for standard brv of 7 $\pm$ 3 and that of brv mutant D 14 $\pm$ 9 reported by Hundley *et al.* (1985). Hence, the observed diminution in the growth rate of the brv mutants was not considered to be a result of changes in VPC/pfu ratios.

Plaque titration of the standard brv and brv mutants was carried out at 37°C in the presence of 5ug/ml pancreatin in Eagles MEM containing 0.5% agar (Matsuno *et al.*, 1977a; Smith *et al.*, 1979; Carpio *et al.*, 1981; Sabara *et al.*, 1982b). Plaques formed under these conditions varied in size. The largest plaques, 2-4 diameter, were observed in cells infected with the standard brv and the brv mutant D. The brv mutants A_4, B_4 and B_5 produced small size plaques of a diameter of 1-2mm for brv mutant A_4 and $\leq$ 0.5mm for the brv mutants B_4 and B_5. If progress of plaque formation relates to virus growth rate to some degree, plaque size depends on cytopathogenicity of the virus strain because a plaque is formed by cell death resulting from virus

infection and the number of cells killed in the infection process in a given time determine the size of a plaque. Plaque purified brv mutants D, A_4, B_4 and B_5 were studied for growth characteristics with comparison and reference to standard brv. Both the brv mutants and standard brv were used to infect cells at m.o.i. 5-10 pfu/cell and the virus yields were assayed for infectivity (Matsuno et al., 1977a; McCrae and Faulkner-Valle, 1981). The brv mutants A_4, B_4 and B_5 produced titres of infectious virus lower than standard brv at all time points during the period of infection, but brv mutant D was 0.5 log units lower than standard brv. In agreement with the observations of Carpio et al. (1981) and McCrae and Faulkner-Valle (1981) the standard brv produced complete CPE between 16 and 24 pi and brv mutant D did as well. However, the brv mutants A_4, B_4 and B_5 did not reach comparable CPE until after 72 hours pi and even then infectivities were much lower than those reached by standard brv.

D Genome transcription in-vivo and in-vitro and genome replication.

In-vivo RNA synthesis of brv mutants D, A_4, B_4 and B_5 was compared with that of standard brv at different temperatures (31°C, 37°C and 39.5°C) in order to find out whether or not the changes in incubation temperature would affect the kinetics of RNA synthesis. At the input of 5-10 pfu/cell, all virus mutants as well as the standard brv incorporated (5,6-^{3}H] uridine to a similar extent during the first 4 hours of infection at the different temperatures.

* The labelled ssRNAs and the dsRNA segments were differentiated by differences in their relative migration on polyacrylamide gels which contained unlabelled genomic RNA of standard brv visualised by silver staining as reference. The finding presented in Figure 6.7. where mainly ssRNA was recorded in low amounts seems to be in contrast to the finding in Figure 6.5. It is explained by differences in sensitivity of the two procedures to detect the ssRNA.

Differences in the rates of RNA synthesis for the brv mutants were observed between 6 and 16 hours post infection (Figure 6.1). At each temperature, the rate of RNA synthesis in cells infected with the brv mutants A_4, B_4 and B_5 were lower than corresponding values for standard brv (Table 6.1) indicating that the brv mutants A_4, and especially B_4 and B_5 were limited in their ability to replicate in host cells, and this defect was most pronounced for mutants B_4 and B_5 at 39.5°C. However, all the brv mutants as well as standard brv produced RNAs at rates which decreased with fall in temperature of virus growth (Figure 6.1). The brv mutant D which at 16 hours pi produced as much CPE in infected cells as standard brv, was observed to synthesise RNA comparable to that of the standard brv at the incubation temperatures 31, 37 and 39.5°C. The observed abnormalities in the growth parameters (infectivity, plaque size, in-vivo RNA synthesis) of the brv mutants A_4, B_4 and B_5 may be inferred to have arisen from the genomic RNA rearrangements in which RNA segment 5 was lost and abnormal rearranged RNA segments E, F, G,G' and H respectively emerged. However, brv mutant D which also lost segment 5, exhibited a growth pattern similar to the standard brv.

Total [^{32}P] labelled RNA synthesised in cells infected with standard brv and the brv mutant A_4 when separated on polyacrylamide gels, showed both ssRNAs and dsRNAs in infected cells when harvested at 18 hours pi. In contrast, the RNA extracted from samples harvested at 6 hours pi contained largely genomic RNAs (Figure 6.5).* This observation signifies failure to detect early the low levels

* In future ssRNA should be removed from the RNA mixture by RNase treatment.

of ssRNAs synthesised during the time after infection by primary and secondary transcription. The rearranged RNA segment E was transcribed and replicated. Both ssRNA and dsRNA forms of RNA band E were found indicating that rearrangements of genomic RNA segment had conserved recognition sequences for in-vivo polymerase to transcribe, and the replicase to replicate rotavirus genome segments (Imai et al., 1983; reviewed Holmes, 1983). Experiments aiming at separating ssRNAs from dsRNAs by CF11 cellulose chromatography (Franklin, 1966) produced pure ssRNA eluting in 15% ethanol; but the fractions eluting in TSE buffer alone contained a mixture of both ds and ssRNAs (Figure 6.6): therefore, in quantifying and comparing the eluted fractions, an allowance had to be given for the mixture in the dsRNA fraction and the ratios of ssRNA:dsRNA were calculated as a minimum.* The CF11 cellulose chromatography performed on total RNA synthesised at 6 and 18 hours pi (Figure 6.7) showed kinetics of RNA synthesis that were dominated at the early time (6 hours pi) by ssRNAs over the genomic dsRNAs. The ratio ssRNA:dsRNA was similar for the brv mutant D and A_4 as for standard brv. There was a transition from ssRNA to dsRNA synthesis later in infection (Figure 6.7). The pattern of RNA synthesis in cells infected with brv mutant A_4 was such that it reached the transition period later than either the fast growing brv mutant D or the standard brv. It would appear as if virus mutant A_4 continued to synthesise a lot of ssRNA until enough mRNA was available for translation of products necessary for the steps of virus packaging and assembly.

In reoviruses (Nonoyama et al., 1974) the transition period was found at between 10-12 hours pi. Observations made in brv infection indicated that the replicative cycle (maximal production of infectious virus) was completed by 12 hours pi (Carpio et al., 1981; McCrae and Faulkner-Valle, 1981).

Bovine rotavirus shares with the reovirus, another member of the Reoviridae family (Matthews, 1979; Joklik, 1983), the characteristic transcription of genomic segments in relative proportions which decreased with an increase in the size of transcribing RNA segment (Skehel and Joklik, 1968; Levine and Samuel, 1980; Gaillard and Joklik, 1985). The amount of segmental ss RNAs transcribed at 18 hours of infection, were unequal in contrast to reovirus (Nonoyama et al., 1974) which synthesised equal amounts (transcription at equal frequency) of its ten genomic segments - between 10-13 hours pi. It appeared that the rotavirus genome transcription was controlled in a way that produced no preferential transcription of any of the eleven genomic segments. This feature also contrasts with in-vivo transcription pattern found in influenza infected cells: a) Immediately after infection polyadenylated transcripts 1-8 were almost equally produced, but between 30 and 90 min. pi, transcript 5 and 8 were synthesised preferentially and at times later than 2.5 hr. pi, transcripts 4, 5 and 7 were made in greatest amount (Hay et al., 1977, Smith and Hay, 1982). The transcripts synthesised in infected cells were present in amounts inversely related to the sizes of the transcribing genomic RNA segments (Table 6.3) of the standard brv and the brv mutants D and A_4.* The amount of

* This is in agreement with previous findings for rotaviruses (Hundley et al, 1985) and for reoviruses (Skehel and Joklik, 1969).

⇒ Between 6 and 16 hrs pi, the amounts of ssRNA increased by 1.5 (mutant D) to 8-15 fold (standard brv and mutant A4) whereas the amounts of dsRNA increased by 40-100 fold in all cases. The difference in the rates of ssRNA synthesis betwween mutant A4 and the standard brv and mutant D needs further investigation.

This statement needs confirmation by testing the in-vitro transcription reaction in presence of cycloheximide.

RNA eluting in pure TSE consisted of ssRNA and dsRNAs and probably some ssRNA hybrids. Hybrid RNAs (association of positive sense to a complementary strand) which could not be excluded in the assay was thought to contribute to the total dsRNA fraction in amounts that might have brought forward the apparent transition observed by 16 hours pi. Given that between 6 and 16 hours pi, the ssRNA and dsRNA both increased by more than tenfold it became compelling to infer that continued ssRNA synthesis was essential for genome replication.

In order to define a role for the continuous mRNA synthesis in rotavirus replication, cycloheximide, an inhibitor of protein synthesis, was added to the overlay of infected cell cultures. The presence of cycloheximide in cells infected with the standard brv and the brv mutants abolished the synthesis of both ssRNA transcripts and genomic dsRNAs. While cycloheximide inhibited rotavirus protein synthesis by occupying ribosomal sites ("freezing"; Ericson et al., 1982), it caused cessation of genome replication without concurrent accumulation of transcripts. The effect of cycloheximide on rotavirus infection revealed that products of in-vivo transcription (ssRNAs) were neither detected nor found to accumulate intracellularly under prolonged exposure to cycloheximide in the presence of [^{32}P] orthophosphate. It is likely that continuous translation of the rotavirus mRNAs is necessary to initiate and maintain genome replication in view of the observed continued synthesis of ssRNAs in-vivo described above.

Genomes of standard brv and of the brv mutants were

transcribed in-vitro at the optimum temperature of 42°C catalysed by virion-associated RNA polymerase (Flores et al., 1982a; Hundley et al., 1985). The pattern of transcription for 5 hours was linear and the products of in-vitro transcription were present in 8 species of ssRNAs corresponding to the genomic segments 4-11. This agrees with the results obtained using brv (Cohen et al., 1977), hrv (Spencer and Arias, 1981; Flores et al., 1982a); and simian SA-11 (Mason et al., 1980, 1983). The large size RNA segments 1-3 were transcribed far less than the rest and were not easily detectable by autoradiography (Hundley et al., 1985). When the RNA polymerase activity of the standard brv and brv mutants D, A_4, B_4 and B_5 were compared at different temperatures, it was clear that in each case, the enzyme activity was maximal at 42°C. Moreover, Cohen (1977) and Cohen et al. (1979) had described that reovirus as well as rotavirus genomes were in-vitro transcribed best between 45-50°C and that the RNA polymerase activity drastically diminished above 50°C and below 37°C. The ssRNA transcripts obtained in-vitro of brv mutants and of standard brv did not exhibit any observable qualitative differences. The observations suggested that the various genomic rearrangements bore no influence on the functioning of the virion-associated RNA polymerase. Having shown that all of the genomically rearranged segments A-H were transcribed in-vitro it must be argued that the genome rearrangements preserved RNA polymerase recognition sites. In this context studies by Antczak et al. (1982, 1983) are of interest: when the authors studied reovirus 5' terminal

sequences for the presence of binding/recognition sites for RNA polymerase and capsid proteins, they concluded that if such sites existed upstream or surrounding the first initiation codons they could not be based on sequence similarity or identity. Although comparable amounts of protein 1.5ug as estimated by the method of Lowry et al. (1951), was used in each case it was realised later that the virus count for this same amount of protein was 30-90x less in the brv mutants B_4 and B_5. This may also be reflected in infectivity titres shown in table 4.1 Hence there was an apparent low RNA polymerase activity associated with the brv mutants which was attributable to differences in the absolute numbers of virion present in in-vitro transcription assay.

The segmented transcripts produced in the in-vitro transcription assay were present in various relative amounts which did not conform to the inverse relationship between transcript amount and size of transcribing genomic segment size (Nonoyama et al., 1974). For instance, the genomic RNA segment 10 which codes for VP10 and VP12 (McCrae and McCorquodale, 1982; Baybutt and McCrae, 1984) was transcribed more efficiently than the smallest RNA segment 11, while the genomic RNA segment 6 was transcribed far less than the next larger RNA segment 5 of the standard brv. These observations contrast with the results of in-vivo transcription of rotavirus genomes described above, and may be explained by the absence or lack of regulatory mechanisms found only in infected host cells (Darnell, 1982). No doubt, differences in the transcriptional efficiencies of each of the RNA segments were responsible for the findings. Drawing an analogy to reovirus, genome transcription

in-vitro occurs unequally for the 10 RNA segments (Levin and Samuel, 1980) while earlier Nonoyama et al. (1974) had shown that reovirus in-vivo transcripts were produced with equal frequencies at 10-13 hours pi.

The RNAs synthesised in a time course in cells infected with standard brv and the brv mutants D, A_4, B_4 and B_5 were analysed by RNA-PAGE and silver staining. Genomic RNA segments of brv mutants and of standard brv were visualised in the RNA extracts obtained 6 hours post-infection. This method of analysis allows genomic RNA to be visualised and largely excludes ssRNAs which are removed together with cell debris, before the dsRNA is extracted from pelletted virus as described by Follett and Desselberger (1983a), and differs from the method of Maniatis et al. (1982) in which cytoplasmic extracts are carefully prepared and extracted for RNA. There was no observable delay in the accumulation of genomic RNAs produced in cells infected with the brv mutants compared to standard brv. The finding showed that the brv mutants were not deficient in replication (Hundley et al., 1985). Nevertheless, the quantity of RNA synthesised at each time point as judged from the intensity of silver staining and assuming stoichiometric binding of silver ions to nucleic acid (Whitton et al., 1983) indicated that the brv mutants A_4, B_4 and B_5 replicated at rates below that of standard brv (Figure 6.1). Whereas maximum intensity of silver staining for RNA from standard brv and brv mutant D occurred at 16 hours, an equivalent intensity was reached much later than 32 hours pi for the viral RNAs produced in cells infected with the brv mutants A_4, B_4 and

B_5. At the earliest time, 6 hours pi, the rearranged genomic RNA bandsA-G of the brv mutants D, A_4, B_4 and B_5 were observed demonstrating that the abnormal RNA segments were replicated as part of the respective genome.

The [^{32}P] labelled RNA extracted from cells which were infected with standard brv and the brv mutants D and A_4 was analysed on polyacrylamide gel. Genomic segments 1-11 of the RNA of standard brv and the genomes of brv mutants D and A_4 were detected after autoradiography. The RNA band E of brv mutant A_4 was clearly separated from the normal segment 4 and the rearranged segment A of brv mutant D migrated as a doublet with the segment 2 (Figure 6.3). Densitometric evaluation (Whitton et al., 1983) of the autoradiograph (Figure 6.3) and comparison of relative molar proportions of segments and bands (Table 6.2) indicated that the genomic segments were replicated in non-equimolar proportions. The rearranged RNA bands were represented in proportions corresponding to those of normal RNA segments of the same size. The rearranged bands A-H of brv mutants D, A_4, B_4 and B_5 contained RNAs ranging from 3.9% to 30.5% more than were found in the standard brv genome (Results, page 87). The observations were interpreted to mean that rotavirus genome rearrangements allowed more than a normal amount of RNA to be incorporated in the genomes of brv mutants in the form of the rearranged RNA segments A-H (Allen and Desselberger, 1985) and that the rotavirus genome size is flexible. However, the high percentage of excess genomic RNA (30.5%) in the twice-plaque purified brv mutant B_5 may represent RNAs of coexisting virus subpopulations; and this

possibility should be excluded by further plaque purification prior to future studies on genome packaging.

Genomic RNA synthesised in infected cells was quantified by densitometry (Whitton et al., 1983) in order to compare relative frequency of segment replication in the standard brv and brv mutants. It turned out that the rotavirus genomic segments extracted from infected cell cytoplasm (Maniatis et al., 1982) were represented in non-equimolar amounts and that the pattern of genome replication was not affected by genome rearrangements. In measuring the quantity of RNA of the 11 dsRNA segments Whitton et al. (1983) found that brv genomic RNAs were present in equimolar proportions in rotavirions. This was also described for virions of hrv (Schnagl and Holmes, 1976), of reoviruses (Shatkin et al., 1968; Zweerink and Joklik, 1970) and of influenza viruses (McGeoch et al., 1976). The differences between RNA produced during replication and that packaged into rotavirions suggest that genome packaging is very strictly controlled.

E Protein synthesis in infected cells.

Studies on the viral proteins synthesis in-vivo were carried out in order to find out whether or not the brv mutants translated the rearranged parts of their genomes. In comparison with the standard brv which synthesised the viral proteins VP1-VP12 (McCrae and Faulkner-Valle; Hundley et al., 1985), the brv mutants D, A_4, B_4 and B_5 produced similar viral proteins except that none of the brv mutants synthesised the VP5, the product of RNA segment 5 (McCrae

and McCorquodale, 1982). The result was consistent with the fact that none of the brv mutant genomes contains the genomic RNA segment 5 (Hundley et al., 1985) [Figure 2.1]. Although the genomes of brv mutants were transcribed both in-vivo and in-vitro to produce mRNAs corresponding to the rearranged RNA segments A-H, only the brv mutant A_4 which synthesised transcript E was able to produce a protein 74.6K designated VP5A. The size of VP5A was compatible with the coding capacity of the RNA segment E (Hundley et al., 1985). In-vitro translation assays (McCrae and McCorquodale, 1982; Arias et al., 1982; Flores et al., 1982a) should be used to explore the coding capacities of the other rearranged RNA bands whose product, if any, were not detected in infected cells.

When molecular weights of intracellular viral proteins were estimated (Shapiro et al., 1967; Sabara et al., 1982b) and compared with published size values for brv proteins (Thouless, 1979; Matsuno and Mukoyama, 1979; Carpio et al., 1981; Sabara et al., 1982b) differences were observed (Table 9.1). Because the nomenclature of rotavirus proteins is not standardised, any comparison of viral proteins assumes similarity of both protein size and designation. As shown in Table 9.1, these vary among authors. For instance the protein called VP5 by McCrae and Faulkner-Valle (1981) and McCrae and McCorquodale (1982) is probably not identical with the 0_3 (outer capsid protein) [Thouless, 1979], the 68K (Sabara et al., 1982b) and may not be analogous to the NCVP2 of SA11 (Arias et al., 1982). In this thesis all viral proteins are named numerically in

order of decreasing size from VP1-VP12 after Mason et al. (1980) who used this nomenclature to describe SA11 rotavirus proteins. The nomenclature (VP) has received acceptance to be applied to name virus coded proteins of the brvs (UK Compton strain) [McCrae and Faulkner-Valle, 1981; McCrae and McCorquodale, 1982; Hundley et al., 1985].

F Virus morphology and structural proteins.

The brv mutant A_4, the only brv mutant which produced a mutant protein VP5A, was chosen for study of morphological and structural changes that might be associated with genome rearrangements. On purification of the [^{35}S] methionine labelled virus particles by isopycnic centrifugation in CsCl gradients (Novo and Esparza, 1981), three bands labelled p, d, and s and found at densities of 1.34, 1.37 and 1.39 g/ml, respectively, contained virus particles (Figure 10.1) representing empty particles, double capsid virions and single capsid particles in respective order. Between standard brv and brv mutant A_4 there was no observable difference in shape and size. While complete particles of the brv mutant A_4 which carries a rearranged band E (2248bp) are expected to be approximately 0.6% heavier than standard brv of total genome size 18680bp (Rixon et al., 1984) based on 16% RNA content in a single capsid particle (Novo and Esparza, 1981), the CsCl gradients were unable to demonstrate this difference between particles of brv mutant A_4 and those of standard brv. It is probable to detect the differences in densities if a mixture of equal proportions of brv mutant A_4 and standard brv were run on the same

gradient.

It was found repeatedly that the brv mutant protein VP5A which was produced in infected cells, was absent from protein profiles of [^{35}S] methionine labelled purified virus particles and it was suggested that VP5A was most likely a non-structural protein. As expected VP5 was found in cells infected only with standard brv; and the VP5 did not appear to be a structural protein, either. A band of protein was observed to migrate a little faster than the intracellular VP5 of standard brv (Figure 10.3). Since this protein band 5C was visible in the protein profiles of virus particles prepared from tissue culture grown standard brv and brv mutant A_4, the protein 5C was regarded as a trypsin cleavage product of the high molecular weight protein VP4 (Clark et al., 1981; Novo and Esparza, 1981; Lopez et al., 1985). Trypsin cleavage of a product encoded by RNA segment 4, a protein of 88Kd MW, is the basis of enhanced infectivity reported for brv (Clark et al., 1981), simian SA11 rotavirus (Graham and Estes, 1980; Espejo et al., 1981; Estes et al., 1981; Mason et al., 1983) and hrv (Sato et al., 1981; Ward et al., 1984b) and generates two fragments of sizes of ~28Kd and ~60Kd (reviewed Holmes, 1983 and Estes et al., 1983).

The status of VP5 as that of a non-structural protein agrees and is supported by previous reports (Newman et al., 1975; Cohen et al., 1979; Novo and Esparza, 1981; Clark et al., 1981; McCrae and Faulkner-Valle, 1981) who also found that the single capsids contained VP1, VP2, VP3 and VP6, and that outer capsid proteins were VP4, VP7 and

probably VP10. To define the reaction of authentic VP5, Mason et al. (1980, 1983), Smith et al. (1980) and Arias et al. (1982) found that the in-vitro translation product of genomic segment 5 from SA11 was a protein NS or NCVP2 of a size of 53Kd and migrated between 88K and 41K corresponding to a position on brv protein profile between VP4 and VP6. In addition, Mason et al. (1983) also showed that the protein 53K was not detected in purified SA11 virions prepared from SA11 virus grown in the absence of trypsin. In other studies (Bridger and Woode, 1976; Rodger et al., 1977; Matsuno and Mukoyama, 1979; Thouless, 1979) which described brv VP5 as a structural protein, there were no reference tracks showing separation of intracellular proteins. Moreover, the authors produced their results using viruses which had been grown in the presence of trypsin. The absence of RNA segment 5 and the VP5 was associated for several brv mutants (A_4, B_4 and B_5) with diminished capacity to replicate in cell cultures, and failure to suppress the production of cellular proteins efficiently. In contrast, brv mutant D was as efficient as the standard brv in the growth parameters (RNA synthesis, infectivity and plaque formation) as assessed in the same assays. Although the available data suggest that rotavirus growth and virulence is controlled by RNA segment 4 (Kalica et al., 1978, 1983; Greenberg et al., 1981; Offit et al., 1986), the brv mutants A_4, B_4 and B_5 which all had a normal RNA segment 4, failed to suppress host cell metabolism to an extent comparable with that of standard brv and brv mutant D. The studies described here show that not only the

absence of the RNA segment 5, but also the nature of segment rearrangements influenced the growth characteristics of the virus mutants. To date no other biological function has been assigned to genomic RNA segment 5.

G Effect of multiplicity of infection on intracellular protein synthesis.

Bovine rotavirus infection at m.o.i. of 5 pfu/cell is known to cause degradation of host cell DNA and to switch off host cell RNA synthesis leading to development of CPE and cytolysis by 12-15 hr. pi (Carpio et al., 1981). These events were reflected in decline in the incorporation of measurable ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-amino acids into cellular DNA, RNA and amino acids, respectively. Degradation of cellular DNA was shown by finding that the DNA isolated from infected cells decreased in size progressively with duration of infection. The effect of high m.o.i. on host cell protein synthesis was tested using the standard brv, and the brv mutants D, A_4, B_4 and B_5. Independent of m.o.i.s used (1, 5 and 40 pfu/cell) the brv mutants A_4, B_4 and B_5 synthesised viral proteins in the presence of much more host cell protein synthesis than was found in cells infected with the standard brv or the brv mutant D(Figure 12.1). Experiments described earlier, had shown that the same brv mutants A_4, B_4, B_5 produced CPE at a rate that took about 72 hours to establish complete cell damage in infected cells, whereas cell infected with standard brv or brv mutant D reached a comparable stage of CPE between 16 and 24 hours pi. Evidently, the genomic

rearrangements of the brv mutant A_4, B_4 and B_5 were associated with alterations in host cell-virus interaction. Proteins synthesised in cells infected with the standard brv and the brv mutants D, A_4, B_4 and B_5 at low and high m.o.i.s were densitometrically scanned (Whitton et al., 1983) and the relative amounts calculated by computer program 'DENS' (P. Taylor, MRC Virology, Glasgow). This compułation assumes equal [^{35}S]-methionine incorporation in the viral proteins. Methionine comprises 2-5% total amino acids of proteins coded by segments 6-11 which have been sequenced. Each of the virus-specific proteins VP1-VP12 synthesised in cells infected with each of the brv mutants was compared with a corresponding protein of the standard brv. Corresponding proteins of the same size were produced in comparable amounts indicating that in-vivo translation of the brv mutant genomic segments was not impaired and the translation frequency for RNA segments of the same size was comparable. The proteins VP1-VP12 produced in infected cells were present in various non-equimolar proportions, a feature known for reovirus (Joklik, 1974; Zweerink and Joklik, 1970). Furthermore there is evidence from reovirus studies (Levine and Samuel, 1980; Gaillard and Joklik, 1985) that in-vitro translation of reovirus mRNAs (transcripts from genomic RNA segments) occurred with different efficiencies. It appears that rotavirus genomes, too, were translated in-vivo at different frequencies. For example, brv VP6 was synthesised in amounts in excess of 30% of total protein produced during infection, while the other 11 virus-specific proteins shared 70%. Notably, the VP4

and VP11 were very much under-represented; both proteins comprised less than 5% (Table 12.1). The non-structural protein VP9 was preferentially produced in cells infected with the brv mutants A_4, B_4 and B_5. And since the brv mutants were found to exhibit a restricted capacity to inhibit host cell metabolism, the presence of excess VP9 in the brv mutants which lack VP5, may have a role to play in the virus/host cell interaction.

In a time course of intracellular protein synthesis, virus-specific proteins VP1-VP12 were detected at the same earliest time of 6 hours pi (Figure 13.1). The viral proteins VP5 and VP5A appeared to be synthesised in constant proportions over 24 hours and did not alter size or intensity as judged from 2 hour pulse-chase experiments. This observation indicates that VP5A as well as VP5 are not processed proteins, in contrast to the VP7 and VP10 which were observed to undergo post-translational modifications (McCrae and Faulkner-Valle, 1981). Major changes were seen in the time course synthesis of the glycoprotein VP10 which after reaching peak synthesis at 8 hours pi, diminished 30 fold by 16 hours pi (Table 13.1). Although the predicted amino acid sequence shows glycosylation sites in the consecutive uncharged N- terminal residues (7-21) and appear to be uncleavable signal sequences (Baybutt and McCrae, 1984) the glycoprotein VP10 did not accumulate intracellularly. Differences observed between the patterns of intracellular accumulation of VP10 in cells infected with standard brv and the brv mutant A_4, may be related to the low levels of mannose in the mutant glycoproteins, This could

have caused disturbances in the functioning of
signal sequences, which are supposed to aid translocation of glycoproteins VP10 across cell membranes (Blobel et al., 1979; Both et al., 1983b; Baybutt and McCrae, 1984). When brv infected cells were labelled with either D-[2-^{3}H] mannose or D-[6-^{3}H] glucosamine, the distribution of the [^{3}H] label was found in VP2, VP6, VP7 and VP10; this observation agrees with data by Baybutt and McCrae (1984) obtained for brv and glycoprotein patterns of SA11 virus reported by Estes et al. (1982). Depending on the strain of brv, the number of glycoproteins tended to vary amongst authors: one glycoprotein, VP7, was described for brv 486 (Kouvelos et al., 1984) and two VP7 and VP10, were reported for calf diarrhoea virus (Cohen et al., 1978; Sabara et al., 1982 and 1985). The glycosylation pattern of viral proteins synthesised in cells infected with the brv mutants D, A_4 and B_4 showed abnormally low levels of [^{3}H] mannose in the VP7 and VP10. Under similar conditions of labelling, the brv mutant B_5 produced VP7 and VP10 which incorportated [^{3}H] mannose to an extent similar to that of standard brv (Figure 15.1). The content of glucosamine in the viral proteins was comparable (normal).

The nature of glycosylation was examined with tunicamycin, an inhibitor of N- linked glycosylation (Heifetz et al., 1979; Hubbard and Ivatt, 1981; Dunphy et al., 1985). Under tunicamycin treatment, the synthesis of VP7 and VP10 disappeared while there was an accentuated increase in the amount of VP12. A precursor relationship between VP10 and VP12 had been described for brv glycoproteins (McCrae and Faulkner-Valle, 1981; Sabara et

al., 1982, 1985) and did not show dependence on the levels of mannose content in the glycoproteins. Failure of the VP2 and VP6 glycosylation to respond to tunicamycin treatment of infected cells indicated that the glycosylation in these proteins differed from that of the N- glycosidic linkages, and probably contained O-linked oligosaccharide moieties (Figure 14.1). Predicted amino acid sequence derived from the nucleotide sequence data of segment 6 (Cohen et al., 1984; Estes et al., 1984a) showed 7 potential glycosylation sites Asn-X-Thre/Ser at positions 131-133, 166-168, 271-273, 299-301, 345-347, 366-368 and 373-375. The two glycosylation sites of the VP10 reside in the N- terminal region at amino acid 8-10 and 18-20 which lie within the region of the signal sequences (Baybutt and McCrae, 1984). The VP7 was shown to contain three possible glycosylation sites located at positions 69-71, 238-240 and 318-320 (Ellerman et al., 1983). Recent evidence points to VP11 being a glycoprotein, because the predicted amino acid sequence shows potential glycosylation sites at amino acid residues 20-22 and at 118-120 (Imai et al., 1983). Earlier, McCrae and Faulkner-Valle (1981) had found that VP11 was sensitive to tunicamycin. Besides, Sabara et al. (1982b) also observed a protein of 16.1Kd in infected cells which shifted on treatment with tunicamycin to 15.5K. In their gel system the VP12 would correspond to a protein of 14.8K migrating just faster than VP11. Intracellular synthesis of the glycoproteins VP7 and VP11 produced so small amounts of protein in each case that events relating to posttranslational modification of these proteins were

uncertain. However, with [^{3}H] glucosamine and [^{3}H] mannose labelling, a protein of a size smaller than VP10 was found made in the cells infected with brv mutant B5 and the standard brv and may well be VP11 (Figure 15.1 and 15.2). Whether or not all potential glycosylation sites were used all the time, and what the determining factors for their usage are, is not yet clearly known (Hubbard and Ivat, 1981).

The significance of N-linked glycosylation of viral proteins was studied in the presence and absence of tunicamycin both in the brv mutants and standard brv. In the absence of glycosylation, infectivity of the virus yield was reduced tenfold for brv mutants D and A_4 as for the standard brv (Table 14.1). Although the mannose content of the VP7 and VP10 of brv mutants D, A_4 and B_4 were reduced, it was unlikely to be the reason for their relatively low infectivity because when brv mutants D and A_4 were treated with tunicamycin, the infectivities fell by 1 log unit; similar to standard brv whose glycosylation pattern was as unchanged as that of brv mutant B_5. Similarly, Sabara *et al.* (1982a) and Petrie (1983b) described substantial reductions (by 3-4 log units) in the infectivity of brv and simian SA11 rotaviruses when viruses were grown in the presence of tunicamycin. However, Petrie *et al.* (1983) using clone 28 of SA11 rotavirus, which lacks glycosylated VP7, found that infectivity under tunicamycin was reduced to an extent similar to clones which contained glycosylated VP7 and VP10. The authors thus dismissed a role for glycosylation of VP7 in virus infectivity. Epitopes

determining virus attachment to host cells have been reported to be specified on VP10 but the amino acid involved in influencing virus adsorption are not known (Sabara and Babiuk, 1984; Sabara et al., 1985).

H Amino sequence relationship between VP5 and VP5A.

Having demonstrated by T1 oligonucleotide mapping technique (Follett and Desselberger, 1983b) that the rearranged RNA band E found in the genome of brv mutant A_4 was closely related to the genomic RNA segment 5 of standard brv, it was hypothesized that VP5A might be correspondingly similar to VP5 provided it were the product of rearranged band E. The Cleveland peptide maps of VP5 and VP5A (Cleveland et al., 1977) produced patterns which were similar, and showed relationships in the small peptides. But the Cleveland peptide mapping method repeatedly produced maps of VP5 and VP5A that were heavily smeary compared with, for instance, VP6 which always yielded good maps under the same conditions of buffer and enzyme V8 protease. The results of these experiments suggested that the two proteins were related because both behaved in the same way when treated with V8 protease, producing peptide maps which were different from those of any other protein included in the assay (Figure 11.1).

Employing the sensitivity and high resolution provided by the two-dimensional oligopeptide mapping technique (MacDonald, 1980), tryptic maps of VP5A and VP5 appeared nearly identical (Figure 11.2). The mutant protein VP5A seems to have emerged as product of the rearranged genomic

band E, and the trypsin-sensitive (cleavage) sites (arginine and lysine) seem to have remained more or less unaltered when compared to those of VP5. This situation provides further evidence that VP5A is coded for by RNA band E in brv mutant A_4. Further evidence will be sought by *in-vitro* translation assays using genomic RNAs as mRNAs.

I *Antigenic relationships between the brv mutants and standard brv.*

Serotypic relationship between standard brv and the mutants D and A_4 was investigated by plaque reduction neutralisation assays (Wyatt *et al.*, 1983a; Hoshino *et al.*, 1984, 1985a). Polyclonal antiserum raised in rabbits against purified virus particles of brv mutant A_4 caused a three times higher plaque reduction in the homologous reaction than in heterogous reations with brv D and standard brv. There was no difference in the reactivities of antisera directed against standard brv and against brv mutant D with any of the brvs tested, and both antisera neutralised equally well the brv mutant A_4. The reactivity of brv mutant A_4 in the assay possibly indicated a antigenic variation, but the minor antigenic variation was much less than $\leq$ 20-fold which difference would have put the brv mutant A_4 into a new serotype (Hoshino *et al.*, 1984).

In an effort to demonstrate the relevance of N-linked glycosylation in antigenicity, antigens were prepared in cells infected with brv mutants D, A_4, B_4 and B_5 with and without tunicamycin treatment. When reacted with each of the antisera directed against standard brv, brv mutant D and

brv mutant A_4 by IP tests, the untreated antigens reacted in most of the major structural proteins. The VP5, VP5A, VP9 and VP12 were not immunoprecipitable whether or not the preparation had been previously exposed to tunicamycin. Tunicamycin abolished IP reaction of VP7 and VP10, thus indicating that without glycosylation the polyclonal antibody failed to react with non-glycosylated forms of the proteins. On the other hand, the antiserum directed against brv mutant A_4 contained antibodies which recognised, besides the mature VP7 antigen, the non glycosylated form of VP7 (Figure 16.3) in all preparations. This pattern of serological reactivities suggests that brv mutant A_4 contains a VP7 with an alteration in epitope(s) to be able to direct synthesis of antibodies against the precursor of VP7. Possibly the absence of mannose in VP7 of brv mutant A_4 uncovers an antigenic site of the VP7 precursor. It were of major implication to find that genomic rearrangements could directly or indirectly be involved in the emergence of changes in the antigen specifying the serotype VP7 (Kalica et al., 1981; Killen and Dimmock, 1982; Greenberg et al., 1983; Ellerman et al., 1983) but further investigations are needed.

J Gene reassortment between brv mutant D and hrv A59.

Rotaviruses of human and animal origin have been described to exchange genes (genomic RNA segments) by reassortment events (Greenberg et al., 1981; Kalica et al., 1981; Garbarg-Chenon et al., 1984; Midthun et al., 1985). Some evidence is available to suggest that gene reassortment among rotaviruses also occurs under natural infections

(Street et al., 1982; Flores et al., 1986). A reassortment technique had been used by Allen and Desselberger (1985) to show that rearranged RNA bands F and G in the genome of hrv UH (Pedley et al., 1984) were genetically equivalent to the genomic RNA segment 11 of brv, and rearranged bands B and D of the hrv genome were equivalents of RNA segment 9 of brv. When cell cultures were coinfected with a tissue culture-adapted, non-plaquing hrv A59 and the brv mutant D (Hundley et al., 1985), five reassortant viruses, B320-1, B320-2, B320-4, B320-6 and B317-1 were isolated. The reassortment experiments were done at very low m.o.i., (10^{-3} pfu/cell of brv mutant D and 10^{4}TCID50 of hrv A59) which prevented brv D from overgrowing hrv A59. No deliberate external pressures, e.g. use of specific antiserum (Knowlton and Ward, 1985), were imposed on the reassortment process to select for reassortant viruses. The genotypes of the reassortants were determined by RNA-PAGE (Garbarg-Chenon et al., 1984; Allen and Desselberger, 1985). In two reassortants, B320-2 and B317-1, the genomic RNA segment 5 of the hrv A59, reassorted into the genome of brv mutant D which concomitantly lost the rearranged RNA band A. It became evident that rearranged band A is genetically equivalent to the normal RNA segment 5 of the hrv A59 genome and that the rearranged RNA segment A was derived from the standard brv genomic segment 5. After several rounds of plaque-to plaque purification, none of the reassortant virus reverted to normal brv genotype. In no case was the RNA segment A of brv mutant D found to return to the normal position of the RNA segment 5 of the standard

brv genome. The studies confirmed that genome rearrangements represented genetically stable genome elements of replication-competent rotaviruses, and that they were capable of genetic interaction. Of the five reassortants, three, B320-1, B320-4 and B320-6 had acquired the genomic segment 8 from hrv A59 genome and all three reassortants retained the rearranged segment A. Under the low m.o.i. conditions, reassortment occurred at a frequency of 14% (among 36 plaques analysed 5 were found to be reassortants) and involved the donation of RNA segments 5 and 8, both of which code for non-structural proteins, at frequencies of 6% and 8%, respectively. Plaque reduction neutralisation assays using antiserum directed against the parental brv mutant D showed that all five brv D/hrv A59 reassortants reacted similarly to homologous neutralisation reaction. This finding indicated that the brv D/hrv A59 reassortants were of the same serotype as the parent brv mutant D. Since it is established that brv gene 8 codes for the (neutralising) serotype-specifying antigen (McCrae and McCorquodale, 1982; Killen and Dimmock, 1982; Ellerman et al., 1983), changes in the serotype of the reassortant viruses would have occurred if the reassorted hrv A59 segment 8 were functionally equivalent to the brv segment 8. Moreover, as Hoshino et al. (1984) had demonstrated that the serotype of brv strains serotype 6 is not cross-reactive with hrvs, it was concluded that RNA segment 8 of hrv A59 does not code for the serotype specifying antigen (VP7) which in brv is encoded on RNA segment 8 (McCrae and McCorquodale, 1982). Therefore, the hrv A59 segment 8

* In future experiments reassortment between brv mutants A4, B4 and B5 on one side and hrv of standard genome composition on the other side should also be investigated.

could not have been exchanged against brv segment 8 Table B after Dyall-Smith et al. (1983a), Dyall-Smith and Holmes (1984), may well be equivalent to either brv segment 7 or 9. Testing of reassortants using different gel conditions is underway to decide this question (U. Desselberger, personal communication).

*

K Prospects.

Genome rearrangements evidently contribute to the vast genetic heterogeneity among rotaviruses and may well be an operational mechanism in the evolution of rotaviruses (Allen and Desselberger, 1985). Recently, Besselaar et al. (1986) described hrvs occurring naturally in asymptomatic infections of neonates in which segment 11 was missing and an RNA segment appeared in an unusual position between RNA segments 5 and 6.

The nature of RNA segment rearrangement is yet to be analysed by molecular cloning and sequencing techniques. The sequences of rearranged bands of genomic RNA of rotaviruses will provide proposals for (a) mechanism(s) by which viruses carrying such rearrangements might have emerged. The use of radiolabelled cDNA probes specific for rearranged RNA bands in Northern blot hybridizations will reveal or exclude the occurrence of mosaic structures in rearranged bands as were described for DI RNAs of influenza viruses (Fields and Winter, 1982). Efforts will be directed to determine the coding capacity of the rearranged RNA segments by in-vitro translations. Studies of RNA packaging in rotavirus particles are a major point, having

found that genome rearrangements create excess RNA packaging well above what is normally encapsidated (Hundley et al., 1985; Allen and Desselberger, 1985). The frequent reassortment of rotavirus segments opens a question as to whether or not exchanges of RNA segments and rearranged RNA bands occur at random or selectively non-random. A large number of reassortants need be isolated in order to determine frequencies of reassortment events and segregation of genes.

Biological functions of RNA segments 1, 2, 3, 5, 11 and of the non structural genes migrating as RNAs 7-9 require elucidating.

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