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GENOMIC VARIATION IN ROTAVIRUSES

By

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SUMMARY

The rotaviruses are a recently defined ubiquitous group of viruses responsible for causing acute-gastroenteritis in human infants and young animals. Biochemical studies have shown that the rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA).

This thesis concerns an investigation of the nature and extent of genomic variation in rotaviruses. A rapid and sensitive method for analyzing the genome profiles of rotavirus field isolates was developed. This is based on the direct extraction of dsRNA from faecal samples followed by radiolabelling with [³²P] pCp using T₄ RNA ligase. This procedure has been further developed to produce a method for generating diagnostic fingerprints from individual species of dsRNA.

A detailed structural study making use of this fingerprinting method has been undertaken on bovine, porcine and human rotavirus isolates. These analyses show that genome segment mobility variations are always associated with detectable changes in nucleotide sequence. They also show that corresponding genome segments with no mobility variation can have sequence changes at least as great as those found in segments showing electrophoretic mobility variation. These results also revealed evidence for genome segment specific regions of terminal sequence conservation.

Evidence for the occurrence of genome segment reassortment between viruses in the field was obtained.

Finally evidence for the existence of a 'new' porcine rotavirus which is antigenically unrelated to previously described rotaviruses and has an unusual pattern for it's 11 genome segments is presented.

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DECLARATION

The work contained in this thesis was the result of original research conducted by myself under the supervision of Dr. M. A. McCrae. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

CONTENTS

	<u>Page No.</u>
Summary	(i)
Acknowledgements	(ii)
Declaration	(iii)
List of Figures	(viii)
List of Tables	(xviii)
Abbreviations	(xix)
<u>Introduction</u>	
Section I - Viral Gastroenteritis/Rotaviruses	1
(a) Viral Diarrhoea	1
(b) Rotaviruses	
(i) Morphology	4
(ii) Pathology/Immunity	6
(iii) Serology	8
(iv) RNA	11
(v) Polypeptides	15
(vi) Genetic Studies	18
Section II - Variation of Influenza A Virus	22
(i) Introduction	22
(ii) Structure and Properties of Influenza A Virus	23
(iii) Epidemiological History	24
(iv) Mechanisms of Antigenic Variation Antigenic Shift	25
Antigenic Drift	27

	<u>Page No.</u>	
(v) Variation in Haemagglutinin	28	
(vi) Structure and Variation in Neuraminidase	33	
(vii) Variation in Non-Structural Proteins	35	
(viii) Conclusion	38	
 Section III - Variation in the Reoviruses, Orbiviruses and Rotaviruses	 40	
Introduction	40	
(a) Variation in the Orthoreoviruses	40	
(b) Variation in Orbiviruses	43	
(c) Variation in Rotaviruses - Rationale of this Work	49	
 Materials and Methods	 54	
Materials	54	
Methods	1. Virus Growth and Purification	56
	2. Faecal Samples Containing Rotavirus Particles	57
	3. Electron Microscopy	57
	4. Extraction of Rotavirus dsRNA From Faecal Samples	57
	5. 3' Terminal Labelling of dsRNA with T₄ RNA ligase	58
	6. TCA Incorporation Following Radioactive Labelling of Nucleic Acids	60
	7. Polyacrylamide Gel Fractionation of dsRNA	60
	8. Ethidium Bromide Staining	61
	9. Preparative Fractionation of dsRNA or Agarose Gels and Extraction of Isolated Species	61
	10. Partial Nuclease Digestion of Isolated Species with RNase T₁ and Partial Alkaline Hydrolysis	61

	<u>Page No.</u>
11. Production of dsDNA size markers	62
12. Polyacrylamide Gel Fractionation for RNA Sequence Analysis and T ₁ ribonuclease Partial nuclease digestion fingerprinting	62
13. Synthesis of Viral mRNA for Use in Sequencing Studies	63
14. Fractionation of dsRNA Species and Gel Elution for RNA Sequence Analysis	63
15. Genome Strand Separation and RNA Sequence Analysis	64
16. S1 Nuclease Digestion	65
17. Oligonucleotide Fingerprint Analysis	65
18. Autoradiography	66

Results

Chapter 1: A Rapid and Sensitive Method for Analyzing the Genome Profiles of Rotavirus Field Isolates	67
Chapter 2: A Sensitive Method for the Production of Diagnostic Fingerprints of the Genome Segments of Rotavirus Field Isolates	85
Chapter 3: Initial Partial T ₁ ribonuclease Digestion Analysis of Five Bovine Rotavirus Isolates	103
Chapter 4: Partial Nuclease Digestion Analysis of Human, Bovine and Porcine Rotavirus Isolates	119
Chapter 5: Partial T ₁ Ribonuclease Digestion Analysis of Five Bovine Rotavirus Isolates	130
Chapter 6: Partial T ₁ Ribonuclease Digestion Analysis of Four Porcine Rotavirus Isolates	153
Chapter 7: Partial T ₁ Ribonuclease Digestion Analysis of Human Rotavirus Isolates	170
Chapter 8: Terminal RNA Sequence Analysis of Selected Rotavirus Genome Segments	179

	<u>Page No.</u>
<u>General Discussion</u>	
(i) Variation in Rotavirus Genome Profiles	195
(ii) Rotavirus Gene Reassortment	197
(iii) Rotaviruses - A Future Perspective	203
Appendix	206
Publications	209
References	210

FIGURES

	<u>Page No.</u>
Figure 1: Negatively stained electron microscope photograph of purified human rotavirus particles	5
Figure 2: Comparative genome profile analysis of two rotavirus isolates with reovirus serotype 1	12
Figure 3: Comparison of the amino acid sequences predicted from the nucleotide sequence of the N-terminal regions of the 13 influenza haemagglutinin subtypes	31
Figure 4: Dendogram showing the relationships between the HA ₁ N-terminal amino acid sequences deduced from genomic sequences of 32 viruses representing the 13 known influenza haemagglutinin subtypes	32
Figure 5: Schematic representation of the method employed by Kalica <i>et al.</i> (1976) to obtain rotavirus dsRNA from stool samples	68
Figure 6: Schematic representation of the rapid and sensitive method developed for obtaining rotavirus dsRNA from stool samples	70
Figure 7: The sensitivity of ethidium bromide for visualizing dsRNA	71
Figure 8: Graph to show the effect of dilution of infected faeces on incorporation in the end-labelling reaction	73
Figure 9(a): Polyacrylamide gel fractionation of an equal amount of the total material from each dilution of infected faeces	75

Figure 9(b): Further polyacrylamide gel fractionation of equal amounts of total material from dilutions 10^{-4} - 10^{-5} of infected faeces to determine the sensitivity limit of the final method adapted for extracting and labelling rotavirus dsRNA	76
Figure 10 Polyacrylamide gel fractionation of an equal number of incorporated counts from each dilution of infected faeces	77
Figure 11(a): Comparative genome profile analysis of 4 pig rotavirus isolates	80
Figure 11(b): Polyacrylamide gel fractionation of 5 human rotavirus isolates on a short 7.5% polyacrylamide gel	81
Figure 12(a): Polyacrylamide gel to show a comparative genome analysis of 5 field isolates of calf rotavirus with the U.K. tissue culture adapted calf rotavirus	82
Figure 12(b): Comparative genome profile analysis of 4 field isolates of calf rotavirus to show evidence of mixed infections	83
Figure 13: Fractionation of the digestion products of 3' terminally labelled bovine rotavirus species 10 RNAs	88
Figure 14: 1.5% vertical agarose gel fractionation of rotavirus dsRNA using tris-acetate buffer as described in Materials and Methods	90
Figure 15: Fractionation of 3' terminally labelled rotavirus dsRNA on a 1.5% vertical agarose gel in tris-acetate buffer	91

Figure 16:-	Preparative fractionation of end-labelled field-isolated bovine rotavirus dsRNA run on a 1.5% agarose gel	92
Figure 17:	Individual isolated species of dsRNA extracted from the preparative agarose gel as described in Materials and Methods, re-run on a 7.5% polyacrylamide gel using the Laemmli discontinuous buffered system	95
Figure 18:	Comparative genome analysis of two field isolates of bovine rotavirus used to illustrate the method for producing 'fingerprints' of the genome segments	97
Figure 19:	Partial nuclease digestion analysis of the species 10 RNAs from isolates A and B	99
Figure 20:	18% polyacrylamide gel showing partial nuclease digestion analysis of the species 5 RNA's from isolates A and B	100
Figure 21:	Polyacrylamide gel to show a comparative genome profile analysis of the five wild bovine isolates used in this study	104
Figure 22:	Preparative fractionation of 3' terminally labelled viral RNA from 4 of the five wild bovine isolates	105
Figure 23:	Partial T ₁ ribonuclease digestion fingerprints obtained for the five species 1 dsRNAs of the isolates under study	107
Figure 24:	Partial T ₁ ribonuclease digestion fingerprints obtained for the species 2-3 dsRNA doublets of the 5 isolates under study	108
Figure 25:	Partial T ₁ ribonuclease digestion fingerprints obtained for the five species 5 RNAs	109

Figure 26:	Partial T ₁ ribonuclease digestion fingerprints obtained for the species 6 dsRNA's of the five isolates under study	110
Figure 27(a):	Partial T ₁ ribonuclease fingerprints obtained for the species 7-8-9 dsRNA triplets of the five isolates under study	111
Figure 27(b):	Partial digestion fingerprints of the species 7-8-9 dsRNA 'triplets' of the five isolates under study run further to allow evaluation of the partial digestion patterns beyond nucleotide position 30	112
Figure 28:	Partial T ₁ ribonuclease fingerprints obtained for the five species 10 dsRNA's	113
Figure 29:	Partial T ₁ ribonuclease fingerprints obtained for the five species 11 dsRNA's	114
Figure 30:	Fractionation of the 3' end labelled virion RNA from the five wild isolates used in this study on a 7.5% polyacrylamide gel containing 6 M urea and using tris-acetate buffer	116
Figure 31:	Polyacrylamide gel to show a comparative genome analysis of the three wild rotavirus isolates used in this study	120
Figure 32:	Preparative fractionation of the total 3' end labelled viral RNA from the 3 rotavirus isolates under study	121
Figure 33:	Partial T ₁ ribonuclease digestion fingerprints obtained for the three species 4 dsRNA's of the isolates under study	123

Figure 34:	Partial T_1 ribonuclease digestion fingerprints obtained for the species 5 dsRNAs and the species 6 dsRNA's of the three isolates under study	124
Figure 35:	Partial nuclease digestion fingerprints obtained for the near-terminal regions of the species 5 and 6 dsRNA's of the three isolates under study	125
Figure 36:	Partial T_1 ribonuclease digestion fingerprints obtained for the species 10 dsRNA's from the three isolates under study	127
Figure 37:	Partial digestion fingerprints obtained for the species 7-8-9 'triplets' and the species 2-3 doublets of the three isolates under study	128
Figure 38:	Polyacrylamide gel to show a comparative genome analysis of the five bovine isolates used in this study	132
Figure 39(a):	Partial T_1 ribonuclease digestion fingerprints obtained for the terminal region of the five species 1 dsRNA's from the isolates under study	133
Figure 39(b):	Partial T_1 ribonuclease digestion fingerprints obtained for the near-terminal region of the five species 1 dsRNA's from the isolates under study	134
Figure 40:	Partial T_1 ribonuclease digestion fingerprints obtained for the terminal regions of the species 5 and the species 6 dsRNAs from the five isolates under study	135
Figure 41:	Partial T_1 ribonuclease digestion fingerprints obtained for the near-terminal region of the species 5 dsRNA's from the five isolates under study	136

Figure 42:	Partial T ₁ ribonuclease digestion fingerprints obtained for the near-terminal region of the species 6 dsRNA's from the five isolates under study	137
Figure 43(a):	Partial T ₁ ribonuclease digestion fingerprints obtained for the terminal region of the species 10 RNAs of the five isolates under study	138
Figure 43(b):	Partial T ₁ ribonuclease digestion fingerprints obtained for the near-terminal regions of the species 10 RNAs of the five isolates under study	139
Figure 44:	Two dimensional T ₁ oligonucleotide finger-print analysis of the species 10 RNAs from isolates C and D	141
Figure 45:	Partial nuclease digestion fingerprints obtained for the near-terminal region of species 2-3 dsRNA 'doublets' of the five isolates under study	142
Figure 46:	Partial nuclease digestion fingerprints obtained for the near-terminal region of species 7-8-9 dsRNA 'triplets' of the five isolates under study	143
Figure 47(a):	Partial T ₁ ribonuclease digestion fingerprints obtained for the terminal region of the five species 4 RNAs from the isolates under study	144
Figure 47(b):	Partial T ₁ ribonuclease digestion fingerprints obtained for the near-terminal region of the species 4 RNA's from the five isolates under study	145
Figure 48:	Two dimensional T ₁ oligonucleotide finger-print analysis of the species 4 RNAs from isolates C, D and E	147

Page No.

Figure 49(a): Partial T_1 ribonuclease digestion fingerprints obtained for the terminal regions of the species 11 dsRNAs from the five isolates under study	149
Figure 49(b): Partial T_1 ribonuclease digestion fingerprints obtained for the near-terminal regions of the species 11 dsRNA's from the five isolates under study	150
Figure 50: Two dimensional T_1 oligonucleotide finger-print analysis of the species 11 RNAs from isolates C and D	151
Figure 51: Polyacrylamide gel to show a comparative genome profile analysis of the four porcine isolates used in this study	154
Figure 52: Preparative fractionation of viral dsRNA from isolates B and D	156
Figure 53: Partial T_1 ribonuclease digestion analysis of the near terminal region of the species 1 RNAs from the four isolates under study	157
Figure 54: Partial T_1 ribonuclease digestion analysis of the near terminal region of the species 4 RNAs from the isolates under study	158
Figure 55: Partial T_1 ribonuclease digestion analysis of the near terminal region of the species 5 RNAs from the isolates under study	160
Figure 56: Two dimensional T_1 oligonucleotide finger-print analysis of RNA species 5 from isolates C and D	162
Figure 57: Partial T_1 ribonuclease digestion of the near-terminal region of the species 6 RNAs from the isolates under study	163

Page No.

Figure 58:	Partial T ₁ ribonuclease digestion analysis of the near terminal region of the species 7-8-9 'triplets' of the isolates under study	164
Figure 59:	Partial T ₁ ribonuclease digestion analysis of the near-terminal region of the species 10 RNAs from the isolates under study	165
Figure 60:	Partial T ₁ ribonuclease digestion analysis of the near terminal region of the species 11 RNAs from the isolates under study	166
Figure 61:	Partial T ₁ ribonuclease digestion analysis of the terminal region of the species 1 → 4 and the 'extra' band of isolate D	168
Figure 62:	Polyacrylamide gel to show comparative genome profile analysis of the four human isolates used in this study	171
Figure 63:	Partial T ₁ ribonuclease digestion analysis of the near terminal region of the species 1 and species 4 RNAs of the four isolates under study	172
Figure 64:	Partial T ₁ ribonuclease digestion analysis of the near terminal region of the species 5 and 6 RNA of the four human isolates under study	173
Figure 65:	Partial T ₁ ribonuclease digestion analysis of the near terminal region of the species 2-3 'doublet' and the species 7-8-9 'triplet' of the four human isolates under study	174
Figure 66:	Partial T ₁ ribonuclease digestion analysis of the near terminal region of the species 10 and species 11 RNA of the isolates under study	175

Page No.

Figure 67:	Partial T ₁ ribonuclease digestion analysis of the terminal region of the species 10 and 11 RNA of the four human isolates under study	177
Figure 68:	Polyacrylamide gel to show a comparative genome profile analysis of the four rotavirus isolates used in this study	181
Figure 69:	Fractionation of partial T ₁ ribonuclease digestion products of the plus and minus fractions from the hybridization reactions described in Table 3	184
Figure 70:	Partial U ₂ ribonuclease digestion analysis of Sp 10 and 11 genome segments from wild isolate 4329 and the U.K. tissue culture adapted calf virus	186
Figure 71:	Partial nuclease digestion sequence analysis at the 3' end of genomic plus strands for RNA species 10 of isolates O.S.U. (A); U.K. Calf (B) and 6597 (C)	188
Figure 72:	Partial nuclease digestion sequence analysis at the 3' end of genomic plus strands for RNA species 11 of isolates O.S.U. (A); U.K. Calf (B) and 6597 (C)	189
Figure 73:	Partial nuclease digestion sequence analysis at the 3' end of genomic minus strands for RNA species 10 of isolates O.S.U. (A) and 6597 (B)	191
Figure 74:	Partial nuclease digestion sequence determination at the 3' end of genomic minus strands of RNA species 11 from isolates O.S.U. (A) and 6597 (B)	192
Figure 75:	Schematic representation of the partial T ₁ digestion and oligonucleotide finger-printing results for the five bovine isolates analyzed in Chapter 5	199

Figure 76: Comparative genome profile analysis
of the Compton atypical porcine-rotavirus-
like-agent with previously described
rotaviruses from several animal species

207

TABLES

	<u>Page No.</u>
Table 1: Orbivirus serological groups	45
Table 2: Recovery of dsRNA from agarose gels	94
Table 3: The effect of changing the hybridization conditions on the strand displacement reaction	183
Table 4: Base sequence homologies (%) between ^{32}P -labelled vRNA segments of Singapore (H2N2) and influenza virus prototypes PR8 (H1N1) and Hong Kong (H3N2)	202

Abbreviations

AHS	African Horse Sickness
BTV	Bluetongue Virus
cDNA	Complementary DNA
DBM	Diazobenzyloxymethyl (paper)
DNA	Deoxyribonucleic Acid
ds	Double-Strand
DS	Double-Shell
EDIM	Epizootic Diarrhoea of Infant Mice
EDTA	Ethylene Diamine Tetracetic Acid
EHD	Epizootic Haemorrhagic Disease of Deer
ELISA	Enzyme Linked Immunosorbent Assay
E.O.P.	Efficiency of Plating
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulphonic Acid
IAHA	Immune Adherence Haemagglutination Assay
m.o.i.	Multiplicity of Infection
NCDV	Nebraska Calf Diarrhoea Virus
NS	Non Structural
'O'	O-agent
O.S.U.	Ohio State University Tissue Culture Adapted Porcine Rotavirus
p.f.u.	Plaque Forming Unit
RNA	Ribonucleic Acid
S.A.11	Simian-11 Rotavirus
ss	Single Strand
SS	Single Shell
TCA	Trichloroacetic Acid
Tris	Tris (Hydroxymethyl) Amino Ethane
<u>ts</u>	Temperature Sensitive

INTRODUCTION

SECTION 1

(a) Viral Diarrhoea

Acute gastro-enteritis is one of the most common illnesses of children and young animals throughout the world. It is estimated that annually there are some 500 million episodes of diarrhoea in children under the age of 5 (Agarwal, 1979), of these it is widely accepted that from 5-20 million cases result in infant death. In addition to its enormous medical importance acute diarrhoeal disease causes severe economic loss in domestic livestock. Bacteriological investigations have revealed that some outbreaks of infantile gastro-enteritis are associated with enteropathogenic bacteria, particularly E. coli (Shepherd et al., 1975), although in a significant proportion of cases (up to 75%) pathogenic bacteria cannot be isolated.

The first well documented cases of acute viral diarrhoea occurred in 1944 and involved a study of laboratory outbreaks in suckling mice at the Harvard Medical School, U.S.A. (Cheever & Mueller, 1947). Bacteriological investigations although implicating *Salmonella* could not prove that these organisms were responsible for the infection. Eventually it was conclusively shown that the disease was caused by a highly infectious ether-resistant virus (Kraft, 1957; Adams & Kraft, 1963; Kraft, 1966). This virus is known as Epizootic Diarrhoea of Infant Mice (EDIM) or Mouse Rotavirus. Electron microscope study of thin sections of gut and bowel revealed the EDIM virus to be approximately 70 nm in diameter, it also appeared to have an entirely cytoplasmic association (Adams & Kraft, 1967); different groups concluded that it had morphological similarities with either bluetongue or reoviruses.

Attempts at adapting the EDIM virus to growth in tissue culture proved to be universally unsuccessful.

In 1958 a morphologically similar virus, now known as SAll, was isolated from a rectal swab of a vervet monkey (Malherbe & Strickland-Cholmley, 1967). This virus was able to grow in tissue culture and following a number of passages, produced a cytopathic effect in primary vervet monkey kidney cells. Another virus giving rise to a similar cytopathic effect in primary vervet monkey kidney cells was isolated (Malherbe & Strickland-Cholmley, 1967) from pooled filtered washings of cattle and sheep intestines from the Johannesburg municipal abattoir. The animal species of origin of this virus isolate is unknown and it has become known as the 'O' agent.

Recently, it has been found that a similar group of viruses with distinctive morphology, and antigenic similarity are associated with diarrhoea in piglets (Lecce et al., 1976), calves (Woode et al., 1974) and humans (Flewett et al., 1973; Bishop et al., 1974; Cruikshank et al., 1974). It has been conclusively shown that these viruses are morphologically and antigenically distinct from both reoviruses and orbiviruses, and they also differ from them in some of their physicochemical properties (Palmer et al., 1977).

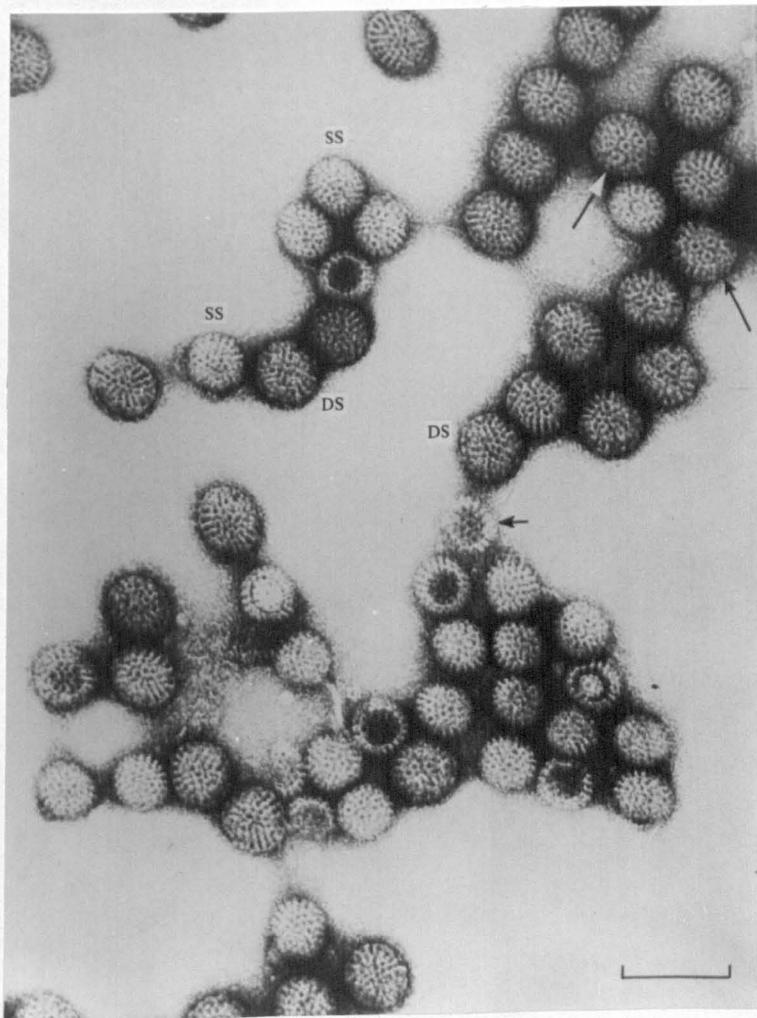
Bacteria-free faecal filtrates from field cases of diarrhoeal disease have been demonstrated experimentally to cause acute gastro-enteritis in colostrum deprived calves (Mebus et al., 1969). Koch's postulates have been satisfied as regards the involvement of the Nebraska calf diarrhoea virus as the causative agent of acute gastroenteritis (Davidson et al., 1975).

Although other viruses have also been found in association with cases of acute viral gastroenteritis (Coronavirus, McClung et al. (1976); Calicivirus, Madeley & Cosgrove (1976); Astrovirus, Madeley (1979); Adenovirus, Uhnoo et al. (1981) and the Norwalk virus, Singh et al. (1981)) the viruses now called Rotaviruses are the major pathogens. Over 90% of children have antibodies by the age of six (Flewett & Woode, 1978), one study (Woode, 1976) indicated that 80% of calf diarrhoea outbreaks in the U.K. were associated with this virus. The name 'rotavirus' (Flewett et al., 1974a) is now commonly accepted for this group of diarrhoea viruses, it describes the distinctive wheel like morphology of intact particles observed in electron micrographs. Morphologically identical human rotaviruses have been observed in the faeces of children with acute gastro-enteritis from the U.K. (Flewett et al., 1973), Chile (Espejo et al., 1980a), Canada (Middleton et al., 1974), India (Holmes et al., 1974), Zimbabwe (Cruickshank et al., 1975) and Australia (Davidson et al., 1975) establishing the worldwide incidence of these infectious agents. The apparently ubiquitous nature of rotaviruses has been confirmed by their observation in the infectious diarrhoea of many other animal species including foals (Flewett et al., 1975), lambs (Snodgrass et al., 1976), rabbits (Bryden et al., 1976), goats (Scott et al., 1978), deer (Tzipori et al., 1976), mice, monkeys and even pronghorn antelopes (Reed et al., 1976). Rotaviruses have also been isolated from chickens and turkeys (McNulty et al., 1980).

(b) Rotaviruses

(i) Morphology. Comparative electron microscope studies have confirmed that rotaviruses isolated from different animal species (humans, calves, pigs and foals) are morphologically indistinguishable from each other and identical to the 'O' agent, SA11, and EDIM virus (Woode et al., 1976). Rotavirus particles (Fig. 1) consist of an electron dense centre about 38 nm in diameter which is icosahedral in structure, surrounded by an inner layer of capsomeres which radiate outwards like the spokes of a wheel (Flewett et al., 1974a). The outer layer of the intact virus particles appears to be attached to these spokes to form a sharply defined rim (Flewett et al., 1973) giving rise to their characteristic wheel-like appearance, hence the name Rotavirus (Rota=Wheel). Complete rotavirus particles are approximately 66-68 nm in diameter (Palmer et al., 1977). The outer shell may be removed physically or enzymically to yield single-shelled particles or 'cores' which are about 10 nm smaller than the intact virions. Field isolated diarrhoeic faeces often contains a mixture of both these particle types, and this may have been one reason why there was initially confusion in the positive identification of a distinct class of viruses causing acute gastro-enteritis. The morphology of rotavirus remains unchanged after treatment with non-ionic detergents, heat, centrifugal force, high salt concentrations and extremes of pH (3 to 10) (Palmer et al., 1977),^{and} in these physicochemical respects rotaviruses most resemble reovirus. However, rotavirus structure like that of Bluetongue virus (BTV) the type 'species' of the genus Orbivirus is not affected by treatment with chymotrypsin which will completely degrade reovirus particles (Joklik, 1974; Luftig et al., 1972). In terms of morphology rotaviruses most resemble BTV rather than reovirus even

Figure 1. Negatively Stained Electron Microscope Photograph of Purified Human Rotavirus Particles (Wa Strain).



Complete double shelled (DS) particles, single shelled (SS) or 'Core' particles and some degraded particles (short arrow) are shown. The long arrows indicate large ring shaped morphological units seen on the surface of some particles. The bar marker represents 100 nm.

though BTV has a very 'fuzzy' outer capsid layer (Verwoerd et al., 1979).

The similarities and differences in morphology and stability for rotavirus compared to reoviruses and orbiviruses supports the concept that the rotaviruses represent a unique new genus. Rotaviruses can be easily detected in diarrhoeic faeces by direct electron microscopy as they are often present at very high concentrations $\sim 10^9$ - 10^{10} particles/gram faeces and their characteristic morphology allows rapid diagnosis. It is therefore somewhat puzzling that these viruses were not detected some 20 years ago when they could have been recognized as the major etiological agents of acute gastro-enteritis.

(ii) Pathology/Immunity

A number of histological studies making use of thin sections derived from biopsy material from the gut of rotavirus infected animals have shown that the virus is invariably present only in the cytoplasm of infected cells (Bishop et al., 1973; Stair et al., 1973). There is variation in the interpretation of other structural artefacts that appear to be virus related (Banfield et al., 1968; Flewett et al., 1974b) although it is agreed that virus particles located in the cytoplasm of infected cells are usually found in dilated cisternae of the endoplasmic reticulum. Thin sections of the gut of rotavirus infected mice, piglets and calves have shown the cells most affected are the epithelial cells located on the sides and tips of villi (Bishop et al., 1973; Holmes et al., 1975).

Although only a few rotavirus isolates have been adapted to routine growth in tissue culture, most 'wild' isolates will go through at least

the preliminary stages of adsorption and replication as shown by immunofluorescence of infected cells allowing limited tissue culture studies of rotavirus pathogenesis in vitro. McNulty et al. (1976) have confirmed that rotavirus particles, in this case bovine rotavirus, also appear to be associated with the cytoplasm of cells infected in tissue culture.

Little is known of immunity to rotavirus in either human or animal populations. The presence of circulating antibodies to rotavirus has been detected by serum neutralization (Thouless et al., 1977) complement fixation (Kapikian et al., 1975) and immunofluorescence (Morishima et al., 1976). The levels of circulating antibodies appeared to decrease with age indicating that adults may become susceptible to re-infection (Flewett & Woode 1978). Re-infection in adults who had antibodies to rotavirus prior to infection has been reported (Ørstavik, 1976). Antibodies can be transmitted across the placenta and detected in infants during their first weeks of life (Flewett & Woode, 1978); this passive immunity may be an important factor in providing early protection.

From the stability and endemic nature of rotavirus it was expected that farm animals would have, like humans, a high level of immunity. This has been confirmed in studies of serum neutralizing titres in several herds of cattle, both in the U.K. where 59 herds of cattle tested were all serologically positive for rotavirus (Woode, 1976), and in Canada where 75% of cattle in 30 herds showed evidence of virus antibodies (Flewett & Woode, 1978). Rotavirus isolates from one animal species can experimentally infect members of some other species; human rotavirus has been reported to infect piglets (Bridger et al., 1975), calves (Mebus et al., 1976) and lambs (Snodgrass et al., 1977). Rotavirus

isolates infecting one animal species are not neutralized by convalescent sera from animals of another species (Thouless et al., 1977); therefore each animal species has at least one unique "serotype" unlike the reoviruses where there are just 3 known mammalian serotypes (Joklik, 1974).

(iii) Serology

It has been comprehensively shown that rotaviruses are antigenically unrelated to either the Reoviruses or the Orbiviruses (Kapikian et al., 1974; Kapikian et al., 1975). The reluctance of rotavirus isolates to grow routinely in tissue culture, together with the relatively poor yields of virus from the tissue culture adapted strains has greatly restricted both serological and biochemical studies on this important virus group. Fortunately most purified wild isolates of rotavirus will at least infect cells in tissue culture and give rise to the expression of viral proteins (Banatvala et al., 1975; Thouless, 1979). Viral infectivity can be measured by indirect immunofluorescence of infected cells (Woode et al., 1976). This method has been used together with complement fixation assays to study the antigenic relationships between rotaviruses isolated from different animal species. Cells infected with calf and mouse rotavirus reacted by immunofluorescence with convalescent serum from calves, mice, children, foals and rabbits (Woode et al., 1976), clearly illustrating that all these isolates share a group or common antigen. It is also possible to carry out "serotyping" of isolates by serum neutralization of virus infectivity. This kind of analysis is used to detect species-specific rotavirus antigens. Virus is mixed with heterologous antisera prior to infecting cells; the subsequent

presence of fluorescent foci following incubation indicates that a virus isolate has not been cross neutralized.

Rotavirus isolates from 7 different animal species; human, calf pig, lamb, foal, mouse and rabbit can be distinguished by serum neutralization (Thouless et al., 1977). There is additional evidence that rotavirus isolates from the same animal species (humans) can be distinguished by serum neutralization. Flewett et al. (1978) have demonstrated that there may be as many as 4 different human rotavirus serotypes. It is not known whether multiple serotypes exist for rotavirus isolates of other animal species although it would not be surprising if the same sort of variation occurred.

The group or common antigen shared by all rotavirus isolates has been demonstrated by indirect immunofluorescence, complement fixation, gel diffusion and immuno-electron microscopy (Flewett & Woode, 1978). A good attempt has been made at locating the antigens responsible for type-specificity and group-specificity in the rotavirus particle (Bridger, 1978).

In natural infections rotavirus particles often occur in two morphological forms, complete virus particles, and incomplete particles which lack the outer capsid layer (Bridger & Woode, 1976; Martin et al., 1975; Palmer et al., 1977). Studies have shown that virus particles lacking the outer capsid layer were agglutinated by antisera with immunofluorescence activity (Bridger, 1978) and by antisera with immunofluorescence and neutralizing activity. Neutralizing activity has been shown to be type-specific (Thouless et al., 1977) and it is thought that the antigens responsible are located on the outer capsid

layer. Since particles with the outer capsid layer were not agglutinated by antisera lacking neutralizing activity but possessing immunofluorescence activity it was concluded that the group specific antigen is masked by the type specific antigen in intact particles and is associated with incomplete particles. The concept that the neutralizing antigen is found on the surface of virions correlates with the observations that only complete rotavirus particles are associated with infectivity (Elias, 1977; Bridger & Woode, 1976).

Complement fixation tests have also been used to distinguish antigenically different rotaviruses (Zissis & Lambert, 1978). It appears that complement fixation differences do not agree with neutralization differences (Flewett et al., 1978), indicating that these two antigenic properties are also unlinked. In addition some rotaviruses have been demonstrated to possess haemagglutination activity (Inaba et al., 1977; Fauvel et al., 1978; Kalica et al., 1978).

At present a whole range of different serological tests are employed to characterize rotavirus isolates. These include neutralization, complement fixation, enzyme linked immunosorbent assay (ELISA), immune adherence haemagglutination assay (IAHA) and haemagglutination. This situation has given rise to a conflicting and very confusing use of terminology. The term "serotype" has been used to describe rotavirus isolates characterized by complement fixation (Zissis & Lambert, 1978), ELISA (Yolken et al., 1978) and immuno-electron microscopy. Kapikian et al. (1981) suggested that these groupings should be designated "subgroups" with "serotype" being specifically reserved for neutralization characteristics. Therefore a great deal of care must be taken in interpreting the literature; lack of clarity in the past has even led to different groups using

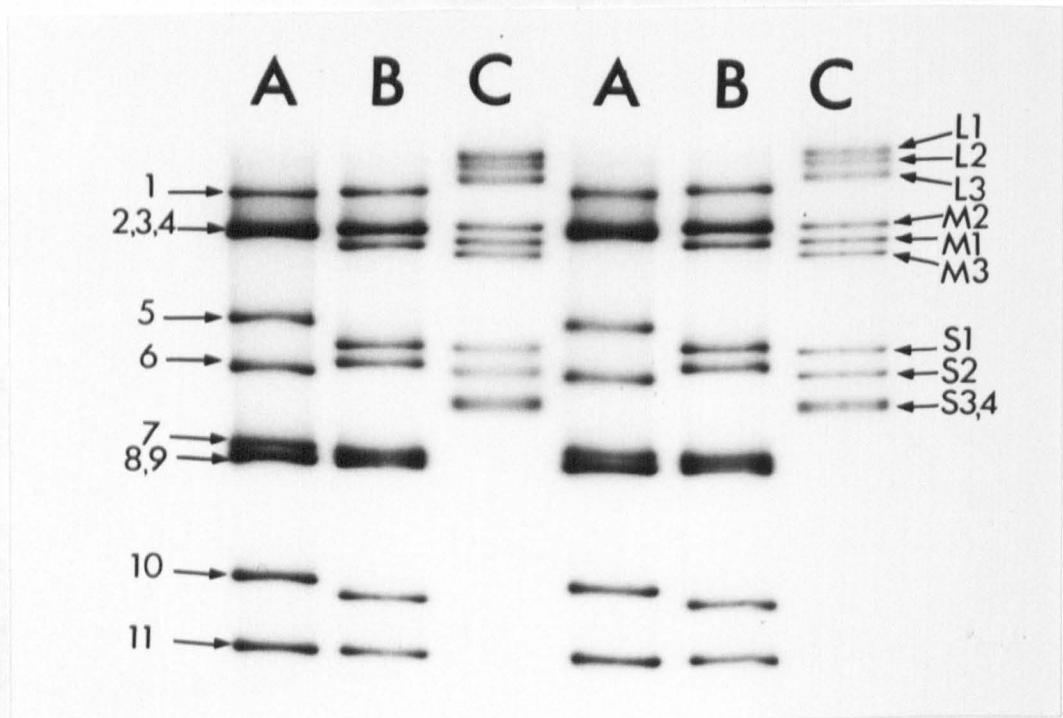
identical terminology for unrelated data in combined publication (Zissis & Lambert, 1978; Thouless et al., 1978).

(iv) Rotavirus RNA

Analysis of the nucleic acid of rotaviruses has revealed the presence of double stranded RNA (Newman et al., 1975). Studies on the thermal denaturation characteristics of calf diarrhoea virus have shown it to have a characteristic melting temperature of 78°C (Rodger et al., 1975). This genomic RNA is segmented and composed of 11 unique individual segments in equimolar amounts (Konica et al., 1976). The segmented nature has been demonstrated by analysis of the RNA by polyacrylamide gel electrophoresis and by contour length determination of human rotavirus species following analysis by electron microscopy. Konica et al (1976) have shown a distribution in length suggesting 4 size classes for the RNA molecules. Polyacrylamide gel electrophoresis of deproteinized rotavirus RNA visualized by staining with ethidium bromide has confirmed the existence of these 4 size classes of molecules. Molecular weight estimations for the individual RNA species have been made by comparison with the dsRNA genome of reovirus (see Fig. 2). The combined molecular weight of the rotavirus genome is thought to be approximately 12×10^6 daltons (Rodger et al., 1975).

Following polyacrylamide gel electrophoresis, comparison of the genomic RNA profiles of rotavirus isolates from the same and different animal species has revealed a high level of variation in the mobilities of corresponding genome segments, even though the overall migrational pattern of the 4 size classes of RNA remains conserved (Verly & Cohen, 1977; Konica et al., 1978b). Rotavirus isolates from the same animal

Figure 2. Comparative Genome Profile Analysis of Two Rotavirus Isolates With Reovirus Serotype 1 (Lang Strain).



Viral RNA was 3' end labelled as described in Materials and Methods. Fractionation was on a 7.5% polyacrylamide gel (Laemli, 1970), electrophoresis was at 20 mA for 16 hrs at 4°C.

Tracks A = U.K. tissue culture adapted calf rotavirus

Tracks B = O.S.U. tissue culture adapted porcine rotavirus

Tracks C = Reovirus serotype 1 (Lang strain)

Rotavirus genome segments are numbered from 1 to 11.

In this gel system the order of reovirus genome segments M1, M2 and S3, S4 are reversed (Sharpe et al., 1978). The reovirus genome segments range in size from $2.8 - 0.6 \times 10^6$ daltons (Joklik, 1974).

species which are serologically similar by complement fixation analysis, have been shown to have different genome profiles. A number of groups have suggested that the analysis of variations in genome segment mobility may be of value in molecular epidemiological studies (Rodger & Holmes, 1979; Kalica et al., 1976). In most cases wild isolates of rotaviruses do not grow in tissue culture, ^{and} Athis fastidious property has curtailed the production of discriminating serological reagents, thus preventing the wide application of serology in epidemiological studies. In contrast, it has been possible to obtain dsRNA profiles from a majority of cases of acute viral gastroenteritis. A widely held view is that simple genome profile analysis may give a much more exacting identity to a particular isolate, because it is based on the relative differences in 11 criteria, than any of the serological tests currently employed.

As a result, molecular epidemiological studies of genome segment variation by one dimensional separation of dsRNA on polyacrylamide gels have been used on large and diverse collections of rotavirus isolates (Espejo et al., 1980b; Rodger & Holmes, 1979; Schnagl et al., 1981). Attempts to correlate individual genome segments to particular antigenic functions by comparing genome profile differences or similarities between serologically distinct isolates have not been very successful, except in the one case of human rotavirus isolates which appear to fall into 2 distinct groups in terms of the relative migrational patterns for their genome segments 10 and 11 (Espejo et al., 1980b). Even this work was initially confounded by error (Konica et al., 1981), although it was eventually shown that ELISA subtypes may correlate with these differences in particular genome segments.

The problem with using differences in electropherotypes as criteria for classifying wild isolates is that the structural basis for these differences is not understood. It is, therefore, very unsatisfactory to make definite conclusions regarding similarities or differences between rotavirus isolates on the basis of an uncharacterized phenomenon. There is an obvious need to investigate the structural basis of these variations.

Rotaviruses have been demonstrated to have an in vitro transcriptase property similar to that shown for reovirus (Skehel & Joklik, 1969); this activity is initiated by enzymic or chemical removal of the outer-shell layer (Cohen, 1977). The transcription products have been shown to confer 100% resistance to ribonuclease when hybridized to an excess of strand separated genomic dsRNA indicating them to be bona fide copies of all 11 genome segments. In vitro transcribed ³²P-labelled ssRNA has been used as a specific hybridization probe to study the genetic relatedness by annealing with genomic dsRNA of homologous or heterologous rotavirus (Matsuno & Nakajima, 1982). The human tissue culture adapted strain Wa was used in this study and it was shown that transcripts of this virus hybridized with the genome of another human rotavirus isolate to the level of 88-100%. No transcripts of Wa appeared to hybridize with the dsRNA from Nebraska calf diarrhoea virus or SAll. Nebraska calf diarrhoea virus and SAll appeared to be 30% related by this method of analysis. This was the first work to appear in the literature attempting to take analysis of rotavirus ds RNA beyond the simple genome profile stage.

Very little is known about viral RNA synthesis during the infectious

cycle of the virus. Production of progeny dsRNA has been investigated by pulse labelling with ^{32}P for sequential periods throughout infection and it was found that (McCrae & Faulkner-Valle, 1981), all genome segments appear to be synthesized at the same rates throughout the infectious cycle. More detailed studies of the kinetics of genome segment synthesis are required before definitive conclusions can be made.

(v) Polypeptides

Many attempts have been made to characterize rotavirus polypeptides in virions and in infected cells (Thouless, 1979; Kalica & Theodore, 1979; Matsuno & Mukoyama, 1979; Espejo et al., 1981; Estes et al., 1981; Urquidi & Esparza, 1981; Smith et al., 1980; Killen & Dimmock, 1982). There is particular interest in identifying the antigenically important proteins which are involved in virus neutralization, infectivity and haemagglutination. Initial studies were greatly restricted by the reluctance of 'wild' isolates to grow in tissue culture, although recently a number of reports have described the successful propagation of fastidious human rotavirus isolates in rotary cell cultures in the presence of trypsin (Sato et al., 1981; Urasawa et al., 1981; Taniguchi et al., 1982).

Both SAll (Mason et al., 1980) and the U.K. tissue culture adapted calf rotavirus (McCrae & Faulkner Valle, 1981) have been adopted by different groups as model systems to help understand the basic virology of rotavirus infection. Unfortunately the use of different tissue culture cell lines, different polyacrylamide gel conditions and 3 different types of nomenclature has resulted in much confusion in the

literature (Killen & Dimmock, 1982). The least contradictory system involves the U.K. tissue culture adapted bovine rotavirus grown in African Green Monkey Kidney Cells (BSC-1).

McCrae & Faulkner-Valle (1981) showed from single-step growth analysis that this virus has a rapid replication cycle at 37°C with maximum virus yield occurring at 10-12 hrs post infection. Protein synthesis was followed in infected cells by incorporation of ³⁵S methionine and resolution of the products on polyacrylamide gels. This method of analysis showed that cellular protein synthesis is rapidly inhibited by the virus and completely switched off at 4 hrs post infection. At least 16 virus induced polypeptides have been detected in infected cells ranging in molecular weight from 20K to 120K. There appears to be no classical early-late switching in the synthesis of these viral proteins although there are some quantitative variations in their rates of synthesis.

Post translational changes were observed for 3 viral proteins following pulse-chase analysis. Experiments making use of tunicamycin, which inhibits glycosylation, and ³H glucosamine showed that 2 of these changes were due to glycosylation. They also allowed the identification of the non-glycosylated precursors of these two glycoproteins. Precursor-product relationships between non-glycosylated and glycosylated proteins have been found for two corresponding sets of translation products for SAll (Mason et al., 1980).

In order to establish which infected cell polypeptides correspond to the virus induced non-structural proteins a number of studies comparing infected cell polypeptides with proteins from purified

virions have been made. With the U.K. tissue culture adapted bovine rotavirus-BSC 1 cells as a model system only one protein has been unequivocally identified as non-structural. Using antisera to double-shelled particles, Ericson et al., (1982) have reported at least 3 non-structural polypeptides in SAll which were not immunoprecipitated from infected cells.

The location of structural polypeptides in the virion has been attempted making use of natural single-shelled particles and by using chemical or enzymic methods to remove the outer shell of the intact particles to produce 'cores'. Because of the variety of treatments and conditions used to produce single-shelled particles many different and conflicting conclusions have been reached regarding the specific location of structural polypeptides in the virus particle. Studies with the bovine rotavirus (McCrae & Faulkner-Valle, 1981) have allowed 4 outershell and 6 inner shell polypeptides to be tentatively identified.

The number of primary genes products seen in infected cells is thought to total 11 (McCrae & Faulkner-Valle, 1981). This is equal to the number of genomic viral RNA segments and suggests that these may be monocistronic as has been demonstrated for the 10 reovirus genome segments (McCrae & Joklik, 1978). Double-stranded RNA genome segment coding assignments have been established by in vitro translation for the U.K. tissue culture adapted bovine rotavirus (McCrae & McCorquodale, 1982). Similar information for SAll is so far incomplete and has only been accomplished for 8 of the genome segments (Sp 1-6, Smith et al., 1980; Sp10 and 11, Dyall-Smith & Holmes, 1981). Both sets of evidence confirm that each genome segment codes for only one polypeptide.

Additionally Dyall Smith & Holmes (1981) have made coding assignments for the Sp10 and Sp11 RNA's of the long and short profile human rotavirus isolates. The long genome profile assignments agreed with SAll and the tissue culture adapted calf rotavirus, the 'short' isolate had the opposite assignments indicating that it's Sp11 RNA probably corresponds to the Sp10 RNA's of the other isolates.

Only one analysis of virus induced polypeptides from rotaviruses infecting different animal species has been made (Thouless, 1977). Although this ^{study} suffered from problems with incomplete host protein synthesis shut off it showed that there is a high level of polymorphism in terms of molecular weight differences for corresponding polypeptides of rotavirus isolates from different animal species - analogous to the heterogeneity seen from genome profile analyses.

(vi) Genetic Studies

The application of genetic studies in other segmented genome virus systems has proved to be invaluable in elucidating RNA-protein coding assignments and also in relating biological functions to viral polypeptides (Palese, 1977; Mustoe et al., 1978).

Different rotavirus isolates can be distinguished by an array of biochemical and serological tests. The first evidence showing that rotavirus isolates can undergo genetic recombination (reassortment) was demonstrated in vitro by Matsuno et al. (1980) with the Nebraska calf diarrhoea virus (NCDV) and SAll. These two tissue culture adapted isolates can be clearly distinguished by serum neutralization, haemagglutination inhibition and also on the basis of electrophoretic

migration of their RNA segments. Reassortment was brought about by mixedly infecting MA104 (Rhesus Monkey Kidney) cells at low multiplicity with NCDV and U.V. irradiated SAll virus. After 24 hr incubation at 37°C reassortants were selected for by the growth of the virus under anti NCDV serum.

One reassortant was isolated, ^{and} from electrophoresis of its RNA it appeared to have at least 5 genome segments derived from the NCDV parent. Serum neutralization tests showed that the recombinant was, as expected neutralized by antiserum to SAll and not by antiserum to NCDV, also the HA activity was inhibited by antiserum to SAll.

Recently there have been two independent reports of the isolation and characterization of temperature sensitive (ts) mutants of bovine rotavirus (Greenberg et al., 1981; Faulkner-Valle et al., 1982). Faulkner-Valle et al. (1982) have demonstrated 5 recombinational groups (from a theoretical total of 11) which fell into 2 classes; efficiency of plaquing (E.O.P.) mutants which still produce virus at the non-permissive temperature, and E.O.P./yield mutants which show a vastly reduced virus yield at the non-permissive temperature. The 5 recombinational groups have been further characterized by upshift and downshift experiments and by analysing RNA and protein synthesis to determine the function or functions defective at the non-permissive temperature. As yet the gene location of the bovine rotavirus ts mutations have not been determined.

Greenberg et al. (1981) reported the existence of 4 recombinational groups amongst ts mutants of the U.K. tissue culture adapted calf rotavirus. These were all E.O.P. and not yield mutants.

Temperature sensitive mutants will be of considerable value in the manipulation of rotaviral genome segments to construct a variety of reassortants which may reveal the genetic determinants of virulence and hence allow the development of live attenuated strains for use as vaccines in man and animals. The study of ts mutations should also contribute to the understanding of the molecular biology of rotavirus replication.

By genotypic analysis of a number of human-bovine rotavirus reassortants it was possible by a process of elimination to determine the genes that were always present in viruses that were specifically neutralized by human rotavirus antiserum or bovine rotavirus antiserum. From these studies Greenberg et al. (1981) have assigned the neutralization antigen to the ninth genome segment in human rotavirus. Similarly the genes coding for the viral proteins reacting in enzyme-linked immunosorbent assay (ELISA) and immune adherence haemagglutination (IAHA) tests to give subgroup types have been identified (Kapikian et al., 1981). Reassortants were recovered that exhibited the IAHA specificity for the human virus and the neutralizing specificity for the bovine virus. The reciprocal reassortants were also recovered demonstrating that these two characteristics were dissociable during reassortment and therefore encoded by different genome segments.

The occurrence of gene reassortment between rotavirus isolates from different animal species in the wild has not been demonstrated. The very marked differences in genome segment patterns observed for different rotavirus isolates are similar to the type of differences seen with natural reassortants of influenza which have been linked

to the emergence of new pandemic strains of this virus (Scholtissek et al., 1978a). In view of the already demonstrated large number of rotavirus serotypes, the occurrence of gene reassortment in the wild would have a profound influence on the strategies that could be adopted for controlling this very important infectious agent by the use of vaccines.

SECTION II

Variation of Influenza A Virus

(i) Introduction

Influenza viruses differ in many respects from the rotaviruses. However, these two representatives of distinct virus families share the property of having segmented RNA genomes. Unlike the rotaviruses, influenza type A viruses have 8 negative-sense single-stranded genome segments (Palese & Young, 1982; Webster et al., 1982). Rotaviruses being relatively recently discovered pathogens are as yet only poorly characterized. In contrast, influenza A viruses are the most extensively studied group of segmented-genome RNA viruses.

The large number of rotavirus "serotypes" (Thouless et al., 1977; Thouless et al., 1978) and the variations observed by genome profile analysis of rotavirus wild isolates (Rodger & Holmes, 1979; Espejo et al., 1980a) have been considered to be analogous to the epidemiological properties of naturally occurring influenza virus isolates. Therefore details of the epidemiological properties of the influenza viruses and the molecular mechanisms of their variation may assist in providing an understanding of rotavirus variation. Influenza viruses can be classified into 3 types - A, B, and C; of these type A influenza has been demonstrated to be responsible for the major influenza pandemics in man this century. Consequently type A influenza has been more extensively studied than types B and C. Influenza A virus was first isolated from pigs in 1931 (Shope, 1931) and subsequently from humans two years later (Smith et al., 1933). Type A influenza s have

also been isolated from horses, seals and birds. In non-pandemic years there is, like rotavirus^{infection} (Davidson et al., 1975) a seasonal incidence of the disease with more human cases reported during the winter months.

(ii) Structure and Properties of influenza A virus

Influenza virus particles are highly pleomorphic having both spherical and filamentous forms (Choppin et al., 1960). These have been shown to be enclosed by an outer lipid envelope, derived from the plasma membrane of the host cell (Klenk & Choppin, 1969, 1970) from which the haemagglutinin and neuraminidase antigens project. These surface antigens, when observed by E.M., are approximately 10 nm in length attached at one end by short hydrophobic amino acid sequences (Skehel & Waterfield, 1975; Gething et al., 1980), ^{and} both of the surface antigens are glycosylated (Wrigley, 1979). Haemagglutinin is the major surface antigen and is thought to account for about 25% of the virion protein (Compans et al., 1970; Schulze, 1970; Skehel & Schild, 1971).

Inside the lipid envelope lies a shell of protein, the matrix or M protein, which is believed to be of structural importance (Landsberger et al., 1978). Within the matrix shell the 8 negative sense single stranded RNA molecules are found in close association with one major protein, the nucleocapsid protein (NP) and 3 high molecular weight proteins P1, P2 and P3, which are required for RNA replication and transcription (Webster et al., 1982). Three virus specified non-structural proteins are found in infected cells; the biological functions of these have not been determined (Lazarowitz et al., 1971; Skehel, 1972; Lamb & Choppin, 1981).

The genome of influenza A virus codes for at least 8 polypeptides, these include besides three P proteins the haemagglutinin and neuraminidase antigens, matrix protein, nucleoprotein and the non-structural protein NS1. Three different methods have been independently developed for the assignment of viral functions and/or proteins to the 8 influenza RNA genome segments (Scholtissek et al., 1976; Palese, 1977; Inglis et al., 1977).

(iii) Epidemiological History

Type A influenza infections follow a pattern in which there is a major pandemic followed by a number of years of much lower incidence. This pattern can be correlated with changes in the viral surface antigens. Influenza A isolates are divided into "subtypes" on the basis of the serological characteristics of their surface antigens, haemagglutinin (HA) and neuraminidase (NA).

The first human influenza A virus isolated was subsequently classified as belonging to the H1N1 subtype. Serological evidence indicates that this subtype was prevalent throughout the world until 1957. Originally the earliest influenza isolates were designated H0 subtypes, ^{but} later evidence (Schild, 1970; Baker et al., 1973; Schild et al., 1980) showed H0 and H1 to be antigenically related and they are now classified as the same subtype. In 1957 followed the largest influenza pandemic since 1918, the increase in incidence of the disease coincided with the emergence of Asian flu. This was an H2N2 subtype which remained circulating until 1968. Immunity to the H1N1 subtype did not afford protection to the new H2N2 subtype. In 1968 another new subtype, Hong Kong (H3N2) emerged, again giving rise to a major pandemic. The H1N1 subtype re-appeared in the population in 1977 as

"red flu" causing a major epidemic amongst members of the population born after 1957 who had not previously been exposed to this virus. Currently variants of both H1N1 and H3N2 subtypes co-circulate.

(iv) Mechanisms of Antigenic Variation

Antigenic Shift

The term "antigenic shift" has been used to describe the emergence of new influenza A virus subtypes. A number of mechanisms have been suggested to account for antigenic shift. One proposal is that the new influenza subtypes may arise by the infection of man with an influenza virus normally associated with a different animal host (Periera, 1969).

This mechanism is not well supported by the epidemiological evidence. Only one such case has been documented; at Fort Dix in 1976 when swine influenza which is endemic for certain pig populations was transferred to military recruits. Five hundred men became infected and one man died (Kaplan & Webster, 1977). The virus isolated was thought to be very similar to that presumed to be the cause of the 1918 pandemic. This coupled to the fact that the majority of the population was susceptible to infection, led to a \$130 million vaccination programme in the U.S. However, despite major concern the Fort Dix swine influenza outbreak did not spread to become an epidemic.

A second proposal to account for antigenic shift is that mutations in the genes coding for the virus surface antigens give rise to changes resulting in new subtypes. A single mutation in an "old" virus might cause the polypeptide chains of either the haemagglutinin or neuraminidase to refold in such a way as to expose completely new antigenic

determinants (see Laver & Webster, 1973). If this were the case then the bulk of the protein in the changed antigenic determinant of the new subtype should be identical with the strain from which it evolved. Laver & Webster (1973) have shown conclusively that the haemagglutinin of the Hong Kong H3N2 subtype is totally unrelated to the haemagglutinin of the Asian H2N2 subtype. Comparative nucleotide sequence studies show there to be only 25% homology between the H3 and H2 genes (Webster and et al., 1982), ^{and} this difference is too large to be accounted for by mutation alone. The lack of intermediate or bridging strains between subtypes (Air, 1981) argues very strongly against mutation as a mechanism for antigenic shift.

The explanation for antigenic shift most strongly supported by the epidemiological evidence is that reassortment of the genes coding for the surface proteins occurs between different virus strains, following mixed infection of a single host (Webster & Laver, 1972a; Laver & Webster, 1972; Laver & Webster, 1973). Influenza virus has a natural host range including mammalian and avian species. Experimental evidence for natural mixed infections with more than one strain of influenza has been reported (Desselberger et al., 1978). Influenza reassortants have also been constructed in the laboratory following mixed infection of cells in tissue culture and selection of reassortant progeny using antiserum (Palese, 1977). The most convincing direct evidence for this hypothesis comes from biochemical results obtained from analysing the appearance of the H3N2 Hong Kong subtype from the prevalent H2N2 (Laver & Webster, 1973; Scholtissek et al., 1978a). These suggest that the H3 haemagglutinin gene was transferred from a duck Ukraine strain to the prevalent H2N2 strain by recombination

to generate the new H3N2 subtype.

The re-emergence of the H1N1 subtype in 1977 can be explained by reassortment of both haemagglutinin and neuraminidase genes. However, the biochemical evidence in this particular case does not support the reassortment hypothesis (Nakajima et al., 1978; Scholtissek et al., 1978; Kendal et al., 1978). Oligonucleotide fingerprinting of this subtype suggests that it is virtually identical to the prevalent H1N1 strain that was circulating up to 27 years previously. Re-emergence of the H1N1 subtype suggests that re-assortment is not the only way by which new subtypes can be generated, ^{and} other mechanisms such as virus latency or persistent infection may contribute to this process. Young & Palese (1979) have demonstrated that some of the H1N1 isolates have genes derived from the cocirculating H3N2 subtype, providing more evidence for natural reassortment of genes between human influenza viruses. However no satisfactory mechanism exists to explain the phenomenon of re-emergence.

From the many thousands of influenza A isolates studied so far there are now known to be at least thirteen distinct HA subtypes (Hinshaw et al., 1982) and nine NA subtypes (Blok , 1981). Of these only three HA subtypes and two NA subtypes have been demonstrated as occurring in human isolates.

Antigenic Drift

In addition to the major changes in the influenza virus surface antigens brought about by antigenic shift, there are many small antigenic differences which accumulate in the surface antigens of

strains belonging to the same subtype. These small changes are termed "antigenic drift". After a pandemic, immunity in the population is high; therefore any virus variants that may arise would have a growth advantage. When antigenic drift has been progressing for some time, the effects of the accumulated small changes allow re-infection of individuals previously infected with the same virus subtype. The mechanism of antigenic drift is thought to involve the selection by the immune system of mutant viruses with slightly altered antigenic determinants. "Antigenic drift" has been demonstrated in the laboratory (Laver & Webster, 1968) by growing influenza A virus in the presence of limiting dilutions of specific antibody.

(v) Variation in Haemagglutinin

Haemagglutinin is the most abundant surface antigen of influenza. It has been demonstrated to have a number of important biological functions; it is involved in the attachment of virus to cells (Klenk et al., 1975) and the stimulation of neutralizing antibody (Laver & Kilbourne, 1966). In infected cells HA is translated as a single polypeptide precursor (Lazarowitz et al., 1971), which has a hydrophobic signal sequence at its N-terminus which allows it to be co-translationally translocated through the membrane of the endoplasmic reticulum. Glycosylation also occurs during this process. After removal of the signal peptide the completed molecule remains anchored in the membrane by its hydrophobic C-terminus. Cleavage of the mature HA (Laver, 1971) is necessary for full infectivity of virions (Klenk & Rott, 1980). In this process one or more amino acid residues are removed, depending on the HA subtype, to generate two polypeptide chains HA_1 (m.w 50K) and HA_2 (m.w 25K).

From crystallographic studies the three-dimensional structure of the Hong Kong (H3) haemagglutinin has been determined (Wilson et al., 1981). The HA glycoprotein has been shown to be a trimeric structure (Wiley et al., 1977; Wilson et al., 1981) with four antigenic sites (Gerhard et al., 1981; Wiley et al., 1981; Webster & Laver, 1980). These antigenic sites were predicted using monoclonal antibodies to probe natural and laboratory generated antigenic variants of the Hong Kong virus.

To date the complete nucleotide sequences of the haemagglutinin(HA) genes from 4 influenza subtypes H1, 2, 3 and 7 have been obtained from molecularly cloned cDNA copies (Winter, Fields & Brownlee, 1981; Gething et al., 1980; Hiti, Davis & Nayak, 1981; Verhoeven et al., 1980; Fang et al., 1981; Porter et al., 1979). Additionally the complete nucleotide sequences of several Hong Kong H3 strains have been determined (Sleigh et al., 1980; Min Jou et al., 1980; Verhoeven et al., 1980). Comparison of the nucleic acid and amino acid sequences of haemagglutinin from the different subtypes has revealed a very high degree of divergence (Webster et al., 1982),^{and} in all comparisons the HA2 subunits appear to be more conserved than their HA1 counterparts.

Sequence relationships among the 3' terminal 350 nucleotides for the haemagglutinin vRNA of the 13 known subtypes of influenza A have been determined (Hinshaw et al., 1982). This region of the haemagglutinin genes codes for approximately 33% of the HA1 polypeptide including the 5' non coding region of the mRNA and the signal peptide coding sequence. Comparison of the nucleic acid and predicted amino acid sequences for the haemagglutinin genes and proteins of the different subtypes of

influenza has revealed them to be very different. However, the presence and relative position of cysteine residues and some key amino acids (gly, thr, val, pro, asp and glu) remains conserved as shown by Figure 3. This underlying level of conservation indicates that all known 13 subtypes may have evolved from a common ancestor. Figure 4 is a dendrogram illustrating the sequence relationships between the HA N-terminal amino acid sequences of 32 virus isolates representing the 13 known subtypes.

From this figure it can be seen that subtypes can be clearly distinguished on the basis of their amino acid sequences, differing by at least 20% with up to 75% difference between the H1 and H3 subtypes. Subtypes appear to be strictly delineated, except in one instance where extension of the data have revealed differences in the N-terminal region of HA1 of two isolates from subtypes H7 (see Fig. 4) of almost 20% (Webster et al., 1982). However the absence of bridging strains suggests that "antigenic" drift may not proceed indefinitely. In view of this evidence the evolutionary origin of the 13 subtypes remains a puzzle. Their existence could be accounted for by antigenic drift over a long period of time.

The sequence analysis of haemagglutinin genes from virus isolates of the same H3 subtype has provided data to allow evaluation of the molecular basis of antigenic drift. Comparative sequence analysis of the haemagglutinin genes of strains of the H3 subtype isolated 11 years apart has shown there to be a 4.7% difference in nucleic acid sequence representing approximately 10% difference in protein sequence. Most changes occurred in variable regions of the HA₁ polypeptide

Figure 3. Comparison of the Amino Acid Sequences Predicted
From the Nucleotide Sequence of the N Terminal Regions of the
Thirteen Influenza Haemagglutinin Subtypes.

H1 (H0,H1)	Met Lys Ala Asn Leu Ile Val Leu Ile Cys Ala Leu Ala Asp Arg Asp Thr Ile Cys Ile Glv Tyr His Ala Asn <u>Agn Ser Thr</u>	11
H2 (H2)	Met Ala Ile Ile Tyr Leu Ile Leu Leu Phe Thr Ala Val Arg Gly Asp Gln Cys Ile Cys Ile Glv Tyr His Ala Asn <u>Agn Ser Thr</u>	21
H3 (Hav5)	Met Glu Arg Val Val Leu Leu Ala Met Ile Ser Leu Val Lys Ser Asp Gln Ile Cys Ile Glv Tyr His Ala Asn <u>Agn Ser Thr</u>	30
H11 (Hav3)	Met Lys Val Leu Phe Ala Ile Ile Ile Cys Ile Arg Asp Asp Gln Ile Cys Ile Glv Tyr Leu Ser Asn <u>Agn Ser Thr</u>	39
H6 (Hav6)	Met Ile Asn Ile Ile Val Val Ile Ile Cys Ile Ala Thr Gly Asp Ser Asp Gln Ile Cys Ile Glv Tyr His Ala Asn <u>Agn Ser Thr</u>	48
H13	Met Asp Ile Arg Bar Ile Val Ile Ser Bar Thr Thr Val Gln Asp Arg Ile Cys Ile Glv Tyr Leu Ser Thr <u>Agn Ser Thr</u>	57
H8 (Hav8)	Met Glu Lys Phe Ile Ala Ile Asn Leu Ala Ser Thr Asn Ile Arg Trp Asp Arg Ile Cys Ile Glv Tyr Gln Ser Asn <u>Agn Ser Thr</u>	66
H9 (Hav9)	Met Glu Thr Lys Ala Ile Ile Ala Ile Leu Leu Met Val Thr Ala Asn Ala Asp Asp Gln Ile Cys Ile Glv Tyr Gln Ser Thr <u>Agn Ser Thr</u>	75
H12 (Hav10)	Met Glu Lys Phe Ile Ile Leu Ser Thr Val Leu Ala Ala Ser Phe Ala Trp Asp Ile Cys Ile Glv Tyr Gln Thr Asn <u>Agn Ser Thr</u>	84
H7 (Hav7)	Met Asn Thr Gln Ile Leu Val Phe Ala Cys Val Leu Ile Lys Ala Lys Glg Asp Ile Cys Ile Glv His His Ala Val Ala <u>Agn Ser Thr</u>	93
H10 (Hav2)	Met Tyr Lys Ile Val Val Leu Val Phe Gly Ala Asn Cys Leu Asp Asp Gln Ile Cys Ile Glv His His Ala Val Pro Asn Gly <u>Agn Ser Thr</u>	102
H4 (Hav4)	Met Leu Ser Ile Thr Ile Leu Phe Leu Leu Ile Ala Cys Glv Ser Ser Gln <u>Agn Ser Thr</u> Cys Leu Glv His His Ala Val Ser <u>Agn Ser Thr</u>	111
H3 (H3)	Met Lys Thr Ile Ile Ala Ile Ser His Ile Phe Cys Leu Val Leu Glg Asn Tyr Ile Val Pro Gly Asp <u>Agn Ser Thr</u> Ala Ile Thr Ile Ile Leu Glv His His Ala Val Pro <u>Agn Ser Thr</u>	120

H1	Thr Val Asp <u>Thr</u> Val Leu Glu Lys <u>Agn Val Thr</u> Val Thr His Ser Val Asn Leu Leu Glu Asp Ser His Asn Gly Lys Leu Ile Arg Leu Lys Ile Ala Pro Leu Glu Cys Ile	71
H2	Lys Val Asp <u>Thr</u> Ile Leu Glu Arg <u>Agn Val Thr</u> Val Thr His Ala Lys Asp Ile Leu Lys The His Asn Gly Lys Leu Ile Cys Ile Leu Asn Glu Ile Pro Pro Leu Glu Leu Glu Cys Ile	80
H3	Gln Val Asp <u>Thr</u> Ile Met Glu Lys <u>Agn Val Thr</u> Val Thr His Ala Glu Asp Ile Leu Glu The His Asn Gly Lys Leu Ile Cys Ile Leu Asn Glu Ile Val Lys Pro Leu Ile Leu Arg Asp	89
H11	Lys Val Asp <u>Thr</u> Ile Ile Glu Ser <u>Agn Val Thr</u> Val Thr Ser Ser Val Glu Leu Val Glu Asn Gly Lys Leu Ile Cys Ile Leu Asn Glu Ile Val Lys Pro Leu Ile Leu Gly Asp	98
H6	Gln Ile Asp <u>Thr</u> Ile Leu Glu Lys <u>Agn Val Thr</u> Val Thr His Ser Val Glu Leu Leu Asn Glu Ile Gly Glu Arg Phe Cys Ile Ile Leu Lys Ile Asn Pro Leu Asp Leu Lys Glu Cys Ile	107
H13	Lys Val Asp <u>Thr</u> Ile Leu Glu Asp Val Pro Val Thr Ser Ser Ile Asp Leu Val Glu The <u>Agn His Thr</u> Cys Ile Glu Asp Gly Ile Ser Ser Pro Val His Leu Glu Asp	116
H8	Thr Val Asn <u>Thr</u> Ile Glu Glu Asn Val Pro Val Thr Cys Ile His His Asn Val Pro Asn His Cys Asn Thr Asp Ile Glu Asp Pro Leu Glu Arg Asp	125
H9	Thr Val Asp <u>Thr</u> Leu Thr Glu Ser Asn Val Pro Val Thr His Thr Lys Glu Leu Leu His Asn Gly His Leu Asp Ile Glu His Pro Leu Val Asp	134
H12	Thr Val Asn <u>Thr</u> Ile Ser Ser Asn Val Val Pro Val Thr His Glu Leu Val Val His Asn Gly His Leu Asp Ile Glu His Pro Leu Val Asp	143
H7	Lys Val Asp <u>Thr</u> Leu Thr Glu Arg Gly Ile Glu Val Val <u>Agn Ala Thr</u> Cys Ile Glu Thr Val Glu Thr Ala Asn Ile Glu Ile Cys Ile Glu Arg --- Pro Thr Asp Leu Glu Cys Ile	152
H10	Ile Val Lys <u>Thr</u> Leu Thr Asp Glu Glu Val Val Thr Ala Cys Glu Leu Val Glu Ser Lys Thr Leu Asp Arg Leu Cys His Lys Glu Arg Lys --- Tyr Lys Asp Leu Glu Cys Ile	161
H4	Met Val Lys <u>Thr</u> Leu Thr Asp Glu Glu Val Val Thr Ala Cys Glu Leu Val Glu Ser Glu His Leu Pro Glu Leu Cys Pro Asp Pro Leu Lys --- Leu Val Asp Glu Cys Ile	170
H3	Ile Val Lys <u>Thr</u> Ile Thr Asn Asp Glu Ile Glu Ile Cys <u>Agn Ala Thr</u> Cys Leu Val Glu Ser Ser Thr Glu Ile Ile Asn Pro His Arg --- Ile Leu Asp Glu Cys Ile Asp	179

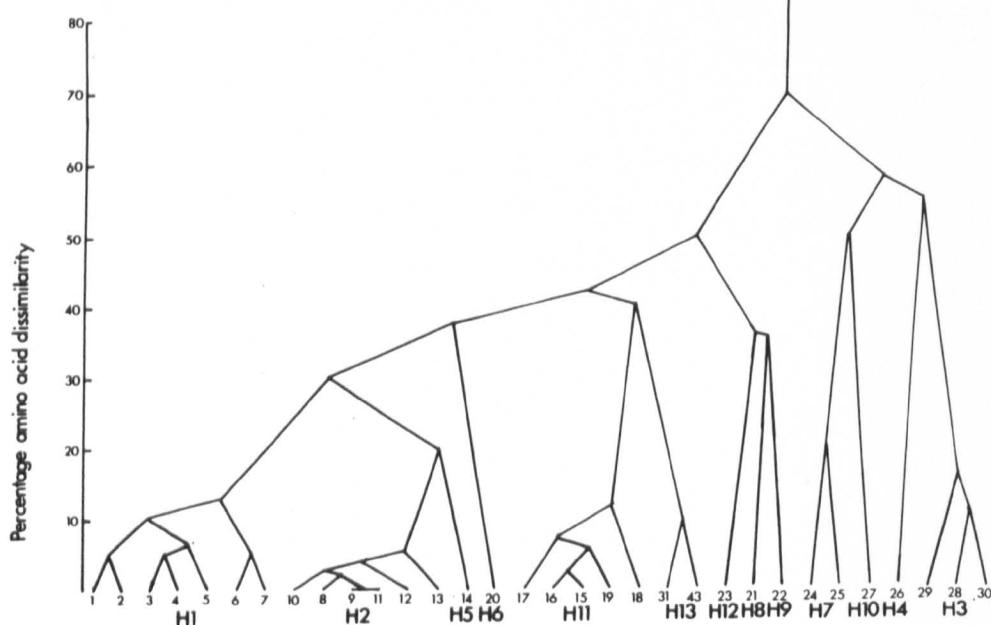
H1	Cys Asn Ile Ala Gly Trp Leu Leu Glu Asn <u>Thr</u> Glu Cys Asp Pro Leu Leu Pro Val Arg Ser Trp Ser Tyr Ile Val Glu The Pro Asn Ser Glu Asn	1
H2	Cys Ser Ser Ala Gly Trp Leu Leu Glu Asn Pro Glu Cys Asp Arg Leu Leu Ser Val Pro Glu Trp Ser Tyr Ile Val Glu Asn Pro Arg Asp Gly Leu Cys Tyr Pro Gly Ser	10
H5	Cys Ser Val Ala Gly Trp Leu Leu Glu Asn Pro Met Cys Asp Arg Leu Leu Val Pro Glu Trp Ser Tyr Ile Val Glu Asn Pro Asn Pro Ile Asn Gly Leu Cys Tyr Pro Gly Ser	19
H11	Cys Ser Phe Ala Gly Trp Ile Leu Glu Asn Pro Met Cys Asp Arg Leu Leu Ile Gly Ile Val Pro Glu Trp Ser Tyr Ile Val Glu Asn Pro Ile Asn Gly Leu Cys Tyr Pro Gly Ser	28
H6	Cys Thr Ile Ala Gly Trp Ile Val Glu Asn Pro Glu Cys Asp Arg Leu Leu Ser Val Pro Glu Trp Ser Tyr Ile Val Glu	37
H13	Cys Ser Bar Phe Ala Gly Trp Ile Val Glu Asn Pro Glu Cys Asp Arg Leu Leu Val Glu Asn Arg Glu Trp Ser Tyr Ile Val Glu	46
H8	Cys Lys Ile Ala Glu Ile Tyr Glu Asn Pro Lys Cys Asp Ile His Leu Lys Val Ile Val Ile Glu Trp Ser Tyr Ile Val Glu Arg Pro	55
H9	Cys Thr Ile Ala Gly Leu Ile Tyr Glu Asn Pro Ser Cys Asp Ile Leu Leu Glu Ile Glu Trp Ser Tyr Ile Val Glu	64
H12	Cys Leu Leu Glu Ile Leu Ile Glu Asn Pro Lys Cys Asp Leu Tyr Leu Asn Gly Arg Glu Trp	73
H7	Cys Glu Asn Leu Glu Gly Thr Ile Ile Glu Asn Pro Glu Cys Asp Ile Leu Glu Phe --- Cys Leu Asp Leu Ile Ile Glu Arg Arg Glu Gly Asn Asn --- Ile Cys Tyr Pro Gly Lys Phe	82
H10	Cys His Pro Ile Gly Ile Ile Ile Glu Asn Pro Ala Cys Asp Leu His Leu Thr Gly Arg --- Trp Glu Thr Ile Ile Glu Arg Glu Asn Ser Ile Asn --- Tyr Cys Tyr Pro	91
H4	Cys Asp Ile Val Asn Gly Ala Leu Leu Ile Ser Pro Ser Cys Asp His Leu Asn Gly Asn --- Glu Trp Asp Val Phe Ile Glu	100
H3	Cys Thr Leu Ile Asp Asn Glu Leu Leu Ile Asp Pro His Cys Asp Gly Phe Glu <u>Agn Cys</u> --- Thr Trp Asp Leu Phe Val Glu Arg Ser Lys Ala Phe Ser --- Asn Cys Tyr	109

The sequences shown are H1, A/PR/8/34; H2, A/RI/5/57; H3, A/Memphis/1/71; H4, A/Duck/Alberta/28/76; H5, A/Shearwater/Australia/75; H6, A/Shearwater/Australia/72; H7, A/Turkey/Oregon/71; H8, A/Turkey/Ontario/6118/68; H9, A/Turkey/Wisconsin/1/66; H10, A/Duck/Manitoba/73; H11, A/Duck/Memphis/546/76; H12, A/Duck/Alberta/60/76. The N-terminal amino acid of HA1 (actual or presumed) is arrowed, potential glycosylation sites are underlined. The boxes indicate amino acid residues conserved through all subtypes. The sequences

have been aligned at cystein residues. Symbols:  most variable position,  next most variable position.

Reproduced from Hinshaw et al. (1982).

Figure 4. Dendrogram Showing the Relationships Between the HA₁ N-Terminal Amino Acid Sequences Deduced From Genomic Sequences of 32 Viruses Representing the 13 Known Influenza Haemagglutinin Subtypes.



The sequences were aligned using amino acids that are invariant in certain portions of sequences (amino acid positions from N-terminus of most subtypes; Cys4, Gly6, Thr18, Val26, Cys42, Cys55, Gly63, Pro65, Cys67, Glu81). The percentage sequence differences of all pairwise comparisons of the aligned sequences starting from the N-terminal Asp or corresponding amino acid

were used to calculate the dendrogram. Thus the positions of each bifurcation in the dendrogram indicate the mean sequence difference of the sequences connected through that point. The sequences used in the analysis are: 1, A/NWS/33 (H1N1); 2, A/PR/8/34 (H1N1); 3, A/Bel/42 (H1N1); 4, A/USSR/90/77 (H1N1); 5, A/Loyang/4/57 (H1N1); 6, A/Swine/Iowa/15/30 (H1N1); 7, A/New Jersey/11/76 (H1N1); 8, A/RI/5⁻/57 (H2N2); 9, A/Tokyo/3/67 (H2N2); 10, A/Netherlands/68 (H2N2); 11, A/Berkeley/68 (H2N2); 12, A/Duck/GDR/72 (H2N9); 13, A/Duck/Alberta/77/77 (H2N3); 14, A/Shearwater/Australia/75 (H5N3); 15, A/Duck/England/56 (H11N6); 16, A/Duck/Ukraine/1/60 (H11N9); 17, A/Tern/Australia/75 (H11N9); 18, A/Duck/Memphis/546/76 (H11N9); 19, A/Duck/New York/12/78 (H11N6); 20, A/Shearwater/Australia/72 (H6N5); 21, A/Turkey/Ontario/6118/68 (H8N4); 22, A/Turkey/Wisconsin/1/66 (H9N2); 23, A/Duck/Alberta/60/76 (H12N5); 24, A/Turkey/Oregon/71 (H7N3); 25, A/Equine/Prague/1/56 (H7N7); 26, A/Duck/Alberta/28/76 (H4N6); 27, A/Duck/Manitoba/53 (H10N7); 28, A/Memphis/1/71 (H3N2); 29, A/Black Duck/Australia/702/78 (H3N8); 30, A/Duck/Ukraine/1/63 (H3N8); 31, A/Gull/Mass/26/80 (H13N6); 43, A/Gull/Md/704/77 (H13N6).

From Hinshaw et al. (1982).

(Palese & Young, 1982). Other comparisons within different subtypes have indicated these changes also accumulate; the rate of accumulation appears to be constant at about 5% nucleotide sequence change/20 yrs (Air, 1981; Webster et al., 1982).

The overall conclusions from comparative nucleic acid and protein sequence analysis of the type A haemagglutinins is that underlying the high level of variations observed between subtypes three basic features remain conserved; the location of key amino acids, the hydrophobic carboxyl terminal amino acids of HA₂, and the amino acid sequence at the N-terminus of HA₂.

(vi) Structure and Variation in Neuraminidase

Neuraminidase, like haemagglutinin, can undergo extensive antigenic variation and is also found on the surface of influenza A virus particles. However antibodies to neuraminidase do not protect against infection. Neuraminidase gains its name from an enzymic activity which catalyzes the cleavage of terminal N-acetylneurameric acid (sialic acid) from specific carbohydrate chains in glycoproteins (Gottschalk, 1957). The functional significance of this activity is not understood (Schulze, 1975; Bucher & Palese, 1975) although it may have a role in preventing virus aggregates from forming. The proteolytic cleavage of haemagglutinin to HA₁ and HA₂ appears to be facilitated by the removal of sialic acid from virus particles. This process is thought to operate by exposing the haemagglutinin cleavage site (Schulman & Palese, 1977; Nakajima & Sugiura, 1980).

Neuraminidase unlike haemagglutinin is held in the viral membrane

by a hydrophobic N-terminus (Webster et al., 1982). It can be released with pronase or by solubilizing in detergent (Drzeniek, 1972). The detergent soluble fraction has been shown to have 4 identical polypeptide chains arranged to form a mushroom-shaped molecule with a molecular weight of 240K (Wrigley, 1979; Fields et al., 1981). Hydrophilic mushroom-head structures can be crystallized for some strains of influenza (Laver, 1978).

The complete nucleotide sequence has been determined for two neuraminidase genes representing the two known human subtypes; N1 (Fields et al., 1981) and N2 (Markoff & Lai 1982). Both these studies revealed the neuraminidase gene to be approximately 1,450 nucleotides in length with only one open reading frame coding for a protein of molecular weight 50K. Cross comparison of the sequences of the two subtypes revealed differences (~ 50%) in primary structure.

Comparative partial sequence analysis up to 340 nucleotides from the 3' terminus have been made for eight of the nine known neuraminidase subtypes (Blok, 1981). For all eight subtypes the N-terminal six amino acids are identical while the next six amino acids are conserved in five of the subtypes studied. Beyond this region of twelve conserved terminal amino acids the predicted protein sequences of the eight subtypes diverge dramatically. The nucleotide sequence differences between neuraminidase subtypes are at least as large as those observed between haemagglutinin subtypes. Comparison of the complete amino acid sequences of neuraminidase subtypes N1 and N2 (Markoff & Lai, 1982) has like haemagglutinin subtypes (Air, 1981) revealed conservation of the positions of cysteine residues. Similarly

potential glycosylation sites are also conserved. However, these N1 and N2 genes differ in nucleotide sequence by numerous point mutations as well as by the insertion and/or the deletion of bases.

Comparative partial sequence analysis for the 3' termini of the neuraminidase genes from several isolates within the two human subtypes have been made (Blok & Air, 1980). Within the N1 subtype six isolates and were compared, only 20 single base differences occurred among the first 200 nucleotides. Similarly 26 single nucleotide differences were observed among the five N2 subtypes studied. From this work it appears that antigenic drift in neuraminidase follows the same pattern as that observed in haemagglutinin. Amino acid substitutions occur at a similar rate, and there are regions in the nucleotide sequence which undergo more point mutations than other regions. Similarly there are certain amino acids which are subject to more changes than others.

(vii) Variation in Non-Structural Proteins

There are six influenza virus genes that do not code for surface antigens; variation among these has not been as extensively studied as for the haemagglutinin and neuraminidase genes.

The matrix protein is coded for by RNA segment 7, (this is the most abundant viral polypeptide accounting for between 33% and 46% of the total virion protein (Compans et al., 1970; Schulze, 1970; Skehel & Schild, 1971). The M genes of an H3N2 (Lamb & Lai, 1981) and an H1N1 subtype (Winter & Fields, 1980; Allen et al., 1980) have been molecularly cloned and sequenced. Lamb & Lai (1981) compared the sequences of the M gene from the H3N2 subtype with that of an H1N1

subtype isolated 38 years previously. This work, in agreement with antigenic studies (Schild, 1972), showed the M gene nucleotide sequences to be highly conserved. Partial sequences of cDNA complementary to the 3' terminus of RNA segment 7 from 5 human strains encompassing the three subtypes H1N1, H2N2 and H3N2 have been compared (Hall & Air, 1981). This study suggests that throughout the 43 year period of isolation of these subtypes the primary sequence of genome segment 7 has remained largely conserved. RNA segment 7 was first shown to have the capacity to code for two mRNA's (Allen et al., 1980; Lamb, Lai & Choppin, 1981) and ^{more recently} 3 mRNA's transcribed from RNA 7 have been isolated. Comparison of the M₁ and M₂ proteins of A/PR/8/34 and A/Udorn/72 have shown that the M₁ proteins differ by only 2.8% whereas the M₂ proteins vary to higher degree (11.3 per cent) (Palese & Young, 1982).

The nucleoproteins, along with the matrix proteins of type A influenza isolates, are serologically cross reactive and are used as type-specific antigens to distinguish between influenza type A, B and C viruses. NP is located inside the virions (Compans & Dimmock, 1969) and therefore is not subjected to the same selective pressures exerted by the immune system on the surface antigens. However, minor antigenic differences have been demonstrated between the nucleoproteins of H1N1 and H3N2 subtypes (Schild et al., 1979), and at least 5 different antigenic sites can be resolved in the nucleoproteins of different human influenza A isolates by the use of monoclonal antibodies (Van Wyke et al., 1980). The biological function of NP is unknown although it is found in close association with the eight single stranded RNA segments to form ribonucleoprotein complexes. The NP gene of APR8/8/34

has been cloned and sequenced independently by 2 groups (Winter & Fields, 1981; Van Rompuy et al., 1981) and the NP gene of human influenza A virus strain A/NT/60/68 has recently been cloned and sequenced (Huddleston & Brownlee, 1982). Comparison of the nucleotide sequences of the NP genes from these different subtypes shows that like the M genes, they are largely conserved.

Hall & Air (1981) also made partial sequence comparisons of RNA segment 8 (NS gene) from the same 5 influenza isolates that were compared for the homology of their M genes. The number of nucleotide and amino acid changes observed for both RNA segments 7 and 8 were similar in order of magnitude to the number of accumulated changes brought about in RNA segments 4 and 6, which code for haemagglutinin and neuraminidase, over the same period of time.

Recent studies have shown that RNA segment 8, like RNA segment 7, codes for more than one polypeptide (Lamb & Lai, 1980). 2 mRNA's coding for 2 non-structural polypeptides have been isolated from infected cells. Full length cDNA clones for the NS genes of fowl plague (Porter, Smith & Emtage, 1980) and 2 influenza strains have been sequenced (Baez et al., 1980; Winter et al., 1981). Comparative analysis of the NS genes of A/PR/8/34 and A/Udorn/72 isolated 38 years apart has revealed a nucleotide sequence difference of only 8.8%. The NS genes are only expressed in infected cells and so their products will not come under the same selective constraints on the surface antigens, yet they too, like the M genes appear to accumulate sequence differences at approximately the same rate as the surface proteins undergoing antigenic drift. This is surprising and suggests that the genes for non-surface proteins are subject to selective pressures

which cause random drift-type point mutations to become fixed.

Although the nucleotide sequences of the three P genes are now known, the extent of their variation has not yet been studied as only one representative sequence is available for each. The individual functions of the polymerase proteins have not been well characterized; they have polymerase activity to synthesize cRNA and to produce mature vRNA.

For the non-surface genes studied so far (NP, NS, and M) comparative nucleotide sequence data has revealed no gross differences analogous to the subtype variations of haemagglutinin and neuraminidase. However, this does not preclude these RNA genome segments from undergoing gene reassortment (Young & Palese, 1979). Natural re-assortment or shuffling of these genes giving rise to specific combinations (or gene constellations) whose products can act together in concert may be an important factor in determining the virulence of particular influenza reassortants.

(viii) Conclusion

Despite many differences, both influenza A and rotaviruses share the characteristic of having a multi-segmented RNA genome. Additionally, both groups of viruses have many 'serotypes' and cause epidemic disease on a seasonal basis. These similarities have led to the belief that variation of influenza A virus which has been extensively studied, may provide a good model system for understanding variation amongst rotavirus isolates.

The long term aim of rotavirus research is to produce effective control measures by the use of vaccines. Before such measures can be introduced a comprehensive knowledge of the natural variation of rotaviruses is required. Early attempts at producing a vaccine against influenza proved ineffective because of the inherent variation of this virus.

Similarly, attempts at vaccinating against rotavirus, the scourvax vaccine, have also proved to be ineffective.

The demonstration of antigenic 'shift' and 'drift' together with the detailed structural characterization of influenza by oligonucleotide fingerprinting, hybridization studies and sequence analysis of cDNA clones has allowed an understanding of the mechanisms for creating diversity amongst influenza isolates. It is hoped that the information presented here for influenza may form a background to aid understanding of, and to provide insight into the nature of rotavirus variation.

Section III

Variation in the Reoviruses, Orbiviruses and Rotaviruses

Introduction

Respiratory Enteric Orphan or REOviruses derive their name from the properties of producing asymptomatic enteric or respiratory infections (Joklik, 1974). Reoviruses belong to the genus 'orthoreovirus' which is representative of the Reoviridae family. For classification as members of the Reoviridae virus isolates must conform to criteria based primarily on morphology and genome size and structure. Additionally virions must possess a double-strand → single-strand RNA polymerase that transcribes the ds RNA genome into mRNA. This section is concerned with a review of current knowledge of variation in the two groups of multisegmented genome dsRNA viruses most closely related to rotaviruses; the orthoreoviruses and the orbiviruses.

Variation amongst the reoviridae has not been as extensively studied as variation in influenza A virus. The principal reason for this being that members of the Reoviridae family have not, until recently, been recognized as the causative agents of diseases of major medical importance, however some aspects of variation among the orthoreoviruses and the orbiviruses have been characterized and may be relevant as an aid to understanding rotavirus diversity.

(a) Variation in the Orthoreoviruses

Reoviruses possess a genome consisting of 10 segments of double stranded RNA with an overall molecular weight of 15×10^6 (Shatkin, 1968). They have been isolated from a wide variety of mammalian

species in all parts of the world (Rosen, 1968) and fall into 3 serotypes (Joklik, 1974) on the basis of HAI* and serum neutralization tests. Reoviruses have also been isolated from birds but these isolates appear to be serologically unrelated to the mammalian reoviruses (Kawamura & Tsubahara, 1966). Electrophoretic analysis of both the ds RNA segments and the viral polypeptides have shown considerable heterogeneity among the three serotypes of mammalian reovirus (Ramig, Cross & Fields, 1977). Separation of the 10 ds RNA genome segments has shown them to fall into 3 size classes L (large, 3 segs), M (medium, 3 segs) and S (small, 4 segs) which are generally conserved despite the variation seen between different isolates (Hrdy, Rosen & Fields, 1979). Each genome segment transcribes a unique mRNA, and each of these mRNA's is translated into a primary polypeptide (designated λ 1,2,3; μ 1, 2, NS; σ 1, 2, NS, 3) (Fields, 1982).

Analysis of the relatedness of the genomic RNA's of the three serotypes by hybridization has revealed that isolates of serotypes 1 and 3 are related to each other by 30-50%, whereas serotype 2 isolates are related to those of the other two serotypes by no more than 10% (Martinson & Lewandowski, 1975). The genetic relatedness between the 3 serotypes has been shown by antigenic studies not to be evenly distributed among the 10 genome segments (Gaillard & Joklik, 1980).

The type specific and most unique of all reovirus proteins is σ 1, This protein has a molecular weight of 45,000 (McCrae & Joklik, 1978) and is a minor component of the reovirus outer capsid shell

* Haemagglutination Inhibition

(Smith, Zweerink & Joklik, 1969). It is estimated that there are no more than 24 copies of $\sigma 1$ present per virion. $\sigma 1$ has been demonstrated to be responsible for a number of important biological functions, it elicits neutralizing antibody (Weiner & Fields, 1977), has the haemagglutinin activity (Weiner et al., 1978), is the reovirus cell attachment protein (Lee, Hayes & Joklik, 1981) and is the primary determinant of reovirus virulence (Weiner et al., 1977). Besides $\sigma 1$, 4 other reovirus proteins also show some detectable type specificity $\lambda 2$, $\mu 1c$, $\sigma 2$ and $\sigma 3$ (Gaillard & Joklik, 1980).

Despite these differences, the antigenic determinants on proteins coded by serotype 2, which by hybridization studies appears to be less related to the other two serotypes are no more different than those on the corresponding proteins of serotypes 1 and 3 (Gaillard & Joklik, 1980). This indicates that in most cases the reovirus antigenic determinants are highly conserved, even though the amino acid sequences of the corresponding genes must be different, since there is no more than 10% homology between their nucleic acid sequences.

The S1 genes of the three serotypes have been analyzed in more detail by comparative sequence studies of the 3' ends of both RNA strands (Li et al., 1980). It would be expected that a number of features associated with these terminal regions, for example RNA polymerase and ribosome binding sites, encapsidation signals, would give rise to conservation of the nucleotide sequence. In fact all 3 S1 genes share a region of at least 6 identical nucleotides at the 5' terminus of their plus-sense strands, ^{and} there appears to be much more extensive

homology at their 3' termini. Terminal sequence determination of the ten genome segments of a reovirus type 3 strain has also indicated regions of terminal homology shared by all the genome segments (McCrae, 1981). In this case four bases are shared at the 5' ends of the plus strands and 7 common nucleotides are found at their 3' termini. Further and more extensive structural studies are required before definitive conclusions can be made about the evolutionary relatedness of corresponding genome segments from different reovirus serotypes.

Reoviruses have been demonstrated to undergo reassortment at high frequency on mixedly infecting cells with different serotypes and selecting for reassortants using temperature sensitive mutants (Sharpe *et al.*, 1978). Reoviruses have provided an ideal system for studying the molecular basis for virulence. The 3 serotypes have distinctive disease patterns in mice; by constructing reassortants the viral genes responsible for particular pathogenic properties can be identified (Fields, 1982).

The occurrence of gene-reassortment in the natural environment, and therefore the contribution of this phenomena for generating diversity among the reoviruses has not been established.

(b) Variation in Orbiviruses

Members of the orbiviruses were originally classified in the arbovirus group because their transmission is associated with arthropod vectors (Casals, 1959). The name orbivirus (orbis-ring) was given because of the characteristic large dough-nut shaped capsomeres seen on the surface of virus particles by electron microscopy (Borden *et al.*, 1971).

The orbivirus group encompasses a wide range of viruses pathogenic for man (Colorado tick fever), domestic animals (Bluetongue (BTV) and African horse sickness), and wild animals (EHD).

The most commonly known infectious agents of the orbivirus group are the viruses associated with bluetongue. This disease, which has been referred to as "Malarial Catarrhal Fever of Sheep" (Hutcheon, 1902) was first suggested to be caused by a virus by Theiler in 1906. In addition to sheep, other ruminants including goats and cattle have been shown to be susceptible to BTV. Epizootiological studies have indicated that BTV can be transmitted by at least 2 culicoides species (biting midges) and the virus has also been reported to be transmitted by Melophagus ovinus, the sheep ked (Howell & Verwoerd, 1971). Bluetongue is the most important disease caused by members of the orbivirus genus; it is essentially a disease of sheep and was only recognized when highly susceptible animals of this species were introduced into enzootic areas.

Orbiviruses are grouped together principally on their morphology (Borden et al., 1971); the obscure historical background and the diverse isolation of orbiviruses has led to much confusion over their classification. Gorman (1979) has suggested a good simple system for their classification - see Table 1. Since BTV and African Horse Sickness (AHS) types were the first orbivirus isolates to be recognized as serologically distinct on the basis of complement fixation tests, isolates representative of these types have been classified as groups A and B respectively. Isolates within either group although sharing the group-common complement fixation antigens can be differentiated

Table 1. Orbivirus Serological Groups

Group	Type	Prototype strains
A	1 to 9	African horse sickness
B	1 to 20	Bluetongue
	21 to 28	Epizootic haemorrhagic disease of deer
	29	Eubenangee
	30	Pata
C	31	Tilligerry
	32	Ibaraki
	1	Colorado tick fever
D	2	Eyach
	1	Palyam
	2	Kasba
	3	Vellore
	4	D'Aguilar
	5	Abadina
	6	Nyabira
E	1	Changuinola
	2	Irituia
F	1	Corriparta
	2	Acado
	3	Bambari
G	1	Kemerovo
	2	Baku
	3	Bauline
	4	Cape Wrath
	5	Chenuda
	6	Great Island
	7	Huacho
	8	Lipovnik
	9	Mono Lake
	10	Nugget
	11	Okhotskiy
	12	Seletar
	13	Sixgun City
	14	Tribec
	15	Yaquina Head
	16	Wad Medani
H	1	Warrego
	2	Mitchell River
I	1	Wallal
	2	Mudjinbarry
J	1 to 5	Equine encephalosis
Ungrouped viruses		
		Lebombo
		Orungo
		Japanaut
		Umatilla

on the basis of serum neutralization and are hence termed "serotypes". There are 9 known serotypes of group A (African horse sickness) and 20 serotypes of group B (Blue tongue). The bluetongue virus group has been additionally complicated following the recognition that Epizootic haemorrhagic disease of deer and the Eubanangee group of viruses originally recognized as separate serogroups, are now known to be serologically related to BTV. Strangely despite their reported serological relatedness to BTV, viruses in the Eubanangee 'subgroup' have not been linked with disease. There are now ten recognized orbivirus groups (A → J) characterized by differences in their complement fixation antigens.

BTV has a genome consisting of 10 segments of dsRNA, as have most members of the orbivirus group with the notable exception of Colorado tick fever which has 12. Electrophoretic separation of the dsRNA genome of the serologically related Warrego and Mitchell River viruses has shown considerable differences in their RNA patterns (Gorman et al., 1977). Similarly heterogeneity of genome segment variation has been reported in the Wallal (Gorman et al., 1978), Eubanangee and Bluetongue serological groups (Gorman et al., 1981). The relatedness of different BTV serotypes has been analyzed at the nucleic acid level by cross hybridization of viral mRNA with the complementary, negative sense-strand of the genomic RNA from the virus under test. Duplexes formed in this way were analyzed by polyacrylamide gel electrophoresis (Huismans & Howell, 1973). These studies showed that different BTV serotypes were almost completely unrelated; in the majority of cases only segment 5 showed homology; cross hybridization experiments with 3 strains of BTV type 4 showed

that even within a serotype there was considerable mismatching of corresponding genome segments. In addition cross hybridization between different subgroups (BTV serotype 10 - group A and AHS serotype 3 - group B) showed less than 5% homology. These studies have demonstrated that BTV isolates show considerable differences in nucleic acid homology both within serotypes and between serotypes of the same complement fixation group. These differences between serotypes can be as large as the differences observed between different orbivirus subgroups.

A more recent study also making use of RNA-RNA hybridization has been undertaken to investigate the degree of relatedness between the genome of BTV 20 and 3 other BTV serotypes (Gorman et al., 1981). Heterologous hybrids were not formed indicating there to be no significant homology between the genomes of these viruses. Similarly cross hybridization of BTV mRNA to Eubanangee complementary minus strand genomic RNA showed no re-association. Eubanangee and BTV serotypes are all related by a shared complement fixation antigen (Borden et al., 1971), yet common nucleotide sequences could not be found that may code for this group-specific characteristic. However, antigenic similarity need not lead to nucleic acid homology and vice versa. From the ten orbivirus genome segments only a few are likely to code for antigenically important proteins. These genome segments may have only minor sequences in common since the antigenic sites of proteins may involve only part of the gene. It is therefore possible that there may be only little correlation between nucleic acid sequence homology and the antigenic properties of orbiviruses. This appears to be analogous to reovirus where the 3 mammalian serotypes which are apparently only distantly related by hybridization share most of

their antigenic determinants.

Sugiyama et al. (1981) have characterized the variation of Bluetongue viruses at the nucleic acid level ^{by} making use of 2-D oligonucleotide fingerprinting of individually isolated genome segments from two BTV serotypes. This study has revealed evidence for gene re-assortment and also for minor changes in the primary structure of genome segments consistent with antigenic drift. Comparison of the fingerprints of corresponding genome segments for isolates of BTV serotypes 10 and 11 showed that four were quite different (segs 2, 3, 5 and 10). Genome segment 8 from both serotypes was virtually identical while the remaining genome segments were, on the basis of this analysis, quite similar. Oligonucleotide fingerprint analysis of another Bluetongue serotype 11 virus isolated 11 years after the type representative isolate revealed this to have a genome segment 3 very similar to that observed in the BTV serotype 10. The remaining nine genome segments were similar to their counterparts of the original BTV 11 isolate. The results indicate that the three BTV isolates appear to share a common gene pool, the presence of the highly conserved species 8 RNA between two serotypes coupled to the subsequent occurrence of BTV 10 seg 3 in a BTV 11 isolate argues strongly for gene reassortment as a mechanism for generating diversity among orbiviruses.

This concept has also been supported by circumstantial evidence indicating multiple infection of hosts with two or more viruses, for example two serotypes of EHD and one of BTV were isolated from a single herd of cattle in Colorado U.S.A. (Barber & Jochim, 1975). However,

no evidence for simultaneous infection of a single animal has been obtained. Reassortants between related orbiviruses have been constructed (Gorman et al., 1978) in an elegant study making use of temperature sensitive mutants to allow selection of recombinant progeny. This has demonstrated conclusively that gene re-assortment can take place between orbiviruses of different serotypes.

Several attempts have been made to facilitate prophylactic immunization against bluetongue (Howell & Verwoerd, 1971); these have met with differing degrees of success. Two approaches to vaccination have been adopted depending on the nature of the outbreaks. These have involved either the very successful use of a monovalent live attenuated vaccine developed from a local predominant virus strain, or where numerous antigenic types of virus are active, the employment of a polyvalent live attenuated vaccine. However effective control under these circumstances has proved to be considerably more difficult to achieve (Howell & Verwoerd, 1971).

The demonstration that BTV can undergo gene re-assortment and that genome segments accumulate minor sequence changes in an analogous fashion to antigenic 'drift' observed for influenza may explain why some vaccines that have been used fail to provide adequate immunity.

(c) Variation in Rotaviruses - rationale of this work

Preliminary studies have indicated the presence of a large number of rotavirus "antigenic types" confined to infecting individual animal species (Thouless et al., 1977). However, variation in the rotaviruses has not been well characterized. This is because of their late

recognition as major pathogens (Lancet-Editorial, 1975) and the problems encountered with their routine adaptation to growth in tissue culture (McNulty, 1978; Flewett & Woode, 1978). As an alternative to serological characterization it has been suggested that analysis of genome profile heterogeneity by polyacrylamide gel electrophoresis may be used as an epidemiological tool (Rodger & Holmes, 1979).

Genome profile analyses were initially very limited in the range of isolates studied because of practical restrictions (Konica et al., 1976). Genome profiles could only be obtained where faecal samples were available in sufficient quantity and containing a high enough concentration of rotavirus to allow them to be purified by conventional methods. Following phenol extraction of the viral RNA, the genome segments were electrophoretically separated on polyacrylamide gels and visualized by ethidium bromide staining. The insensitivity of this staining method coupled to the inefficiency of the extraction procedure led to only few reports of rotavirus genome segment diversity by 1980.

The first objective of this work was to develop a sensitive technique that could be rapidly and reproducibly applied to a wide range of faecal samples and so facilitate a comprehensive molecular epidemiological analysis of rotavirus genome profile heterogeneity. This would enable the nature and extent of genome segment diversity among rotavirus isolates, and the frequency of mixed infections to be determined. In 1979, at the inception of this project, there was no information available regarding the structural basis of variations in rotavirus genome profiles. Similar variations had been observed

among the closely related reoviruses (Hrdy et al., 1979); and orbiviruses (Gorman, 1979). One study made in 1972 involving the construction of hybrid dsRNA molecules between reovirus ts mutants indicated that the relative mobility of corresponding genome segments could be affected by very minor (single base) changes (Ito & Joklik, 1972). Additionally co-migrating corresponding species of RNA from different isolates may have completely different primary structures. It is also possible that co-migrating dsRNA species from different isolates may code for proteins of completely different biological functions. Clearly differences observed in comparative genome profile studies require further structural analysis before definitive conclusions can be drawn about them.

These considerations led to the second objective of this thesis which was to develop a technique that would allow definitive conclusions regarding the variation of rotaviruses at the nucleic acid level. Results obtained in this way may indicate whether gene reassortment is a mechanism by which "new" naturally occurring strains of rotavirus might be generated.

A number of methods were considered as ways for analyzing the primary structure of dsRNA genome segments. These broadly fell into two groups and included either the use of specific hybridization probes or the application of "fingerprinting techniques".

The hybridization approach has been made use of by a number of groups. One such method was to use the endogenous RNA polymerase activity present in rotavirus "cores" to synthesize in vitro single-stranded RNA copies of the dsRNA. Following denaturation, test dsRNA

was hybridized to the ss RNA specific probes and treated with S1 nuclease, the product then being analysed by polyacrylamide gel electrophoresis. This technique has been used to characterize laboratory generated re-assortants (Flores et al., 1981) and also to study the relatedness of corresponding genes from different human and animal rotavirus strains (Matsuno & Nakajima, 1982). Another method has been to use cDNA prepared from genomic dsRNA to probe genomic RNA from different isolates that has been fractionated on polyacrylamide gels and transferred to DBM paper (Street et al., 1982; Schroeder et al., 1982). This hybridization approach has proved to be rather less informative than had been hoped. Most of the problems are of a practical nature and involve finding the ideal conditions for hybridization. The method also suffers from the interpretative problem that if a genome segment from an isolate under test is completely different in primary sequence from the hybridization probe then it will not be detected; therefore a positive answer will be indicated by a negative result. However, the advantage of using the hybridization approach is that it can be simultaneously applied to screen a large number of samples. The (future?) use of molecular clones of protein coding regions from individual rotavirus genome segments as hybridization probes should increase the scope of this technique.

Oligonucleotide fingerprinting can be discounted as a rapid and simple method for analyzing the structure of individual rotavirus genome segments for several reasons. Firstly, wild isolates do not grow in tissue culture therefore the dsRNA for analysis would have to be labelled in vitro following total T_1 ribonuclease digestion.

Secondly, the number of diagnostic spots obtained for the smaller RNA species by this method is low and therefore less significant, making definitive comparisons difficult. Finally each isolate has eleven genome segments, to analyze a few isolates by this method would require a considerable undertaking in materials, time and patience with no guarantee of any useful information being generated. The considerations regarding both the hybridization and the oligonucleotide fingerprinting approaches led to an idea for the development of a method to characterize rotavirus genome segments based on RNA sequence analysis and making use of partial digestion of individual isolated genome segments with a base-specific ribonuclease.

This thesis describes the development of two methods allowing both the analysis of rotavirus genome profiles from field isolates and the structural characterization of the variation observed among them. It describes the application of these techniques to a collection of rotavirus field isolates and the subsequent demonstration of gene re-assortment as a possible mechanism for generating diversity. As a result of these analyses, detailed structural characterization of the rotavirus genome has also revealed evidence for regions of RNA species-specific terminal conservation.

A detailed knowledge of the structural basis of genome segment variation observed amongst rotavirus isolates will be central in forming the policies towards producing effective prophylactic control of this important infectious agent.

MATERIALS AND METHODS

Materials

All materials not listed were obtained as AnalaR grade from BDH Chemicals Ltd., Poole, Dorset.

Agar Aids, Bishops Stortford, Herts.

Electron Microscope grids.

Biorad Laboratories Ltd., Richmond, California, U.S.A.

Ammonium persulphate

Temed

N-N'-methylene bis acrylamide

Sodium dodecyl sulphate

Boehringer Corporation, Lewes, East Sussex.

U₂ ribonuclease

Bethesda Research Laboratories, Science Park, Cambridge.

Polynucleotide kinase

DNA polymerase I

Hpa II

Taq II

Eastman-Kodak, Rochester, New York, U.S.A.

X-omat H) X ray film

X-omat S) X ray film

DX 80 - X ray film developer

FX 40 - X ray film fixer

Flow Laboratories, Irvine, Ayrshire, Scotland.

Glasgow's modifications of Eagle's medium(GMEM)

Gibco Bio-Cult Ltd., Paisley, Scotland.

Foetal calf serum

Hopkins and Williams, Chadwell Heath, Essex.

Repelcote

Pharmacia Fine Chemicals Ltd., Hounslow, Middlesex.

Sephadex G50

P-L Biochemicals Inc., P.O. Box 98, Northampton.

T₄ RNA ligase
Physarum ribonuclease I
T₁ ribonuclease

Radiochemical Centre, Amersham International, Bucks.

[³²P] pCp 300 and 3,000 Ci/mmol
[γ ³²P] ATP 3000 Ci/mmol
[α ³²P] dCTP 3000 Ci/mmol
[5,6³H] UTP 40-60 mCi/mmol

Serva Feinbiochemica, Heidelberg, W. Germany.

Latex beads

Sigma London Chemical Co. Ltd., Poole, Dorset.

Agarose
Dithiothreitol
Nucleotide triphosphates
S1 nuclease
Trizma Base
HEPES
Yeast tRNA

Neil Turner and Co. Ltd., Kings Lynn, Norfolk.

Tape for constructing all gel sandwiches

Whatman LabSales Ltd., Maidstone, Kent.

CF11 cellulose

Worthington Enzymes - agents Flow Laboratories.

Pancreatic ribonuclease

Methods

1. Virus Growth and Purification

The Compton U.K. tissue culture adapted bovine rotavirus was obtained from Dr. M. Thouless; the Ohio State University (O.S.U.) tissue culture adapted porcine rotavirus was obtained from Professor E. H. Bohl. Confluent 80 OZ roller bottles of BSC-1 cells were infected with a multiplicity of infection (m.o.i.) of approximately 0.1 p.f.u./cell for virus growth. Following adsorption (1 hr) the cells were overlayed with 35 mls GMEM containing trypsin at 10 µg/ml. Virus growth was allowed to continue for 3 days at 37°C by which time all the cells had become detached from the glass.

Virus was purified using a modification of the purification procedure for reovirus (Smith et al., 1969). In summary, cells were disrupted by freeze-thawing and then homogenized with a 1/4 volume of Freon 113 (trichlorofluoroethane). The phases were separated by low speed centrifugation and the aqueous phase decanted. The freon phase was re-extracted by homogenization three times using 25 ml of resuspension buffer (50 mM Tris-HCl pH 8.0, 10 mM NaCl, 3 mM CaCl₂ and 1.5 mM B-mercaptoethanol). The aqueous phases were combined and the virus was concentrated by centrifugation (100,000 g 30 mins 4°C). The virus pellet was resuspended in resuspension buffer, loaded onto a preformed CsCl gradient ρ 1.2 - 1.4 and centrifuged at 100,000 g and 4° for 2 hrs. The ρ 1.36 virus band was collected by side puncture, concentrated by centrifugation and resuspended in 50 mM Tris pH 8.0.

2. Faecal Samples Containing Rotavirus Particles

'Wild' rotavirus isolates analyzed in this thesis have been supplied from four sources:-

a) Mr. Tony Scott,

E.M. Diagnostic Unit,

Central Veterinary Laboratory, Weybridge.

b) Dr. Janice Bridger,

Institute For Research On Animal Diseases, Compton.

c) Mr. Ian Chrystie,

St. Thomas's Hospital, London.

d) Dr. Margaret Thouless,

East Birmingham Hospital, Birmingham.

3. Electron Microscopy

Provisional diagnosis of rotavirus was carried out by identifying rotavirus particles in faecal samples. Particles were visualized in the JEM 100S following negative staining (2 mins) of infected faeces with 2% phosphotungstate pH 7.0 on 300 mesh formvar coated grids. Virus particle concentrations were determined from 'random field' ratio counts against standard concentrations of 0.109μ diameter latex beads.

4. Extraction of Rotavirus dsRNA From Faecal Samples

Workable volumes (0.1-1 ml) of rotavirus infected faeces were diluted

to 5 ml in 0.1 M acetate buffer pH 5.0, made 1% for SDS and extracted with an equal volume of acetate buffer saturated phenol at 60°C to remove DNA. The aqueous phase of this extraction was then further deproteinised by extraction with water-saturated phenol at 20°C. Following ether extraction (4X) of the aqueous phase, nucleic acid and remaining protein were precipitated from the samples at -20°C by the addition of 2 volumes of ethanol. The ethanol precipitate was resuspended in 1 mM EDTA pH 8.0 and single-stranded RNA precipitated by adding LiCl to a final concentration of 2 M and storage overnight at 4°C. The 2 M LiCl supernatant was then made 4 M for LiCl to precipitate dsRNA which was collected by centrifugation at 20,000 g for 30 min following overnight precipitation at 4°C. The 4 M LiCl pellet was resuspended in 0.5 ml of 1 mM EDTA pH 8.0 and concentrated by ethanol precipitation before being finally resuspended in 20-50 µl of 1 mM EDTA.

5. 3' Terminal Labelling of dsRNA With T₄ RNA Ligase

Viral dsRNA was obtained from purified virions by extraction with water saturated phenol (2X) at 20°C. Following ether extraction (4X) of the aqueous phase, dsRNA was precipitated overnight with two volumes of ethanol at -20°C. The ethanol precipitate was resuspended in 20-50 µl of 1 mM EDTA pH 8.0.

Viral dsRNA from both purified virions and infected faeces was labelled at its 3' termini using [³²P] pCp and T₄ RNA ligase as described by England & Uhlenbeck (1978). The reaction mixture was made up in the following way:-

3 μ l dimethylsulphoxide (DMSO)

2 μ l 50 mM dithiothreitol

2 μ l 750 mM Hepes pH 8.3

2 μ l 150 mM MgCl₂

2 μ l 75 nM ATP

3 μ l glycerol

4 μ l sample

0.5 - 10 units T₄ RNA ligase - the volume of distilled water was
total reaction mixture = 30 μ l. adjusted 4-11.5 μ l to compensate for
the amount of ligase used.

5 - 50 μ Ci [³²P] pCp was dried in siliconized (0.75 ml) μ fuge tubes prior to adding the reaction ingredients. The reaction was allowed to proceed for 19 hrs at 4°C followed by 10 mins at 37°C. Differing amounts of T₄ RNA ligase, [³²P] pCp, and dsRNA were used depending on the experimental requirement for incorporated counts.

Several methods were used to separate the labelled dsRNA from unincorporated isotope at the end of the reaction. Of these the most suitable was the use of Franklin columns (Franklin, 1966). The labelling reaction was terminated by addition of SDS to 0.1% and then 2 volumes of TNE (10 mM Tris buffer pH 7.4, 0.1 M NaCl, 1 mM EDTA) were added. Ethanol was then added to a final concentration of 35% and the sample applied to a column of Whatman CF 11 cellulose equilibrated with TNE + 35% ethanol. After 35% and 15% ethanol washes to remove the unincorporated isotope, the residual labelled dsRNA was eluted in TNE buffer.

6. TCA Incorporation Following Radioactive Labelling of Nucleic Acids

2 μ l aliquots were taken from the radiolabelled dsRNA samples, resuspended in 50 μ l 1 mM EDTA, following ethanol precipitation with 5 μ g cold yeast tRNA carrier from the Franklin column dsRNA fractions. These were air dried, ^{and} following this they were given five 5 minute washes in ice cold 5% TCA, two ten minute washes in ethanol and two washes in ether.

Filter papers were air dried and the radioactivity determined with a Packard Tri-carb scintillation counter using toluene containing 2-5-diphenyloxazole (PPO) (5 g/litre) as scintillation fluid. Loading of equal TCA incorporated counts gave radioactivity balanced tracks following polyacrylamide gel electrophoresis of the dsRNA.

7. Polyacrylamide Gel Fractionation of dsRNA

The individual species of dsRNA were fractionated on 7.5% polyacrylamide gels using the Laemmli discontinuous buffer system (Laemmli, 1970).

Electrophoresis on 20 x 20 cm gels was carried out at 20 mA for 16 hrs ^{and} at 4°C. ^A these gels were dried down under vacuo prior to autoradiography.

In preparative fractionation of dsRNA for use in the sequence studies, electrophoresis on 20 x 40 cm gels was carried out at 35 mA for 24 hrs.

The 7.5% polyacrylamide gel shown in Figure 30 was run using the Tris-acetate buffer described by Horuichi et al. (1975). A 10X stock of this buffer had the following ingredients: 400 mM Tris, 200 mM sodium acetate, 20 mM EDTA glacial acetic acid added to pH 7.4. The gel matrix contained 6M urea which had to be eluted for 1 hr in distilled water before drying under vacuo.

8. Ethidium Bromide Staining

Gels containing nucleic acid were stained with ethidium bromide at 5 µg/ml for 1 hr. Nucleic acid was viewed on an Ultra-violet light box. Photographic record were made using a Polaroid land camera with Polaroid type 665 land film.

9. Preparative Fractionation of dsRNA on Agarose Gels and Extraction of Isolated Species

Labelled dsRNA was fractionated by electrophoresis on a 1.5% agarose gel containing 6 M urea using a Tris-acetate continuous buffer system (Horiuchi *et al.*, 1975). Electrophoresis was conducted for 16 h at 30 mA. The RNA species were located by autoradiography of the wet gel and excised with a scalpel. RNA was recovered from the excised slices using a 'freeze-squeeze' procedure in which the slice was first placed in a sterile disposable 2 ml syringe with a 25 gauge needle which was partially plugged with glass wool and the plunger firmly pressed down. The syringe was frozen at -70°C for 2 h and then the needle pushed through the top of a 1.5 ml Eppendorf microcentrifuge tube. This assembly was centrifuged at 1300 x g and 4°C for 1 h to squeeze the RNA in solution out of the gel slice into the Eppendorf tube. The recovered RNA was concentrated by ethanol precipitation and resuspended in 20-50 µl of 20 mM Tris, pH 7.4, and stored at -20°C.

10. Partial Nuclease Digestion of Isolated Species with RNase T₁ and Partial Alkaline Hydrolysis

Isolated species of dsRNA were denatured at 50°C in 90% dimethyl

sulphoxide as described by McCrae & Joklik (1978). Following denaturation, 8-10 µg of yeast tRNA were added both to act as carrier in later precipitation steps and to provide an equalizing background of nucleic acid to facilitate the calibration of the partial nuclease digestion conditions. The RNA was then immediately ethanol-precipitated. The dried pellet was resuspended in 4 µl of 20 mM Tris pH 7.4, and digested with 10^{-4} units T_1 ribonuclease per µg of RNA for 15 min at 37°C. Random cleavage of isolated species RNA was achieved by partial alkaline hydrolysis carried out in 4 µl of 50 mM bicarbonate buffer, pH 9.0, at 90°C for 3 min.

11. Production of dsDNA Size Markers

The DNA size markers used in this thesis were produced by digestion (2 hrs) of 2 µgs of the plasmid PBR 322 at 37°C provided by Dr. M. A. McCrae with 2 units of the restriction endonucleases Hpa II or Taq II as described by Sharp et al. (1973). The resulting DNA fragments were labelled with the klenow fraction of DNA polymerase I and 10 µ Ci [^{32}P] dCTP as described by Sanger et al. (1977). The reaction was stopped by addition of 2X column buffer containing 100 mM Tris pH 8.0, 0.2% SDS and 1M NaCl. This mixture was passed through a 5 ml sephadex G-50 column to remove unincorporated isotope. The void volume peak was pooled and precipitated with ethanol.

12. Polyacrylamide Gel Fractionation For RNA Sequence Analysis and T_1 Ribonuclease Partial Nuclease Digestion 'Fingerprinting'

Following digestion, an equal volume of gel sample buffer (Donis-Keller

et al., 1977) was added to the digests and the samples heated at 100°C for 1 minute. The oligonucleotide fragments generated by digestion were then fractionated on 12-20% polyacrylamide gels using TBE buffer (90 mM Tris, 90 mM Boric acid, 2.5 mM EDTA) as described by Maxam & Gilbert (1977). These gels were pre-run at 1.6 KV for 1 hr before loading and fractionation was from 2½ to 8 hrs depending on the gel concentration and region of RNA under analysis. After electrophoresis gels were covered with cling film and exposed 'wet'.

13. Synthesis of Viral mRNA For Use in Sequencing Studies

Rotavirus mRNA was synthesized in the in vitro transcription reaction following activation of the virion associated RNA dependent RNA polymerase (Cohen, 1977). This enzyme was activated by incubating purified virions in 5 mM EDTA at 37°C for 15 minutes. Following activation, mRNA synthesis was performed for 5 hrs at 37°C, using the reaction conditions of Mason et al. (1980). mRNA synthesis was followed by measuring the increase in incorporated [5,6³H] UTP at hourly intervals.

14. Fractionation of dsRNA Species and Gel Elution For RNA Sequence Analysis

3' terminally labelled dsRNA was fractionated on long 6% 20 x 40 cm gels using the discontinuous buffer system as described by Laemmli (1970). Samples were mixed with an equal volume of gel buffer and heated at 70°C for 2 minutes prior to electrophoresis. Individual RNA bands were localized by autoradiography, excised with a scalpel and the RNA eluted as described by McCrae & Joklik (1978).

15. Genome Strand Separation and RNA Sequence Analysis

(a) The individually isolated genome segments in 1 mM EDTA were denatured in 90% DMSO at 50°C for 20 minutes then mixed with 50 µg of the unlabelled viral mRNA. Hybridization to achieve displacement of the plus strand was performed in 0.204 M NaCl, 34 mM Tris HCl and 6.59 mM EDTA for 48 hrs at 25°C. Following this reaction single- and double-strand RNA molecules were fractionated by Whatman CF 11 cellulose column chromatography. Fractions containing the separated labelled strands were pooled, yeast carrier tRNA added and precipitated at -20°C with two volumes of ethanol. The dsRNA fractions were denatured in 90% DMSO at 50°C for 20 minutes then ethanol precipitated.

(b) For RNA sequence analysis the following reaction conditions were used:-

1. T_1 ribonuclease, 5×10^{-5} units/µg RNA for 15 mins in 100 mM Tris-HCl buffer pH 7.4, 10 mM EDTA at 37°C.
2. U_2 ribonuclease, 5×10^{-5} units/µg RNA for 15 mins in 50 mM acetate buffer, 2 mM EDTA pH 4.5 at 37°C.
3. Pancreatic ribonuclease, 2×10^{-8} units/µg RNA for 5 mins in 100 mM Tris-HCl buffer pH 7.4, 10 mM EDTA at 37°C.
4. Physarum ribonuclease I, 1 µl of 25 units/ml per µg RNA for 15 mins in 10 mM acetate buffer, 1 mM EDTA pH 5.0 at 37°C.
5. Ladder production was achieved by heating the terminally labelled RNA in 50 mM-bicarbonate buffer pH 9 at 90°C for 3 minutes.

Digestions were all performed in 4 µl, following digestion the

samples were mixed with an equal volume of sample buffer described by Donis-Keller et al. (1977), heated at 100°C for 1 minute before loading on sequencing gels.

16. S1 Nuclease Digestion

Dried ethanol precipitates of RNA samples for analysis were resuspended in the assay mixture (50 µl). Digestion was performed at 37°C in 50 mM sodium acetate pH 4.5, 5 mM zinc chloride using 1000 units/ml of S1 nuclease. Digestion was for thirty minutes, ^{and} resistance to S1 nuclease was estimated by comparing TCA precipitable counts remaining in the digested sample with those in the control sample treated in the same way without S1 nuclease.

17. Oligonucleotide Fingerprint Analysis

Individually isolated dsRNA samples were denatured in 80% DMSO at 50°C for 15 mins, ^{and} the single strands were then precipitated with ethanol using 5 µg of glycogen (kindly given by Dr. J. Clewley) as carrier. RNA samples were totally digested with 10 units of T₁ ribonuclease for 30 minutes at 37°C. Following this digestion the oligonucleotide fragments were labelled with [γ ³²P] ATP as described by Frisby et al. (1977). Solid urea was added to give a final concentration of 6 M and then 15 µl of a solution containing 6 M urea, 50% W/V sucrose, 0.2% xylene cyanol, 0.2% bromophenol blue and 15 mg bacterial tRNA per ml (kindly given by Dr. J. Clewley). Before loading on the first dimensional gel, the sample was incubated at 60°C for 2 minutes.

The two dimensional electrophoresis was the procedure of De Wachter & Fiers (1972). Briefly, the first dimension was run in a 10% polyacrylamide gel in 6 M urea 25 mM citric acid pH 3.5. Electrophoresis was at 900V, 50 mA, until the bromophenol blue dye had moved 19 cm from the origin. The second dimension was run in a 21.8% acrylamide gel in 0.1 M Tris-borate pH 8.3, 2.5 mM EDTA. Electrophoresis was at 50 mA per gel until the bromophenol blue had moved 21 cm from the first dimension gel strip. After electrophoresis each gel was autoradiographed at -70°C to obtain the oligonucleotide fingerprint.

18. Autoradiography

Polyacrylamide (Laemmli) gels dried onto Whatman 3 MM paper under vacuo, 'wet' sequencing gels and oligonucleotide fingerprint gels bearing [³²P] were covered with cling film and exposed to X-omat H or X-omat S using lightning plus image intensifying screens at -70°C. Exposure times varied from 1 to 14 days depending on the amount of incorporated radioactivity loaded on the gels. When the exposure time permitted improved resolution was achieved when the intensifying screen was omitted, increasing exposure times 5 fold.

Film was developed for 5 min in freshly made Kodak DX80 (diluted 1 + 3) and fixed for 5 mins in freshly made FX40 (diluted 1 + 3). The film was washed thoroughly in running tap water (30 mins) before air drying.

RESULTS CHAPTER 1

The greater part of this Chapter has appeared in press

(J. Virol. Methods, 2 (1981) 203-209)

Chapter 1

A Rapid and Sensitive Method for Analyzing the Genome Profiles of Rotavirus Field Isolates

Introduction

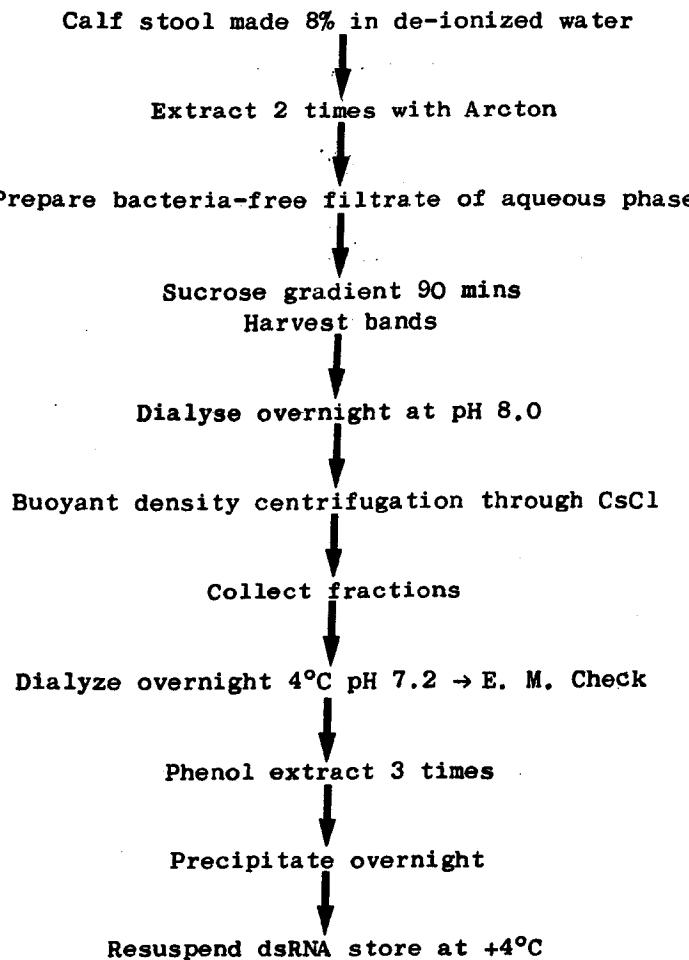
Several suggestions that analysis of rotavirus genome profiles by polyacrylamide gel electrophoresis may prove useful for molecular epidemiological purposes have been made (Konica *et al.*, 1976; Rodger & Holmes, 1979). At the time this project was started genome profile studies could only be undertaken when rotavirus was available in sufficient amounts to allow highly inefficient, labour intensive and elaborate virus purification procedures. A basic summary of the method employed by Konica *et al.* (1976) is shown schematically in Figure 5. The use of ethidium bromide which is a relatively insensitive stain for detecting dsRNA further limited this approach.

In order to survey the polymorphism of migration of dsRNA genome segments from wild rotavirus isolates it was decided to develop a rapid and reproducible method for preparing rotaviral dsRNA from a large number of infected stool samples. A second requirement was to devise a very sensitive technique to allow visualization of that dsRNA following gel fractionation.

Results

The approach adopted towards meeting these requirements was dictated by the need to find a simple method that involved the fewest possible steps. Attempts were made to phenol extract double-stranded RNA directly from virions in faecal samples. These proved highly

Figure 5. Schematic Representation of the Methods Employed by
Kalica et al. (1976) to obtain Rotavirus dsRNA from Stool
Samples



successful and eliminated the need for elaborate virus purification procedures. An initial hot phenol (60° pH 5) extraction was also included to reduce the levels of cellular or bacterial DNA in the sample. Without this step subsequent purification of the dsRNA was hindered by very large precipitates. Following phenol extraction, the rotavirus dsRNA in the aqueous phase required further purification from contaminating single-stranded nucleic acids and proteins. To achieve this a number of methods were investigated. The best results were obtained using differential (2M and 4M) high salt precipitation at 4°C . The final method adopted for rotavirus dsRNA purification is summarized schematically in Figure 6.

Ethidium bromide staining of rotavirus dsRNA was attempted to ascertain the sensitivity of this technique. The O.S.U. strain of pig rotavirus was grown in tissue culture and virus particles purified as described in materials and methods. Differing amounts of rotavirus dsRNA measured spectrophotometrically were fractionated by polyacrylamide gel electrophoresis and analyzed by staining with ethidium bromide (see Figure 7). From this analysis the minimum amount of dsRNA to give a detectable gel profile by staining with ethidium bromide at 5 $\mu\text{g}/\text{ml}$ for 1 hr was 1-2 μg .

As an alternative to using ethidium bromide, a method for detecting rotavirus dsRNA was developed based on the in vitro radiolabelling of reovirus dsRNA as described by Uhlenbeck & England (1978) and McCrae (1981). Labelling of 2 μg dsRNA from the O.S.U. rotavirus isolate gives 10×10^6 incorporated counts/minute. Quantitative analysis has shown that 10×10^3 incorporated counts/min are

Figure 6. Schematic Representation of the Rapid and Sensitive Method Developed for Obtaining Rotavirus dsRNA from Stool Samples

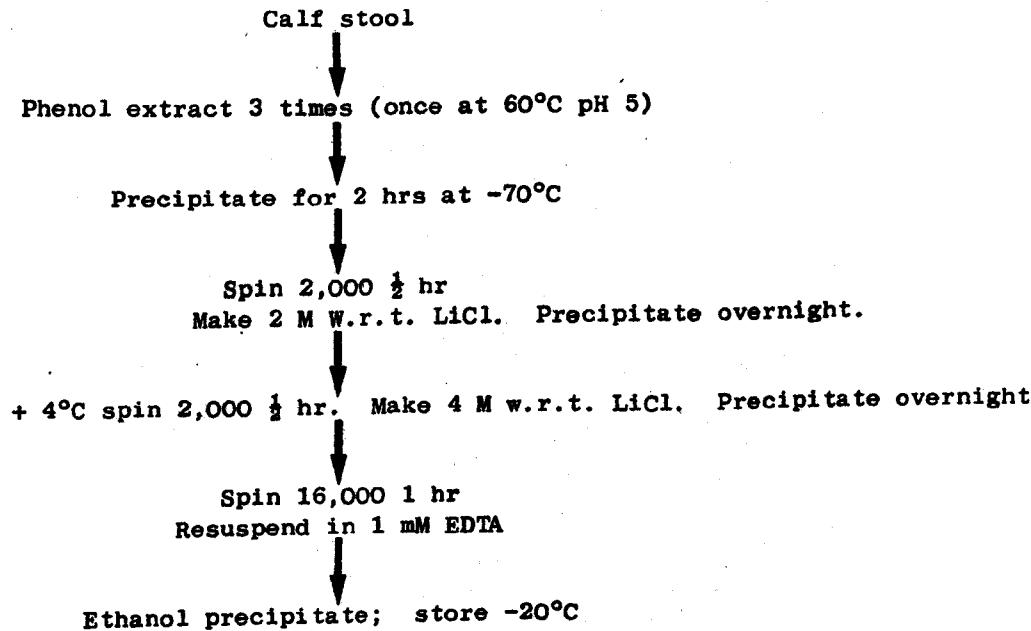


Figure 7. The Sensitivity of Ethidium Bromide Staining for Visualizing dsRNA.



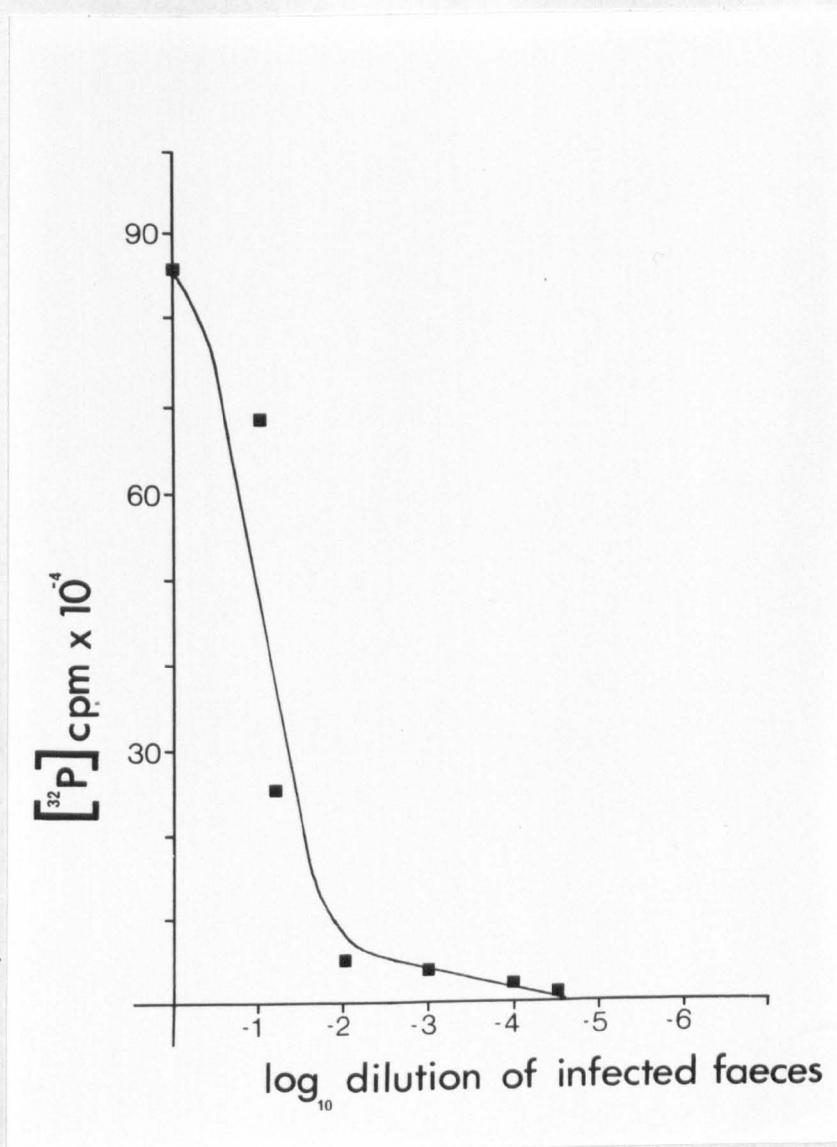
Polyacrylamide gel fractionation of differing amounts of pig rotavirus dsRNA (as assayed by O.D.₂₆₀) stained with 5 µg/ml of ethidium bromide and visualized with U.V. light: 1) 20 µg dsRNA; 2) 10 µg dsRNA; 3) 2 µg dsRNA; 4) 1 µg dsRNA; 5) 0.2 µg dsRNA; 6) 0.1 µg dsRNA; 7) 0.01 µg dsRNA; 8) no sample. The higher molecular weight RNA species stain more intensely because of their increased length. The minimum amount of dsRNA to give a detectable gel profile using this method of staining is 1-2 µg. Electrophoresis in this and the other gels in this Chapter was at 20 mA for 16 hrs at 4°C.

sufficient to give a rotavirus genome profile following autoradiography overnight. Therefore, using the same amount of double-stranded RNA 1,000 times more genome profiles can be obtained by radiolabelling.

The major problem encountered for producing genome profiles using radiolabelling was in obtaining radioactivity balanced polyacrylamide gels. To overcome this difficulty removal of unincorporated radiolabel from the sample following the in vitro reaction was necessary. A number of methods were investigated to facilitate this requirement. Separation of unincorporated label from the dsRNA was best achieved by the use of a cellulose CF11 column chromatographic procedure as originally described by Franklin (1966). In this system only dsRNA binds to the cellulose in the presence of 15% ethanol, unbound nucleic acid including the unincorporated radioactive label and single-stranded RNA is eluted off in buffer containing 15% ethanol. Double-stranded RNA is obtained by washing through with buffer containing no ethanol.

The sensitivity of the combined techniques for extracting and labelling rotavirus dsRNA were determined by serially diluting a sample of a calf rotavirus isolate containing 10^{11} virus particles/ml with non-infected calf faeces. Each sample was then subjected to analysis. The use of non-infected calf faeces as a diluent ensures that the same amount of faecal material was extracted in each case. Following phenol extraction and radiolabelling each sample was assayed for the number of counts incorporated into nucleic acid. The results of this analysis are shown in Figure 8. This figure illustrates that even at 10^{-4} dilution of the original faeces significant incorporation

Figure 8. Graph to Show the Effect of Dilution of Infected Faeces on Incorporation in the End-Labeling Reaction.



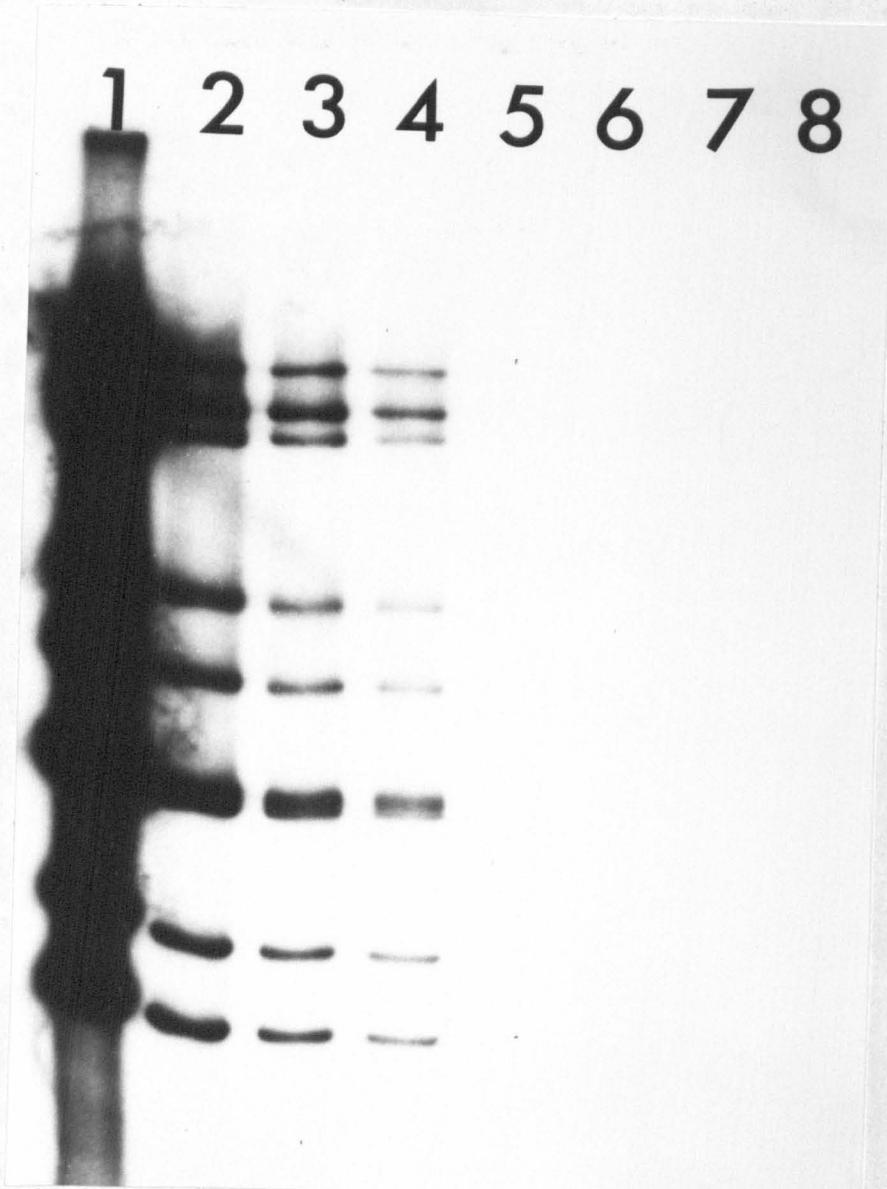
3' end labelling of the various diluted and extracted samples was carried out as described in Materials and Methods. Incorporation into TCA precipitable counts was measured by precipitating an aliquot of the 0% ethanol eluent of each Franklin column with cold 5% TCA.

(20,000 c.p.m.) above that obtained for the control faecal sample containing no rotavirus (1,300 c.p.m.) was achieved.

To show that these incorporated counts were in rotavirus dsRNA two types of gel analysis of the samples were performed. Figures 9a and 9b show the results of running 25% of the incorporated counts from each of the labelling reactions on adjacent gel tracks. This analysis indicated that the fall in incorporated counts with infected faeces dilution (Figure 8) was due directly to the presence of less rotavirus dsRNA at each dilution; and consequently showed that incorporation in this method was mainly into rotavirus dsRNA. The second type of analysis involved running a fixed number of incorporated counts from each dilution on adjacent gel tracks. The results (Figure 10) showed that the relative incorporation of [³²P]Cp into rotavirus dsRNA did not vary with dilution, again indicating that the majority of incorporation was into rotavirus dsRNA.

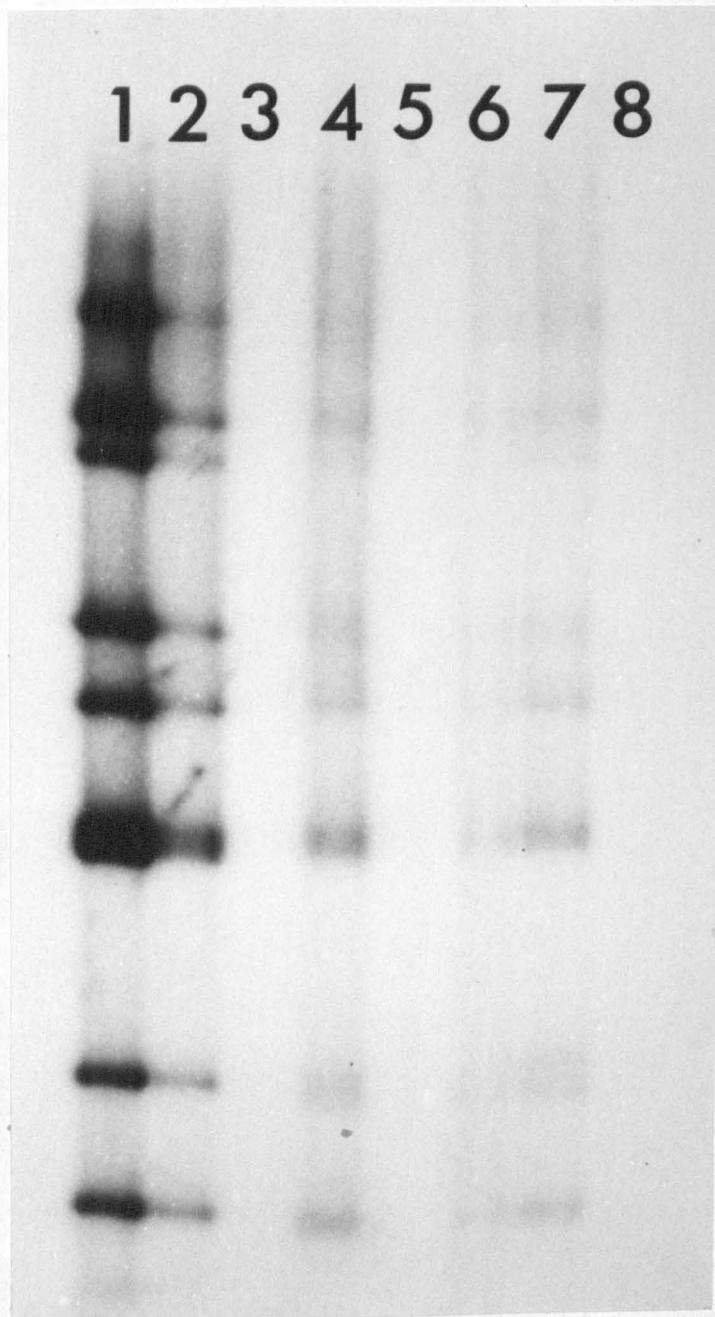
The experiment described above also allowed some quantitation of the sensitivity of the combined methods to be carried out. The extractions for the above dilution experiment were all performed on 2.5 ml of faeces, and since the original infected sample contained 10^{11} particles/ml, then in the undiluted sample the dsRNA from 2.5×10^{11} particles was extracted. Figures 9 and 10 show that it was easily possible to obtain a dsRNA gel profile using a 10^{-4} dilution of this sample, and so we can conservatively conclude that this method can be reproducibly used to obtain genome profiles from as little as 2.5×10^7 virus particles. It is possible to make some approximate comparisons between the sensitivity of this method and that currently used in work

Figure 9(a). Polyacrylamide Gel Fractionation of an Equal
Amount of the Total Material from Each Dilution of Infected
Faeces.



Bovine rotavirus dsRNA was extracted and labelled as described in Materials and Methods. 25% of the total sample for each dilution was run in successive gel tracks of a short (20 cm) 7.5% polyacrylamide gel. Track 1) undiluted infected faeces; 2) 10^{-2} dilution of infected faeces; 3) 10^{-3} dilution of infected faeces; 4) 10^{-4} dilution of infected faeces; 5) 5 x 10^{-4} dilution of infected faeces; 6) 10^{-5} dilution of infected faeces; 7) 10^{-6} dilution of infected faeces; 8) material from uninfected gnotobiotic faeces.

Figure 9(b). Further Polyacrylamide Gel Fractionation of
Equal Amounts of Total Material from Dilutions of 10^{-4} to 10^{-5}
of Infected Faeces to Determine the Sensitivity Limit of the
Final Method Adopted for Extracting and Labelling Rotavirus
dsRNA.



25% of the total sample for each dilution was run in successive gel tracks. Track 1) 10^{-4} dilution of infected faeces; 2) $10^{-4.1}$ dilution of infected faeces; 3) $10^{-4.2}$ dilution of infected faeces; 4) $10^{-4.3}$ dilution of infected faeces; 5) $10^{-4.4}$ dilution of infected faeces; 6) $10^{-4.5}$ dilution of infected faeces; 7) 10^{-5} dilution of infected faeces; 8) material from uninjected gnotobiotic faeces.

Figure 10. Polyacrylamide Gel Fractionation of an Equal Number of Incorporated Counts from Each Dilution of Infected Faeces.



Samples were labelled and incorporated counts determined as described in Materials and Methods and in the legend to Figure 9(a). 3,000 incorporated counts from each sample were run on a short (20 cm) 7.5% Laemli polyacrylamide gel. Track: 1) undiluted infected faeces; 2) 10^{-1} dilution of infected

faeces; 3) 2×10^{-1} dilution of infected faeces; 4) 10^{-2} dilution of infected faeces; 5) 2×10^{-2} dilution of infected faeces; 6) 10^{-3} dilution of infected faeces; 7) 10^{-4} dilution of infected faeces; 8) material from uninfected gnotobiotic faeces. Numbering of the genome segments shows that on these short gels segments 2, 3 and 7, 8, 9 comigrate.

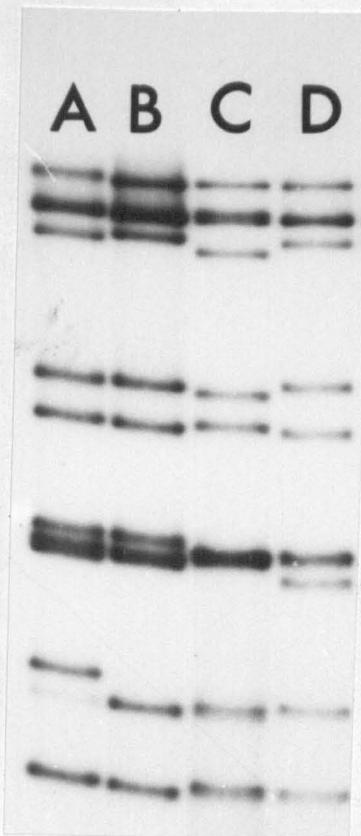
which involved detecting the viral dsRNA using ethidium bromide staining. The minimum amount of dsRNA required to give a detectable gel profile using ethidium bromide staining is 1-2 µg. Making the reasonable assumption that the viral RNA constitutes about 10% by mass of the virion, a figure comparable with that seen with other reoviridae (Joklik, 1974), and that 1 mg of virus protein constitutes approximately 1×10^{13} virus particles (Smith *et al.*, 1969); then by calculation 10-20 µg of virus or $1-2 \times 10^{11}$ virus particles are required to obtain a single gel profile using ethidium bromide staining. This means that this detection method is in the region of 5000-10,000 times more sensitive than those currently employed. Finally, the above calculations do not take into account the fact that using the 20,000 c.p.m. incorporated using 2.5×10^7 particles multiple gel profiles can be produced whilst $1-2 \times 10^{11}$ particles are required for a single gel profile using ethidium bromide staining. This sensitive and reproducible method for analyzing the electrophoretic profile of rotavirus genomic RNA, has the advantage that it can simultaneously be applied to a large number (20-50) of samples. The established methodology for analyzing the dsRNA of wild isolates of rotavirus involved purification of virions from infected faeces by arcton extraction and caesium chloride centrifugation before phenol extraction of the dsRNA, which was then visualized on polyacrylamide gels by staining with ethidium bromide (Rodger & Holmes, 1979). This procedure was very labour intensive and very insensitive so that molecular epidemiological studies of wild isolates have been limited to a few samples where infected faeces were available in quantity (Rodger & Holmes, 1979). The method described removes the requirement for virus

purification before analysis, thereby greatly reducing the work involved in producing the genome profile. Also the use of in vitro labelling to mark the genome segments has increased the sensitivity of detection some 5000-10,000 fold. These two facts combined mean that very small quantities and/or very low level infections can be subject to genome profile analysis. It should therefore now be technically feasible to carry out much wider molecular epidemiological surveying of the genome profiles of wild isolates of rotavirus to examine the degree of virus variation occurring with these virus infections.

During the course of this thesis approximately 200 isolates were analyzed by this method for producing genome profiles. Examples of some comparisons of genome profiles of human, pig and calf rotavirus isolates are shown in Figures 11 and 12. There is variation in migration rates for all the genome segments, this variation being as great between isolates from the same animal species as between isolates from different animal species. Clearly the genome profiles cannot in most cases be related to the animal species from which the rotaviruses were isolated. Figure 11a shows four pig rotavirus genome RNA profiles, two of which (tracks A and B) were isolated during a single outbreak on the same farm (infact isolates A and B originate from a single litter of piglets). This illustrates that rotavirus samples isolated in the same place at the same time can have different genome profiles.

The genome profile analysis of five human isolates is shown in Figure 11b. Track A is a 'short' profile isolate, the others being 'long' profile isolates. These short genome profile isolates have not been observed in any other animal species. Long and short profile

Figure 11(a). Comparative Genome Profile Analysis of 4 Pig Rotavirus Isolates.



Fractionation was on a short 7.5% polyacrylamide gel.

Track A = 2758 (Nantwich)

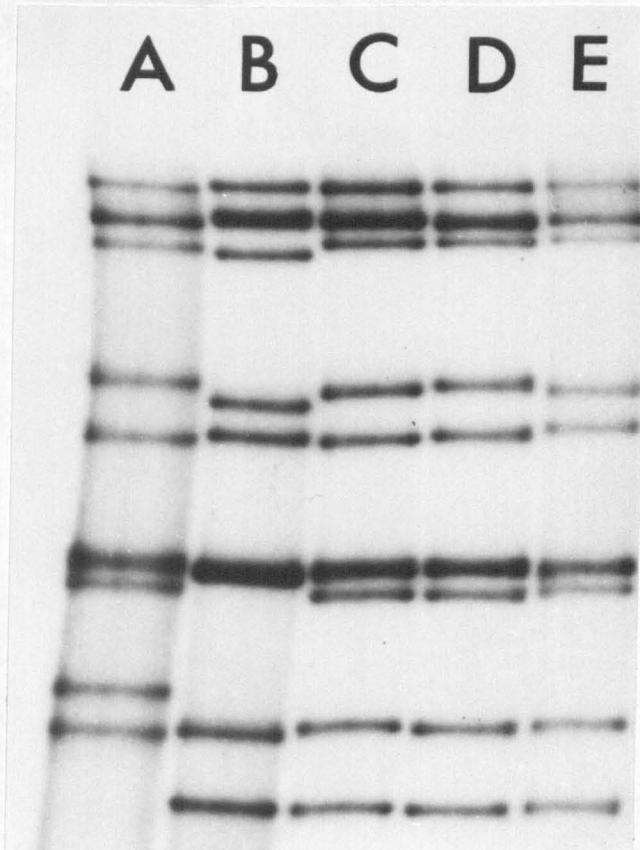
Track B = 2759 (Nantwich)

Track C = 23015 (Reading)

Track D = 21438 (Reading)

Mobility variation are evident for most of the corresponding genome segments. Isolates in tracks A and B originated from the same litter (see text).

Figure 11(b). Polyacrylamide Gel Fractionation of Five Human Rotavirus Isolates on a Short 7.5% Polyacrylamide Gel.



Mobility variations exist for most of the corresponding genome segments. Track A shows a 'short' profile isolate, tracks B → E are 'long' profile isolates. Place of origin given in brackets.

Track A = Parsons (London)

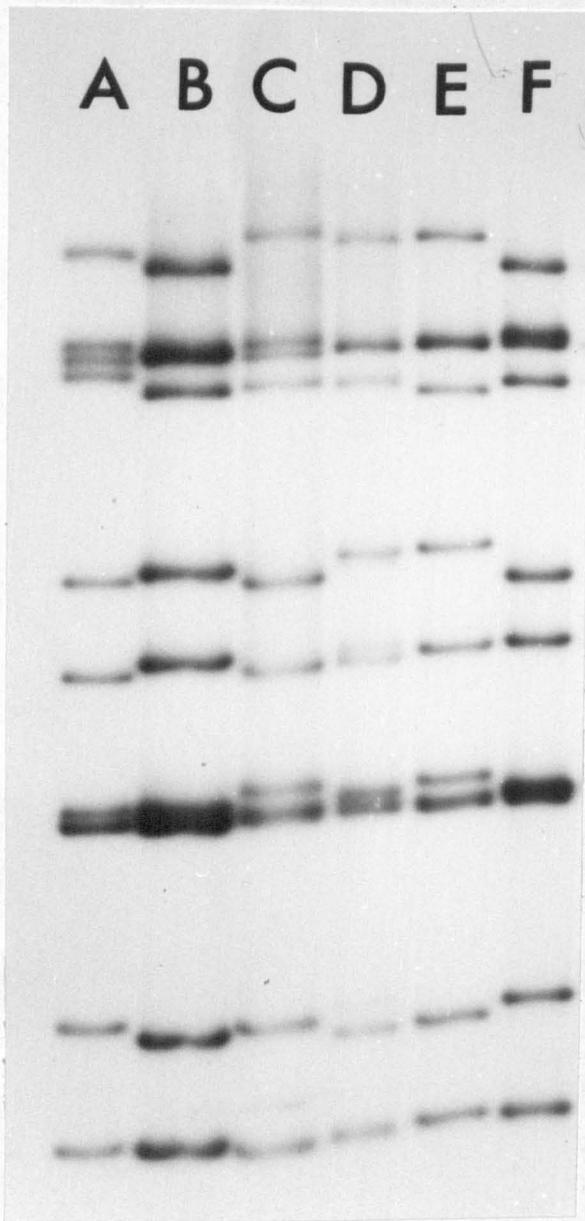
Track B = Norman (London)

Track C = Mekim (London)

Track D = Hook (London)

Track E = Lam (London)

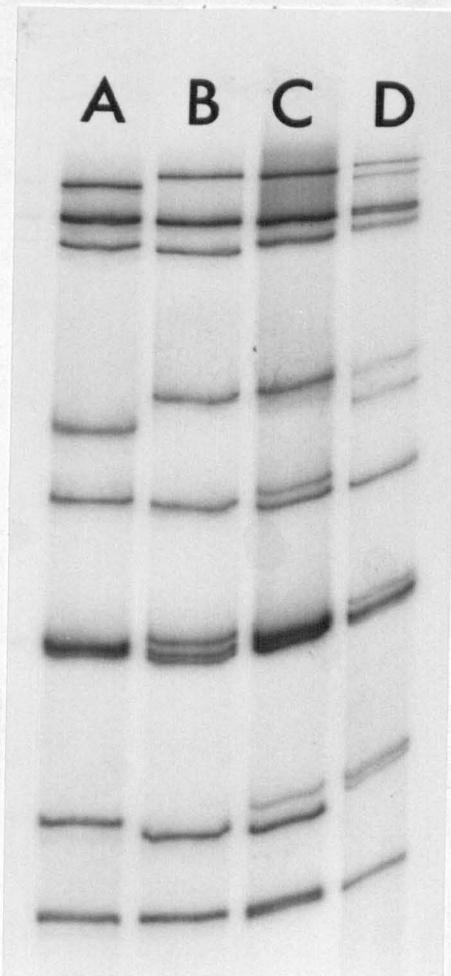
Figure 12 (a). Polyacrylamide Gel to Show a Comparative
Genome Analysis of 5 Field Isolates of Calf Rotavirus with the
U.K. Tissue Culture Adapted Calf Rotavirus.



Extraction of either infected faeces or purified virus (tissue culture adapted virus) and subsequent 3' end labelling were both carried out as described in Materials and Methods. Samples were analyzed on a high resolution (40 x 40 cm) 7.5% polyacrylamide gel. The field isolates were from 5 geographically distinct locations given in brackets.

- Track A = U.K. tissue culture adapted calf rotavirus (Compton)
Track B = 5635 (Exeter)
Track C = 3973 (Reading)
Track D = 4756 (Penrith)
Track E = 3665 (Northants)
Track F = 4329 (Shrewsbury)

Figure 12(b). Comparative Genome Profile Analysis of 4 Field Isolates of Calf Rotavirus to Show Evidence of Mixed Infections.



3' end labelled virus RNA was fractionated at 20 mA for 16 hrs on a 20 x 20 cm 7.5% polyacrylamide gel.

Track A = 4329 (Shrewsbury)

Track B = 3665 (Northampton)

Track C = 4756 (Penrith)

Track D = 3973 (Reading)

The isolates in tracks C and D show genome profiles with more than 11 segments indicating the occurrence of mixed infections.

human isolates can also be distinguished serologically on the basis of differences detected by enzyme linked immunosorbent assay (Konica et al., 1981).

Figure 12a shows the genome profiles of six calf rotavirus isolates, again a high level of genome segment variation can be observed. Figure 12b also shows the genome profiles of 4 calf rotavirus isolates, tracks C and D show the presence of at least 12 distinct dsRNA genome segments indicative of infections with more than one genome profile. Of the rotavirus isolates analyzed during the course of this thesis approximately 10% of the samples have shown similar evidence for mixed infection. Mixed infection is a necessary pre-requisite for the occurrence of gene re-assortment which may be an important mechanism for generating new pathogenic strains of rotavirus.

Simple genome profile analysis can now be used on a wider range of samples by application of the rapid and reproducible technique described. Studies have revealed that the level of variation in migration of corresponding genome segments is high regardless of the species of origin of the rotavirus isolates. However, it has not been possible to make definitive molecular epidemiological conclusions from these studies as the structural basis of genome segment variation is unknown. Hence interpretation of the variation between genome profiles can only be speculative.

RESULTS CHAPTER 2

Most of the work in this Chapter has appeared in press

(*J. Virol. Methods*, 3 (1981) 261-269).

Chapter 2

A Sensitive Method for the Production of Diagnostic Fingerprints of the Genome Segments of Rotavirus Field Isolates

Introduction

The analysis of migration patterns of dsRNA genome segments from a number of rotavirus isolates has shown major variations in the migration of each segment. This variability in migration of dsRNA segments has been seen in rotavirus isolates from different animal species, in rotavirus isolates from the same animal species, and in isolates collected from the same and different locations. Therefore comparisons of the RNA profiles from virus isolates of different origins have not identified the host species or the geographic location of isolation. An understanding of the structural basis of rotavirus genome segment migration was required before definitive conclusions could be drawn about similarities or differences seen between genome profiles. Therefore an essential requirement was to find a technique that would allow the diagnostic characterization of individual rotavirus genome segments.

Diagnostic fingerprints of isolated dsRNA species could have been produced using the classical two dimensional T_1 oligonucleotide fingerprinting procedure of De Wachter & Fiers (1972) following in vitro labelling as described by Frisby et al. (1977). However, considering that rotaviruses have eleven genome segments this approach has the major disadvantage that it is very labour intensive and so could have only been applied to a very limited number of isolates.

Alternatively cDNA may have been synthesized in vitro from either

double-strand denatured rotavirus genomic RNA or from mRNA. The cDNA could then have been used to study genetic relatedness by annealing with dsRNA from other rotavirus isolates. Although the hybridization approach could have been applied to a much larger number of samples than would have been possible by oligonucleotide fingerprinting, it was envisaged that serious interpretation problems may have arisen for isolates where genome segments were unrelated or only partially related to the hybridization probes.

These considerations led to an idea for the development of a new technique to characterize dsRNA based principally on RNA sequence analysis of 3' terminally labelled viral dsRNA as described in Materials and Methods. In brief outline, the proposed technique would rely on the isolation of individual terminally-labelled genome segments which could then be subjected to partial digestion with a base specific ribonuclease. To produce a diagnostic partial digestion "fingerprint" for a particular genome segment the nuclease generated fragments would then be electrophoretically separated in one dimension on a polyacrylamide gel. This approach should allow the reliable analysis of a wide range of samples.

Results

The primary objective in the development of this technique was to find a rapid and simple way to isolate individual dsRNA genome segments. Polyacrylamide gel electrophoresis using the Laemmli (1970) discontinuous buffered system has been employed by several groups to achieve good fractionation of rotavirus dsRNA genome segments. Although polyacrylamide is a very effective separating medium the high

concentrations used in the fractionation of dsRNA created problems for methods of recovering these nucleic acids. A number of methods for recovering RNA from polyacrylamide were investigated. It was found that electro-elution which gives a high yield of recovered RNA was very labour intensive. Other methods of elution such as passive diffusion or physical/mechanical forces which could be simultaneously applied to a large number of samples gave very poor recoveries.

Therefore attempts were made to denature and digest isolated dsRNA species in excised polyacrylamide gel slices. However, under these conditions ribonuclease digestion proved impossible to control as shown by Figure 13. This figure shows attempts at digestion of denatured dsRNA species 10 from a bovine isolate in excised gel slices over a 10^5 dilution range of T_1 ribonuclease concentrations. In excised gel slices denatured dsRNA was extremely resistant to ribonuclease digestion.

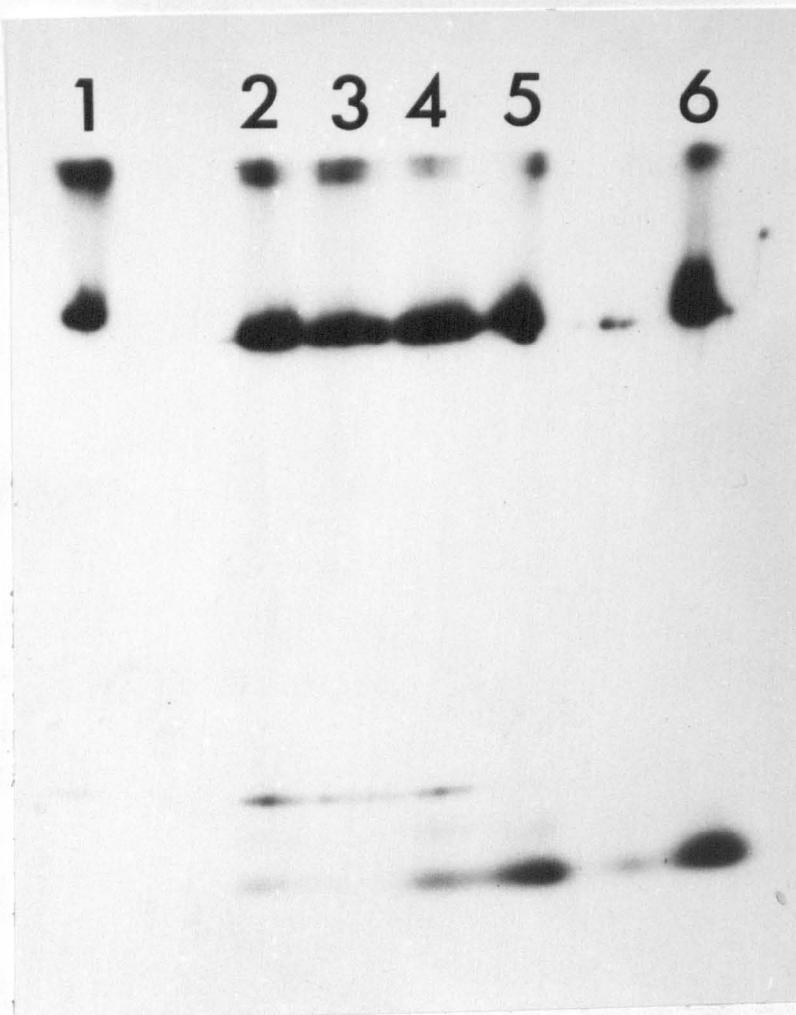
Because of the problems encountered in recovering dsRNA from polyacrylamide its use as a preparative fractionation system was abandoned.

Agarose was investigated as an alternative separating medium. Agarose has the benefits of being easier to handle and gives much better recoveries of nucleic acids from gel slices. However its major disadvantage as a separating medium is a loss of resolution.

A number of different buffer systems (Tris-phosphate, Tris-HCl, Tris-borate, glycine-sodium hydroxide and Tris-acetate) and agarose

Figure 13. Fractionation of the Digestion Products of 3'

Terminally Labelled Bovine Rotavirus Species 10 RNA's.



This 7.5% polyacrylamide gel was run at 20 mA for 5 hrs.

Individual gel slices containing dsRNA were subjected to treatment with 90% DMSO at 50°C for 30 mins following ds denaturation these slices were placed in 25 µl of digestion buffer at different enzyme concentrations. Both digestion buffer and gel slices were loaded in the same well for analysis after

30 minutes incubation at 37°C.

Track 1 = Control with no T_1 ribonuclease

Track 2 = T_1 ribonuclease at 0.2 units/ml

Track 3 = T_1 ribonuclease at 2 units/ml

Track 4 = T_1 ribonuclease at 20 units/ml

Track 5 = T_1 ribonuclease at 200 units/ml

Track 6 = T_1 ribonuclease at 2,000 units/ml

gel concentrations were studied to find the best conditions for rotavirus dsRNA separation. All suffered from the same artefactual problem of the specific loss of particular genome segments during electrophoresis. The tris-acetate buffered system in 1.5% agarose was the gel separating system that showed this effect the least (see Figure 14). Figure 14 shows this disappearance effect in the tris-acetate gel system. This effect increased with increasing electrophoresis time. The same sample run on an acrylamide gel showed all genome segments to be present in equimolar amounts. The addition of tRNA to the sample or the pre-running of tRNA through the sample well appeared to reduce the effect (see Figure 15) indicating that this phenomenon may be due to some form of non-specific loss.

The problem was eventually alleviated by the use of 6 M urea in the separating gel giving rise to partially denaturing conditions during dsRNA fractionation.

Figure 16 shows the fractionation of rotavirus RNA achievable on a 1.5% agarose gel containing 6 M urea and using the Tris acetate buffer system. Using agarose, individual resolution of species 2,3, and 7,8,9, was not possible. However, since characteristic fingerprints could be generated from a mixture of species the ability to isolate only eight discrete fractions did not represent a major drawback of this system. Several methods were attempted as ways for recovering dsRNA from these preparative agarose gels, ^{and} the use of the "freeze-squeeze" technique for recovery (see Materials and Methods) proved to be both the most rapid and efficient of the methods tried.

Figure 14. 1.5% Vertical Agarose Gel Fractionation of Rotavirus dsRNA Using Tris-Acetate Buffer as Described in Materials and Methods.



3' terminally labelled rotavirus genomic RNA was loaded at different times.

Electrophoresis Time

Track A = 1 hr

Electrophoresis Time

Track B = 2½ hrs

Track C = 4 hrs

Track D = 5½ hrs

Track E = 7 hrs

Track F = 8½ hrs

Electrophoresis was at 40 mA at 4°C.

Figure 15. Fractionation of 3' Terminally Labelled Rotavirus dsRNA on a 1.5% Vertical Agarose Gel in the Tris Acetate Electrophoresis Buffer.



Electrophoresis was at 40 mA for 4 hrs at 4°C. Both pre-running with tRNA added to one electrophoresis well or added to the sample reduced the disappearing band effect. The gel was pre-run for 1 hr prior to loading.

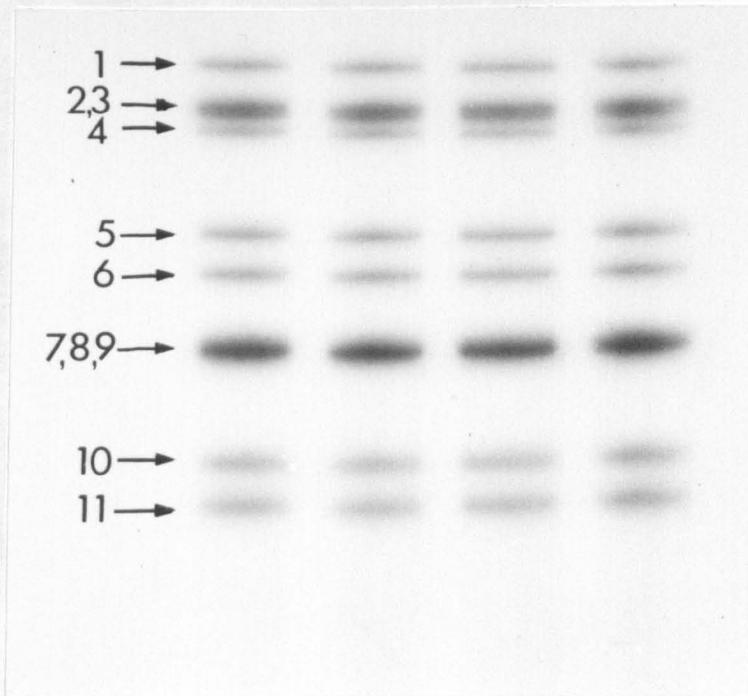
Track 1 = 20 µg tRNA loaded at the beginning of pre-run time

Track 2 = no tRNA added

Track 3 = 20 µg tRNA added to the dsRNA sample

Track 4 = no tRNA added.

Figure 16. Preparative Fractionation of End-Labelled Field-Isolated Bovine Rotavirus dsRNA Run on a 1.5% Agarose Gel.



This gel contained 6M urea and the tris-acetate buffered system was used. Migration was from top to bottom; numbering the genome segments shows that on this system segments 2, 3 and 7, 8, 9 co-migrate. Electrophoresis was at 30 mA for 16 hrs at room temperature.

Table 2 shows the recovery obtained on eluting RNA species from agarose gels as described in Materials and Methods. The recovery values of 30-35% were based on the total number of counts loaded onto the gel, although not all of the radioactivity was incorporated into dsRNA. If recovery values were estimated on the basis of the counts recovered from those detected in excised gel slices by Cerenkov counting then the recovery value increased to 50-70%.

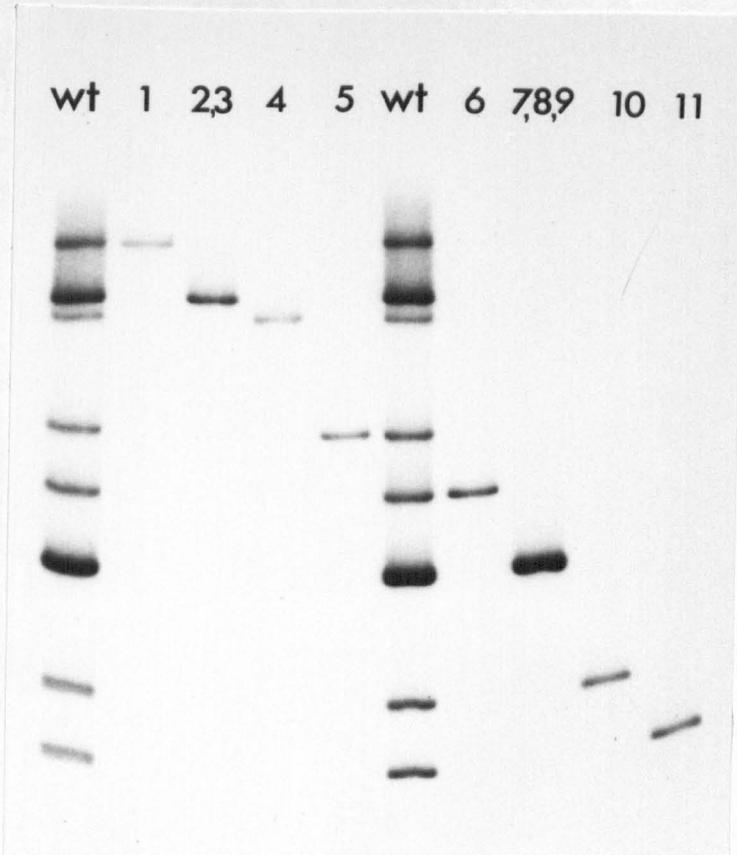
Having established that agarose was a suitable medium for resolving genome segments and that it allowed a good yield of isolated fragments to be easily obtained, it was important to check that the recovered RNA was not badly degraded and that the change in gel buffer system did not affect the relative migration pattern of RNA species. Figure 17 shows the isolated dsRNA species re-run on a 7.5% polyacrylamide gel using the Laemmli discontinuous buffer system. It shows that the isolated species were obtained without cross-contamination or detectable degradation and had the same relative mobilities in the two gel systems.

Having established a rapid and reproducible method for isolating individual 3' end labelled dsRNA genome segments the next requirement was a method for producing partial digestion of the dsRNA. The most specific nuclease is T_1 ribonuclease which cleaves only on the 3' side of G residues. For this reason partial digestion with T_1 ribonuclease was chosen to generate a series of oligonucleotide nucleotide fragments of which only those retaining the 3' end labelled nucleotide were seen following gel fractionation and autoradiography. Before digestion could be achieved the ribonuclease-resistant double stranded RNA was

Table 2. Recovery of dsRNA from Agarose Gels

Species of ds RNA	Total counts of each species loaded on the gel ($\times 10^{-4}$) based on total loading of 22×10^6 c.p.m.	Total counts of each species recovered from gel slice ($\times 10^{-4}$)	% recovery
1	218	69	32
2,3	436	120	27.5
4	218	65	29.8
5	218	77	35.3
6	218	89	40.8
7,8,9	654	215	32.8
10	218	67	30.7
11	218	74	33.9

Figure 17. Individual Isolated Species of dsRNA, Extracted
From the Preparative Agarose Gel as Described in Materials and
Methods, Re-Run on a 7.5% Polyacrylamide Gel Using the Laemmli
Discontinuous Buffered System.



Isolated species are numbered consecutively across the top of the autoradiograph; the whole wild type genome for comparison is designated 'wt'. Electrophoresis was at 20 mA for 16 hrs at 4°C.

denatured by treatment with 90% DMSO at 50°C for 20 mins.

In conjunction with partial T_1 digestion, partial digestion with alkali was also used as a means of achieving random cleavage of the RNA chain and thereby generating the so-called 'ladder' of oligonucleotides, all differing in size by a single base (Donis-Keller et al., 1977). The separate application of these two treatments to an isolated species of dsRNA after denaturation allowed the positions of the G residues up to approximately 100 bases from the 3' end of each RNA strand, i.e. a total of 200 nucleotides of any given species, to be uniquely positioned. The G position pattern generated will obviously be completely diagnostic for any given species of RNA, and could be expected to allow both major and very minor changes in RNA sequence to be identified.

The optimum conditions for partial digestion were found by adjusting the background tRNA levels and the T_1 ribonuclease enzyme concentrations. Uniform reproducible digestions were obtained by digestion against a high background of tRNA with a relatively high ribonuclease concentration (see Materials and Methods for conditions). For initial studies the partial digestion products were fractionated on 18% thin polyacrylamide gels (Maxam & Gilbert, 1977; Sanger & Coulson, 1978).

To illustrate the results achievable with the technique two field isolates of bovine rotavirus were taken for detailed analysis, Figure 18 shows simple genome profile analysis of these two isolates on an analytical polyacrylamide gel.

Figure 18. Comparative Genome Analysis of Two Field Isolates
of Bovine Rotavirus (A and B) Used to Illustrate the Method
for Producing 'Fingerprints' of the Genome Segments.

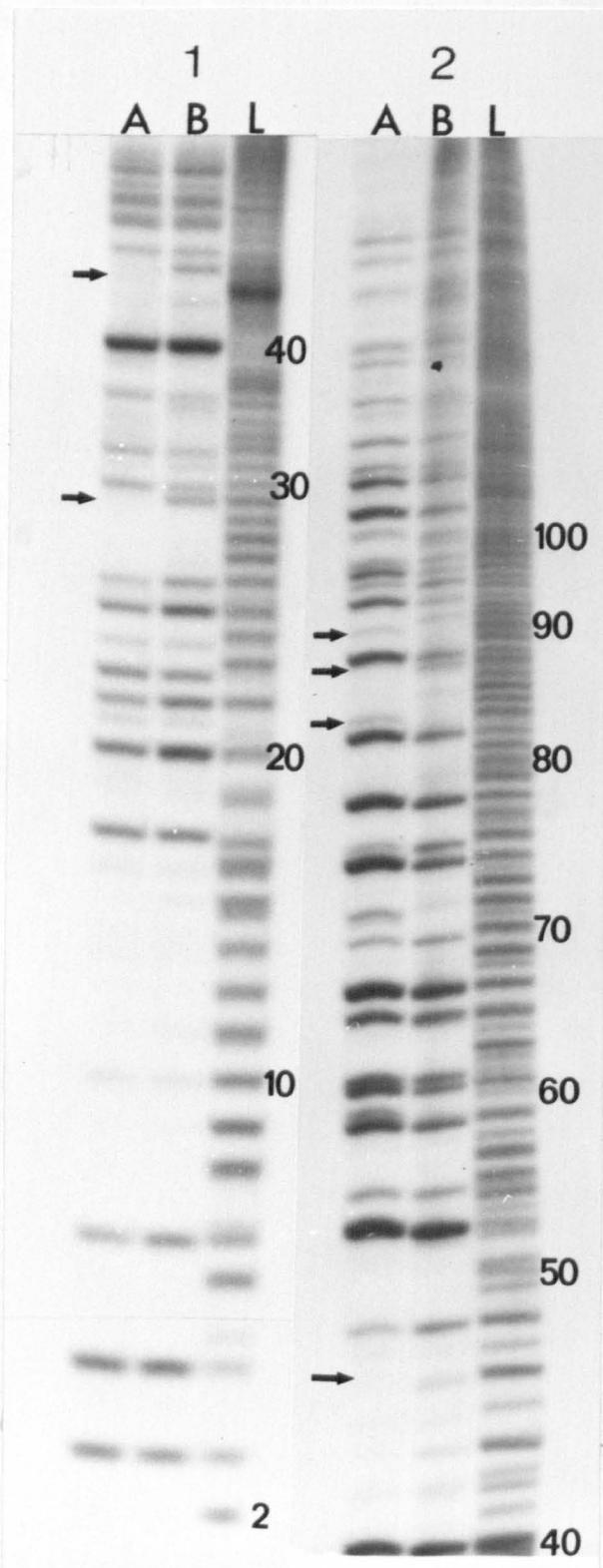


These samples were run on a 7.5% polyacrylamide gel using the Laemmli discontinuous buffered system. Genome segments are numbered from top to bottom. Electrophoresis was at 20 mA for 16 hrs at 4°C.

Figure 19 shows the nuclease digestion patterns obtained on comparing the two species 10 RNA's which do not co-migrate in the genome profile analysis (Figure 17). From two gel loadings at least 100 nucleotide positions from the terminus can be easily discerned. Several differences between the two corresponding RNA species are arrowed. Figure 20 shows the partial nuclease digestion patterns for the corresponding species 5 RNA's. These RNA species which have a greater mobility difference on simple genome profile analysis (Figure 18) also have more banding differences between their fingerprints. Comparison of Figures 19 and 20 show similarity in patterns for only their terminal 7 nucleotide positions consistent with the evidence for conserved terminal sequences of 8 nucleotides for all rotavirus genome segments (McCrae & McCorquodale, 1982b). Beyond this position the fingerprints for both sp10 RNA's are totally different from those of both the sp5 RNA's. These results confirm that partial nuclease digestion analysis produces highly diagnostic fingerprints for individual rotavirus genome segments.

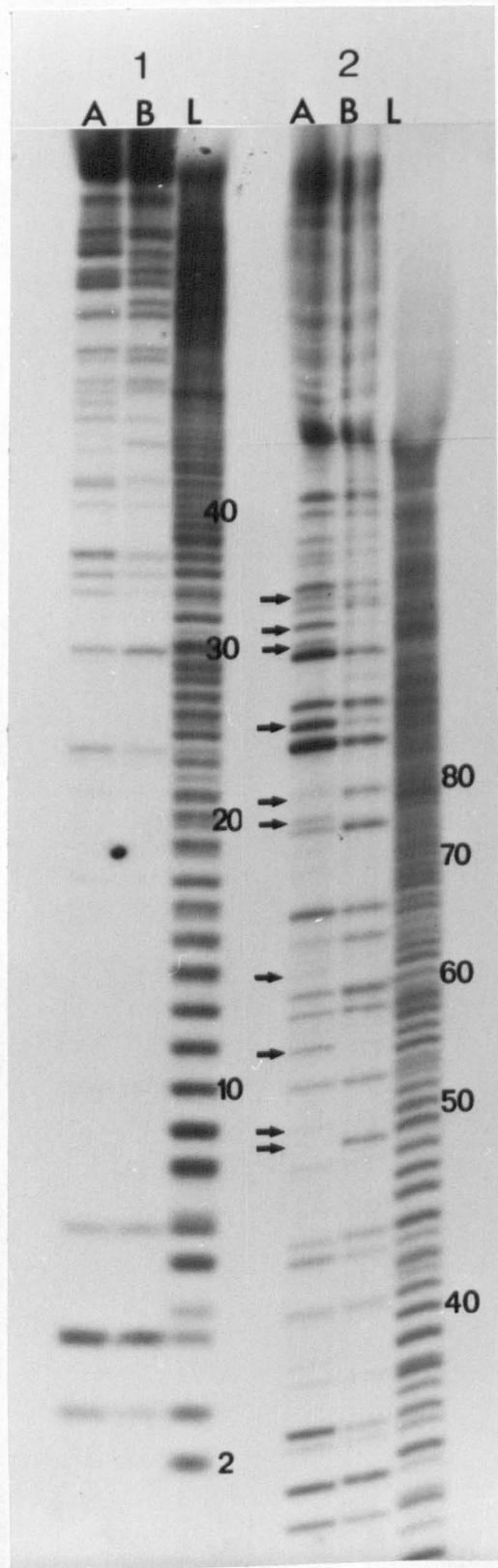
The widespread application of this partial nuclease digestion fingerprinting method required that it had a high sensitivity, permitting fingerprints to be generated with the minimum amount of isolate possible. The minimum number of counts required to produce a diagnostic T_1 pattern is approximately 3000 c.p.m., this requiring a 10 day exposure to visualize the pattern by autoradiography. Since the ladder track pattern is not RNA species-specific, it can be produced from any RNA species that is available in large amounts. Therefore, using the above figure together with the recovery value of 30% achieved on gel extraction of a given species (Table 2),

Figure 19. Partial Nuclease Digestion Analysis of the Sp10
RNA's from Isolates A and B.



The tracks labelled L are the partial hydrolysis reference ladder tracks, the nucleotide positions are numbered up to position 100. This 18% polyacrylamide gel (20 x 40 cm) was loaded twice, loading 1 was run for 3½ hrs and loading 2 was run for 8 hrs at 1.6 KV. Tracks A and B contain the partial digestion products of the Sp10 RNA's from isolates A and B, the two loadings begin to overlap at nucleotide position 40. Differences in banding patterns between these two corresponding genome segments are marked with arrows. The terminal nucleotide is not observed in the reference ladder by this method of hydrolysis.

Figure 20. 18% Polyacrylamide Gel Showing Partial Nuclease
Digestion Analysis of the Sp5 RNA's From Isolates A and B.



Tracks L are the partial hydrolysis reference ladder tracks, the nucleotide positions are labelled up to position 80. This gel was loaded twice, loading 1 has been run for 3½ hrs and loading 2 has been run for 6½ hrs at 1.6 KV. Tracks A and B contain the partial digestion products of the species 5 RNA's from isolates A and B, the two loadings overlap beyond nucleotide position 30. Differences in banding patterns between these two corresponding genome segments are marked with arrows.

approximately 10,000 c.p.m. per RNA species are required for fingerprint production. Since there are 11 species of viral RNA, then approximately 110,000 c.p.m. is the minimum amount of incorporated material required to produce a complete fingerprint analysis of a given isolate. Calibration of the sensitivity of the 3' end labelling technique (Results, Chapter 1) allowed the conclusion that a fingerprint analysis requiring 110,000 incorporated cts/min into the dsRNA rotavirus genome could be performed on a 1 ml sample of faeces containing a minimum of $2\text{--}5 \times 10^9$ virus particles/ml. This means that it should be possible to produce fingerprints from the majority of rotavirus field isolates.

Discussion

The original method for analyzing genome profiles of rotavirus field isolates has been extended to provide a rapid, reproducible and sensitive technique for the production of diagnostic fingerprints of rotavirus genome segments. The technique developed relies on the use of partial nuclease digestion with a base-specific nuclease to generate a diagnostic set of overlapping oligonucleotides labelled at their 3' termini. This method has a number of advantages for epidemiological surveying work over the more traditional two-dimensional oligonucleotide fingerprint analysis that has been employed in other virus systems (Desselberger *et al.*, 1978; Walker *et al.*, 1980). The production of the fingerprint pattern is achieved using a single dimension gel fractionation which is much less labour-intensive, making it practically feasible to apply it to a wider range of virus isolates. The fingerprinting method operates by defining the location of G residues in the

RNA relative to the termini. Therefore, as with the traditional two-dimensional method the number of diagnostic bands obtained is proportional to the G content of the RNA but is independent of its length.

The method requires only a simple single dimension gel analysis to define exactly the G positions relative to the termini for a given species of dsRNA. This will facilitate the reliable comparison of results obtained at widely different times in the same laboratory and, more important, it should allow results obtained in different laboratories to be similarly compared.

RESULTS CHAPTER 3

Chapter 3

Initial Partial T₁ Ribonuclease Digestion Analysis of Five Bovine

Rotavirus Isolates

Introduction

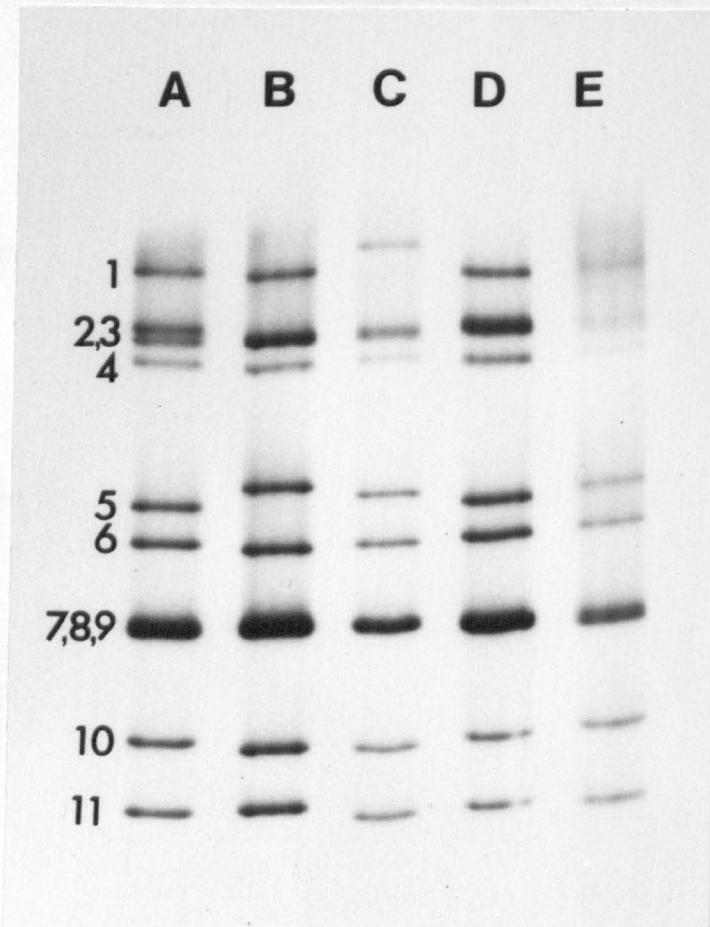
With no serological data available the choice of rotavirus isolates to be analyzed by one-dimensional partial nuclease digestion had to be made on a number of arbitrary criteria. Bovine isolates were chosen for the preliminary studies as a large collection of these samples was available. Isolates giving only 11 genome segments were analyzed, ^{since} A these were considered to represent single infections. To maximize the chance of finding variation, isolates were selected on the basis of differences in their genome profiles and for differences in the time and geographic location of their isolation.

Results

Five isolates were chosen for analysis, ^{since} A this was considered the maximum feasible number of samples that could be handled at one time. One dimensional genome profiles of 3' end labelled dsRNA extracted from the five isolates labelled A-E are shown in Figure 21. Electrophoretic variation can be observed for genome segments 1, 2, 4, 5, 6, 10 and 11. For all five isolates the 7, 8, 9 complex migrated as a tight unresolved triplet.

Preparative fractionation of the dsRNA segments for four of the rotavirus isolates is shown in Figure 22 under the partially denaturing conditions of the tris-acetate-urea preparative agarose gel system relative mobility differences observed between different

Figure 21. Polyacrylamide Gel to Show a Comparative Genome
Profile Analysis of the Five Wild Bovine Isolates Used in this
Study.



Viral RNA was 3' end labelled as described in Materials and Methods. Samples were run on a 20 cm 7.5% polyacrylamide gel at 20 mA for 16 hrs. The isolates were from geographically distinct locations within the U.K. given in parentheses.

Track A = Isolate A (483 Truro)

Track B = Isolate B (4331 Exeter)

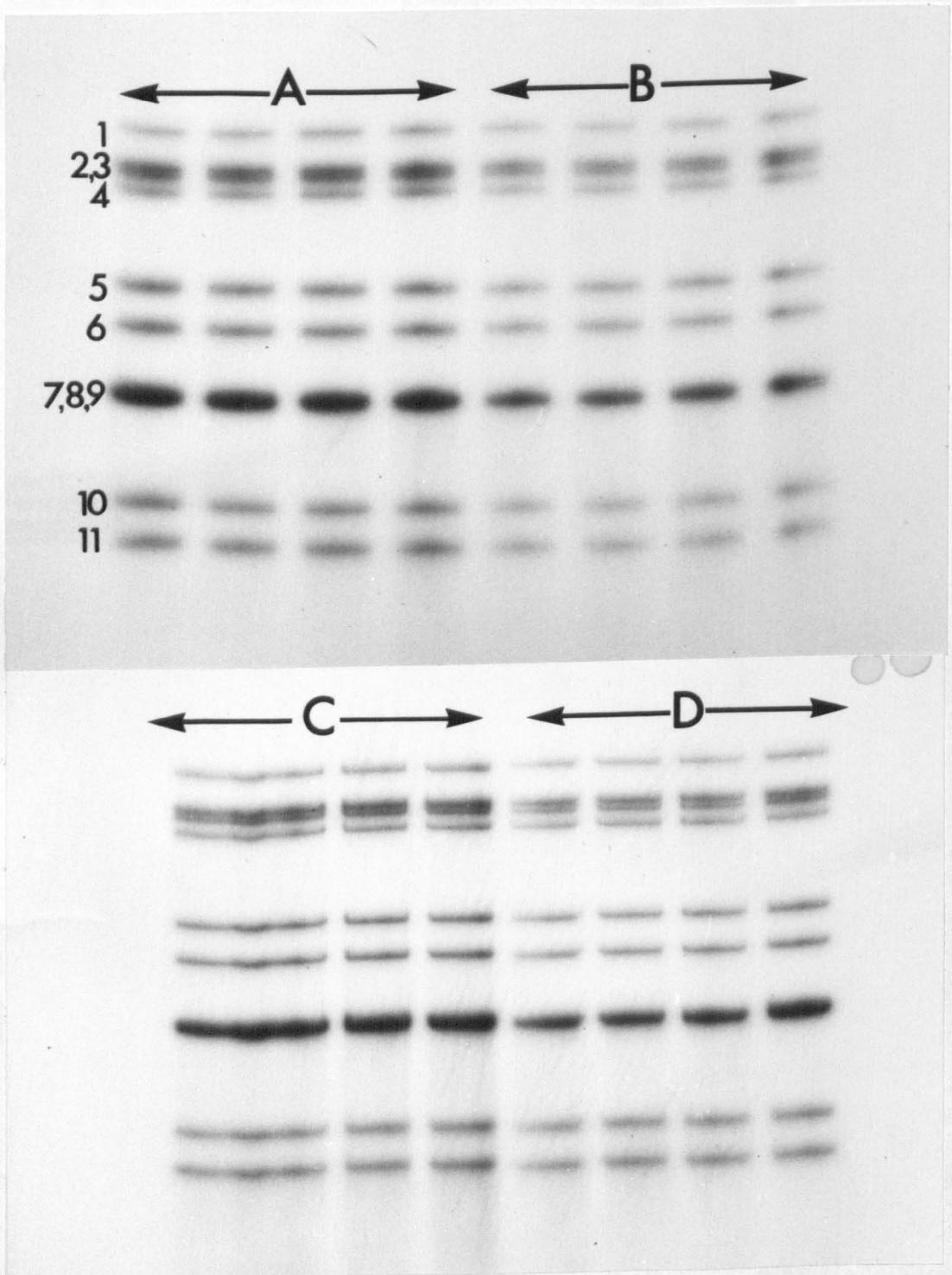
Track C = Isolate C (2855 Carmarthen)

Track D = Isolate D (4329 Shrewsbury)

Track E = Isolate E (118 Penrith)

Migration was from top to bottom, the genome segments of wild isolate A are numbered from 1 to 11. Differences in migrational rates can be observed for most of the corresponding genome segments.

Figure 22. Preparative Fractionation of 3' End Labelled Viral
RNA From Four of the Five Wild Bovine Isolates.



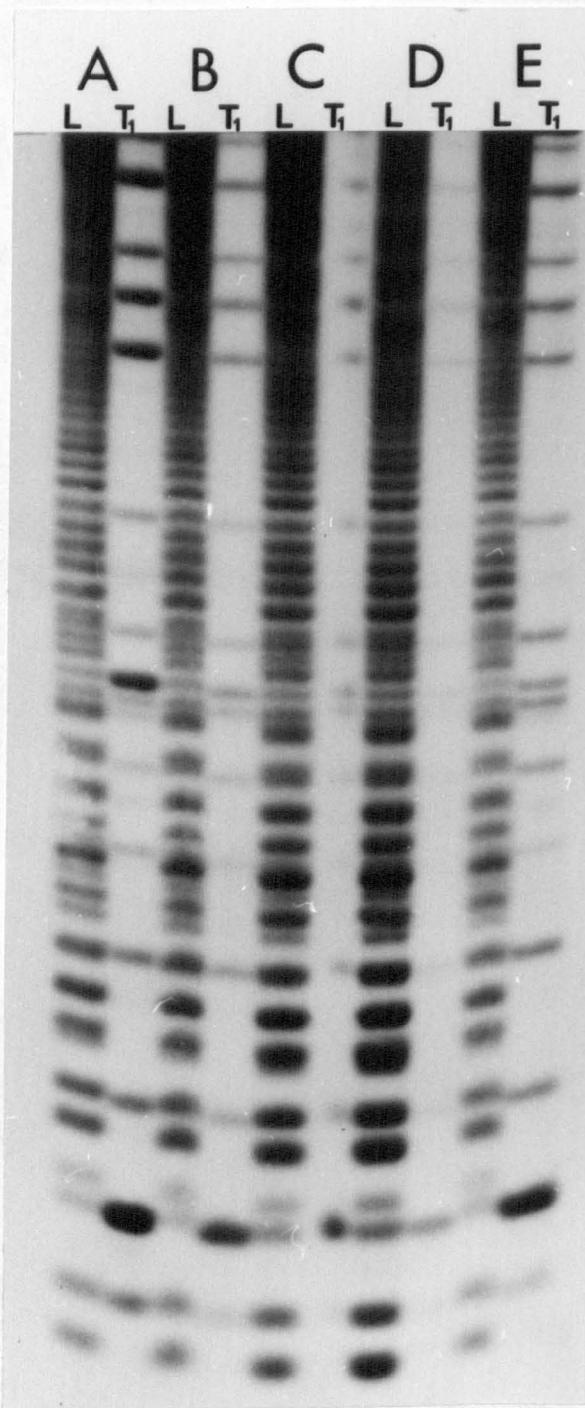
The isolates (labelled A-D) were each loaded across four gel tracks on these two preparative 1.5% agarose gels. Electrophoresis was at 30 mA for 16 hrs at room temperature. Migration was from top to bottom, the genome segments of wild isolate A are labelled from 1 to 11. Relative migrational differences observed for corresponding genome segments in Figure 21 disappear on preparative fractionation.

isolates on polyacrylamide disappeared. Eight discrete fractions of each genome were extracted from these preparative gels providing 40 samples for one-dimensional partial nuclease-digestion analysis.

Figs 23-29 show the one-dimensional partial nuclease digestion patterns of the terminal 2-40 nucleotides for seven of the eight fractions extracted, the autoradiograph of the Sp4 RNAs was too faint to allow it to be reproduced photographically. All genome segments studied had the same pattern for the terminal seven nucleotides indicating the absolutely conserved terminal region (McCrae & McCorquodale, 1982). Beyond this common region the analyses showed that each set of genome segments had a unique map of terminal G residues derived from both RNA strands which allowed their individual and hence highly diagnostic characterization. This is best illustrated by cross comparing figures and by Figure 29 where partial digestion products of a segment 1 were mistakenly run with four species 11 RNA's.

Despite differences in migrational rates on polyacrylamide corresponding genome segments had very similar partial nuclease digestion maps for their terminal 2-40 nucleotides. One simple explanation for this remarkable similarity in banding patterns was that the nucleotide sequences in the terminal regions of corresponding genome segments are highly conserved. Unfortunately the only recovered dsRNA samples with enough incorporated counts to allow further analysis of near terminal regions was the "triplet" comprising RNA species 7, 8, and 9. Figure 27b shows this gel analysis covering the region of 30-100 terminal nucleotides - no more banding differences are evident.

Figure 23. Partial T_1 Ribonuclease Digestion Fingerprints
Obtained for the Five Species 1 dsRNAs of the Isolates Under-
study.



The five individually isolated species 1 RNAs are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 21. Each sample has been divided into two parts, the tracks marked L are the partial hydrolysis reference ladders for each sample, the tracks marked T_1 are the partial T_1 ribonuclease digestion patterns obtained for each sample. This 18% 20 x 40 cm gel was run at 1.6 KV for 2½ hrs. Unfortunately on this particular gel the T_1 partial digestion products for most of the samples were very faint especially so for isolate D. However this figure serves to illustrate both the absolutely conserved region of terminal nucleotides (< 10) and the surprising very high similarity in banding patterns observed beyond this region. These isolates have considerable mobility differences for their species 1 RNA's on simple genome profile analysis (Figure 21).

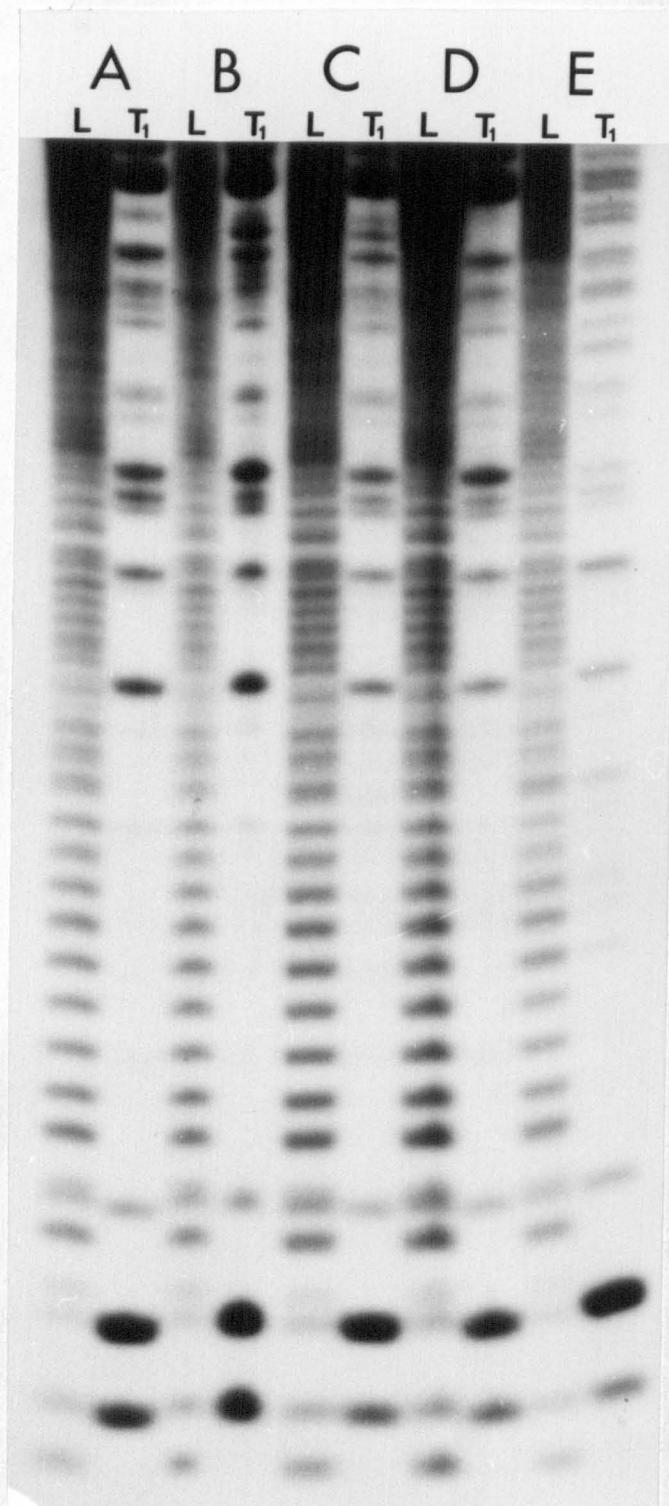
Figure 24. Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Species 2 and 3 dsRNA "Doublets" of the Five
Isolates Under Study.



The five samples are labelled A to E the running conditions and arrangement of sample is as described in the legend to Figure 23. The samples on this gel have been slightly under-digested hence the smaller (< 30) nucleotides oligonucleotide fractions are less well represented. This figure illustrates a high level of banding pattern similarity for the terminal 2-40 nucleotides from the combined digestion of RNA species 2 and 3.

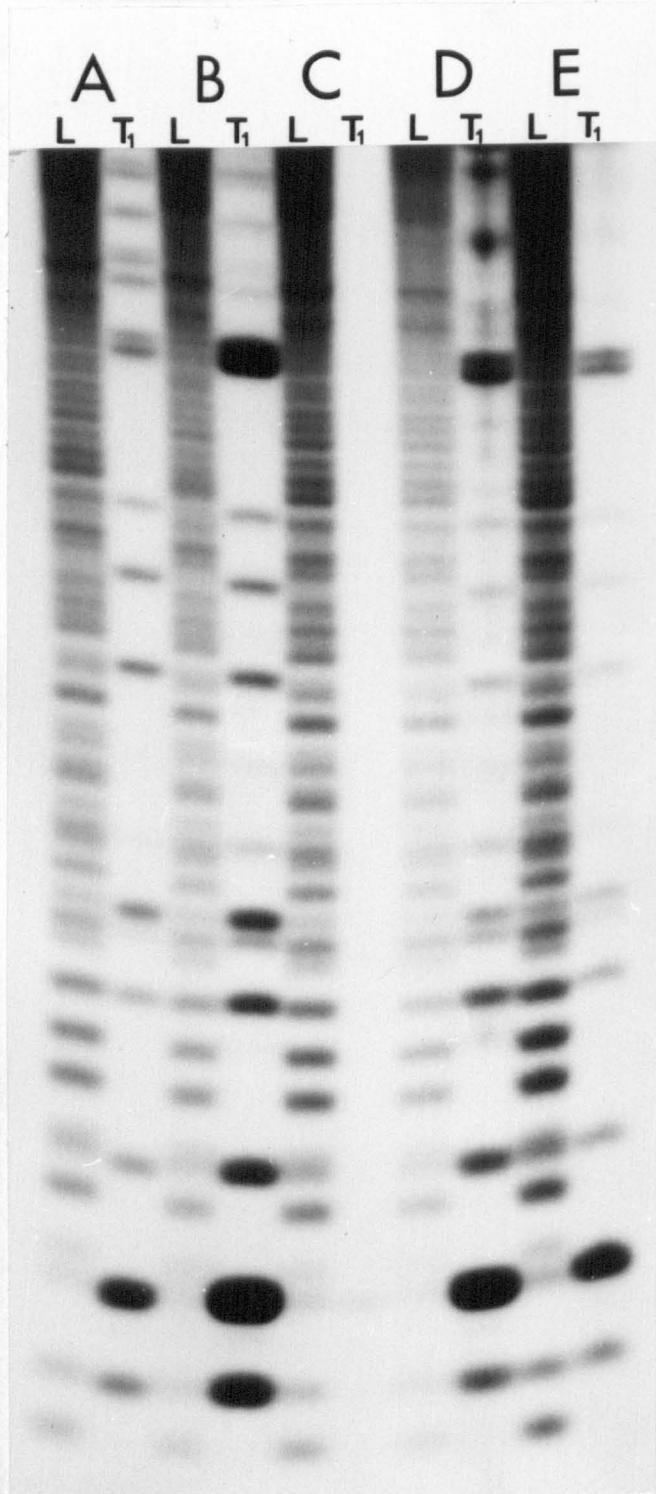
Figure 25. Partial T₁ Ribonuclease Fingerprints Obtained For
the Five Species 5 RNA's.



The five individually isolated species are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 21. The layout and the running condition of the samples is as described in the legend to Figure 23. This figure clearly illustrates the absolutely conserved terminal region (< 10 nucleotides) and also the similarity in banding patterns beyond this region shared by all the species 5 RNA's.

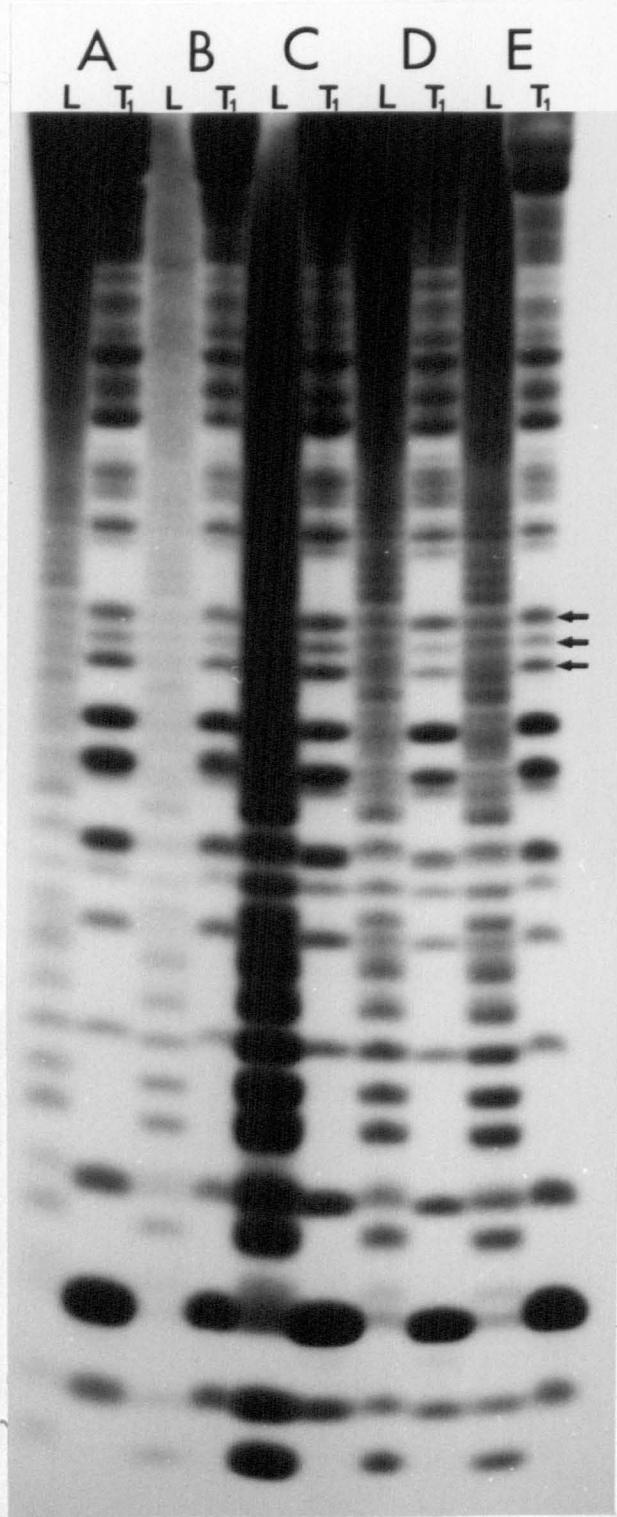
Figure 26. Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Species 6 dsRNA's of the Five Isolated Under
Study.



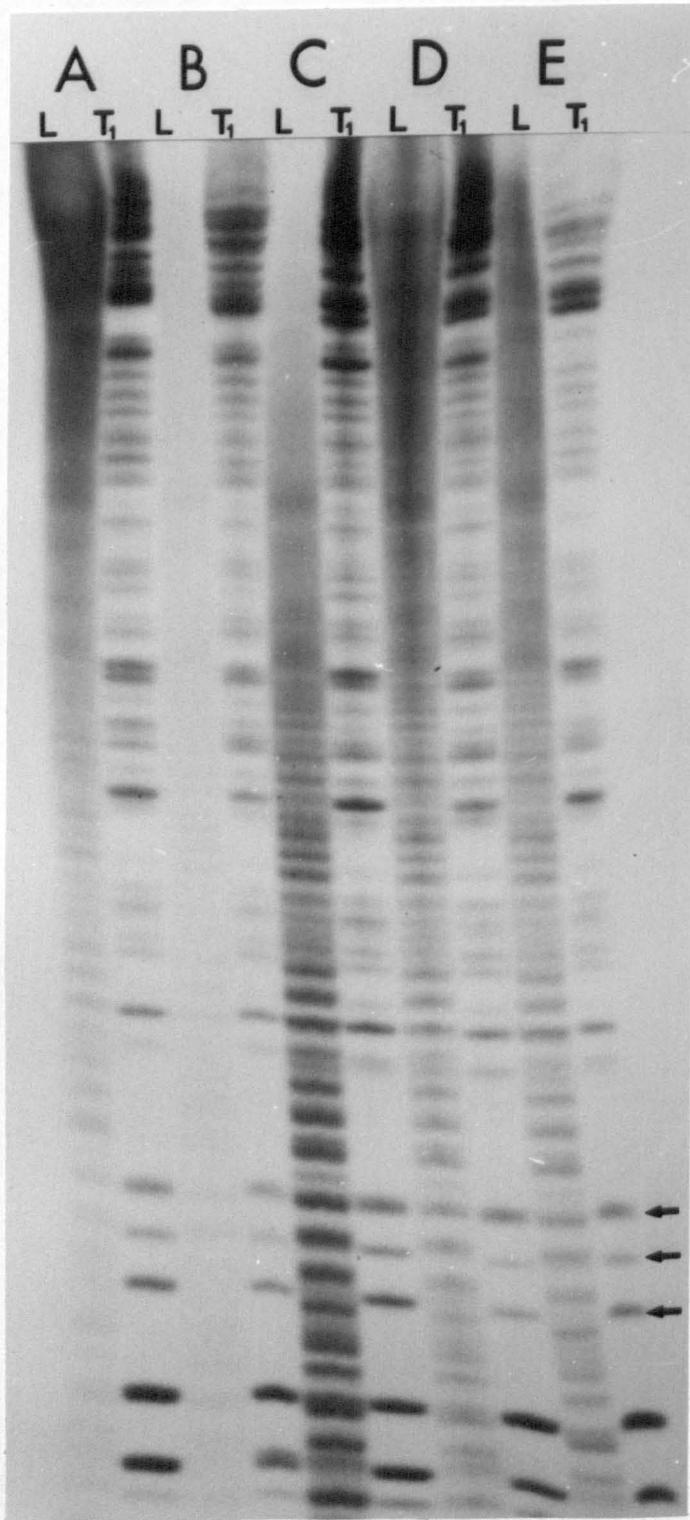
The five individually isolated species 6 RNA's are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 21. The layout and the running conditions of the samples is as described in the legend to Figure 23. In this figure the partial digestion of RNA species 6 from isolate C was lost in preparation, the other tracks show again a high level of banding pattern similarity beyond the absolutely conserved terminal region.

Figure 27a. Partial T_1 Ribonuclease Fingerprints Obtained for
the Species 7, 8, 9 dsRNA "Triplets" of the Five Isolates Under-
study.



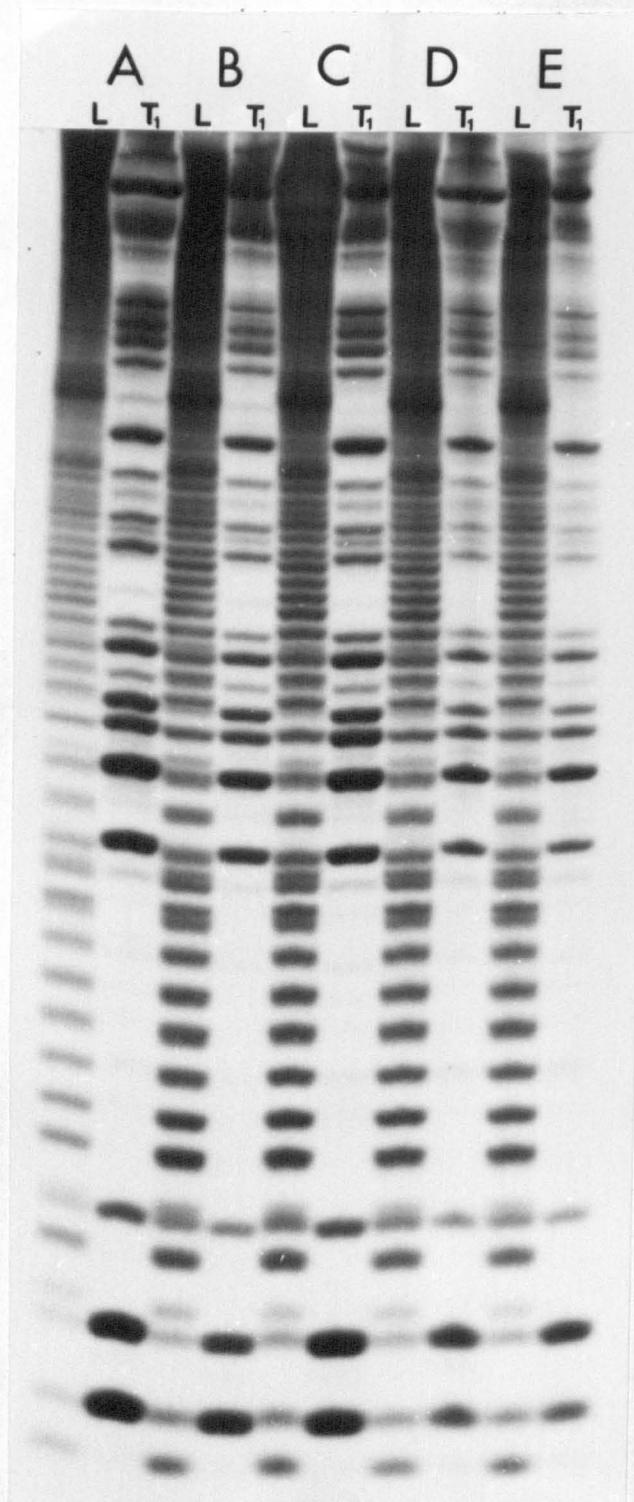
The five samples are labelled A to E and^{are} arranged in the same order as the genome profiles illustrated in Figure 21. The layout and running conditions of the samples is as described in the legend to Figure 23. This figure illustrates a high level of banding pattern for the terminal 2-40 nucleotides from the combined digestion of RNA species 7, 8, and 9, the absolutely conserved terminal region is also evident for these genome segments. The three small arrows on the right of digestion track E mark the beginning of Figure 27b.

Figure 27b. This Figure Shows the Partial Digestion Fingerprints
of the Species 7, 8, 9 dsRNA "Triplets" of the Five Isolates
Understudy Run Further to Allow Evaluation of the Partial
Digestion Patterns Beyond Nucleotide Position 30.



The three arrows indicate the triplet of bands arrowed in Figure 27a and allow the two figures to be matched up. Beyond this position it can be seen that the banding patterns obtained for the "triplets" by partial nuclease digestion remain very similar. This 18% gel was run at 1.6 KV for 6 hrs.

Figure 28. Partial T_1 Ribonuclease Fingerprints Obtained for
the Five Species 10 dsRNA's.



The five individually isolated species are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 21. The layout and the running conditions of the samples is as described in the legend to Figure 23. In this figure the partial digestions and ladder formation have worked perfectly. The absolutely conserved terminal region can be clearly discerned, beyond this the banding patterns for all these RNA species are again very similar.

Figure 29. Partial T₁ Ribonuclease Fingerprints Obtained for
the Five Species 11 dsRNA's.



The five individually isolated RNA species are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 21. The layout and running conditions is as described in the legend to Figure 23. A species I RNA sample was mistakenly run in track D (compare to Figure 23), the ladder track of isolate C did not work. This figure illustrates that the species 11 dsRNA's are also very similar beyond the region of absolute terminal conservation.

Under partially denaturing conditions on preparative agarose gels no migrational differences were observed between the genome profiles of isolates A, B, C and D. This observation together with the partial nuclease digestion results indicated that the differences observed on fractionation in 'native' acrylamide gels may have been due to only very minor variations in their structure.

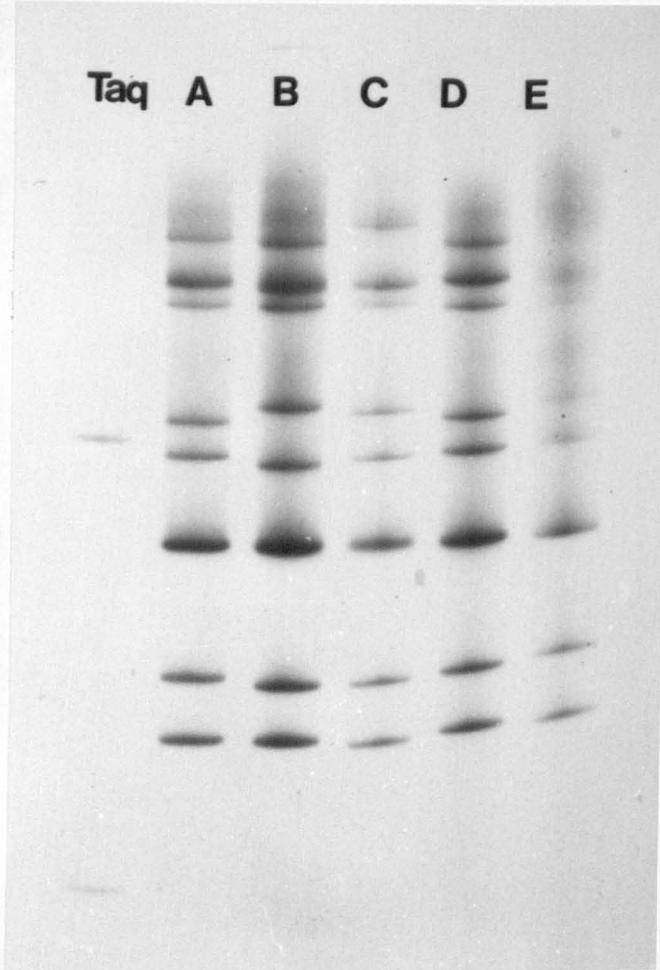
In order to test whether the change in the gel matrix, buffer, and running conditions had caused genome profile differences to disappear the five dsRNA profiles were analyzed under the same conditions used for the preparative agarose gels but utilizing acrylamide as the separating medium. Double-stranded DNA size markers were also included to give an approximate estimation of the molecular weights of the dsRNA genome segments. Figure 30 shows that electrophoresis using acrylamide as the separating matrix and under partially denaturing conditions the differences in genome profiles previously observed on analytical fractionation were real and remained.

Discussion

Partial nuclease digestion analysis of 5 bovine isolates with different genome profiles did not reveal major differences in the terminal 2-40 nucleotides between any of the corresponding species of dsRNA including those which had different migrational rates in either tris-glycine-SDS or continuous tris-acetate acrylamide gels.

Between some corresponding genome segments, minor differences in G positions were observed. However each genome segment appeared to have a characteristic terminal region for both strands of its dsRNA. Whether

Figure 30. Fractionation of the 3' End Labelled Virion RNA
From the Five Wild Isolates Used in this Study on a 7.5%
Acrylamide Gel Containing 6M Urea and Using Tris Acetate
Running Buffer (See Materials and Methods).



Electrophoresis was at 36 mA for 28 hrs at 4°C. The track marked Taq contains a Taq II digest of the plasmid PBR322 giving two fragments the largest being approximately 1.4 Kb and running with the rotavirus RNA species 5 and 6, the smaller fragment is 0.6 Kb and runs below the smallest RNA species. The genome profiles are labelled A to E and arranged in the same order as

in Figure 21.

This figure illustrates that changing the running buffer and using 6 M urea in the gel matrix does not cause the loss of heterogeneity among genome profiles (see text) as observed in Figure 22.

these terminal regions represented conserved sequences which were dramatically different past a certain point, or whether corresponding dsRNA segments with different migrational rates on simple genome profile analysis had very similar primary sequences remained to be determined.

From the DNA size markers(see Figure 30) estimates of the molecular weights of the rotavirus genome segments could be made; Sp11 the smallest genome segment comprises approximately 800 bases, the largest Sp1 RNA's are approximately 4 Kb. Partial nuclease digestion analysis allowed characterization of 1/8th of the primary sequence for the smallest Sp11 RNA (50 bases from either end) to 1/40th for the largest RNA species. Clearly there are great possibilities for variation in the remaining 39/40 → 7/8 regions of each rotavirus genome segment.

Further analysis of Sp 7, 8, 9 RNA's as a combined triplet indicated that those species of RNA were also very similar for up to 100 bases for either strand. However this was not a good example as all three RNA species co-migrated on one-dimensional analysis and may have been identical anyway. Also, as these maps were constructed from six terminally labelled strands any differences in banding patterns were difficult to interpret as differences in one strand may have been masked by similar banding patterns from any of the other five.

The basis of migrational variation of corresponding genome segments on polyacrylamide gel electrophoresis is not understood. The main conclusion from this work was that corresponding segments of bovine rotavirus dsRNA that do not co-migrate can have similar partial nuclease digestion patterns for their terminal 40 bases. It was expected to find some terminally conserved sequences because of the functions shared by

all the genome segments e.g. encapsidation signals, polymerase binding sites and ribosome binding sites.

Whether the apparent 2-40 terminal banding similarity for corresponding genome segments reflected genuine RNA segment specific terminal conservation or whether non-comigrating corresponding genome segments have highly similar primary sequences remained to be determined.

RESULTS CHAPTER 4

**Some of the work in this Chapter has been submitted
for publication, Virology (1982)**

Chapter 4

Partial Nuclease Digestion Analysis of Human, Bovine and Porcine

Rotavirus Isolates

Introduction

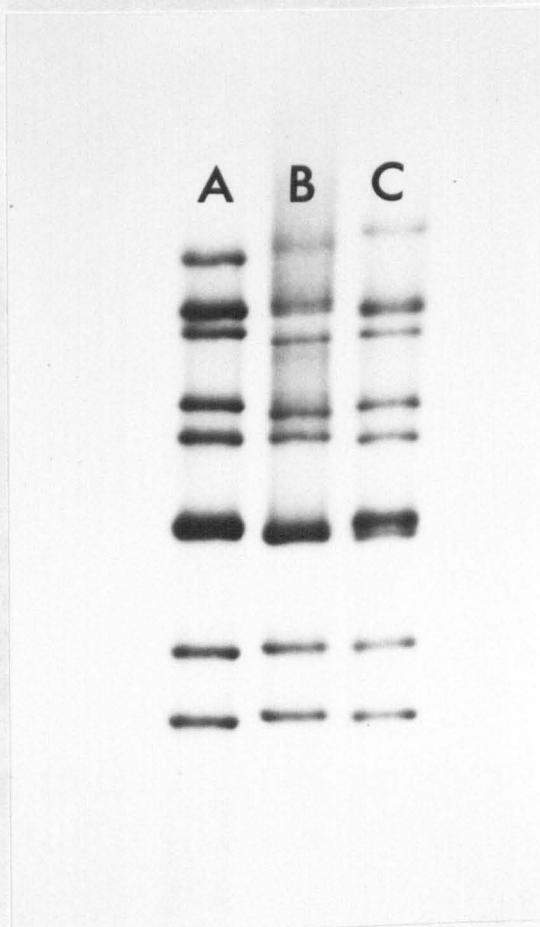
Partial nuclease digestion fingerprint analysis of the bovine rotaviruses showed no major banding pattern differences in the terminal 2-40 nucleotide regions for any of the corresponding genome segments. The results of terminal nucleotide sequence analysis of the S1 genes (Li *et al.*, 1980) of the closely related three mammalian reovirus serotypes has revealed their nucleotide sequences to be very divergent. However it was possible that the bovine isolates analyzed were very similar as they all originated from the same animal species. Therefore to maximize the chances of finding variation by the partial nuclease digestion method of analysis, 3 rotavirus samples isolated from different animal species were selected for analysis; these were assumed to be serologically different with respect to their surface antigens (see Thouless *et al.*, 1977).

Results

The genome profiles of the 3' terminally labelled dsRNA of these human, pig and calf isolates are shown in Figure 31. Clear differences in electrophoretic variation can be observed for segments 1, 4, 5, 10 and 11.

These samples were subjected to preparative fractionation on a tris-acetate agarose gel (see Figure 32). This figure shows that the profile differences observed on analytical fractionation disappeared

Figure 31. Polyacrylamide Gel to Show a Comparative Genome Analysis
of the Three Wild Rotavirus Isolates Used in This Study.



Viral RNA was 3' end labelled as described in Materials and Methods. Samples were run on a 20 cm 7.5% polyacrylamide gel at 20 mA for 16 hrs. These samples were from three animal species isolated at different times and from distinct locations within the U.K given in brackets.

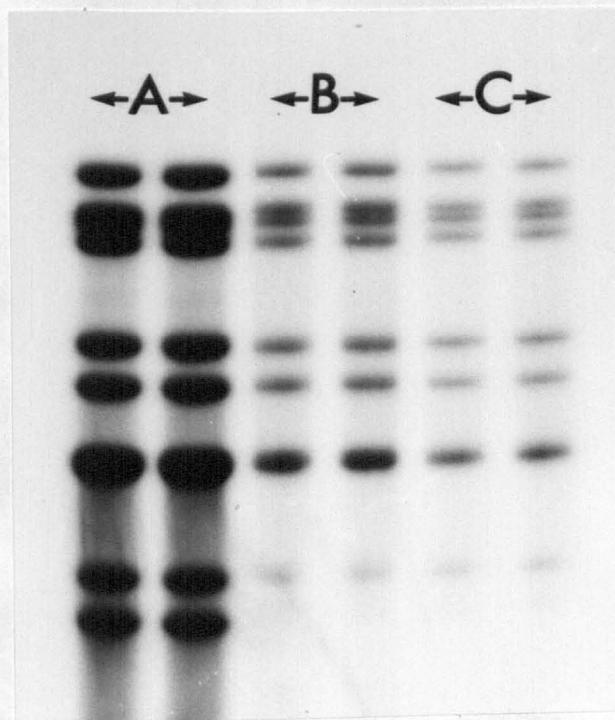
Track A = Bovine isolate A (4329 Shrewsbury)

Track B = Human isolate B (Glen Mallin Birmingham)

Track C = Pig isolate C (28338 Reading)

Clear differences in migrational rates can be seen for genome segments 1, 4, 5, 10 and 11.

Figure 32. Preparative Fractionation of the Total 3' End Labelled Viral RNA From the Three Rotavirus Isolates Under Study.

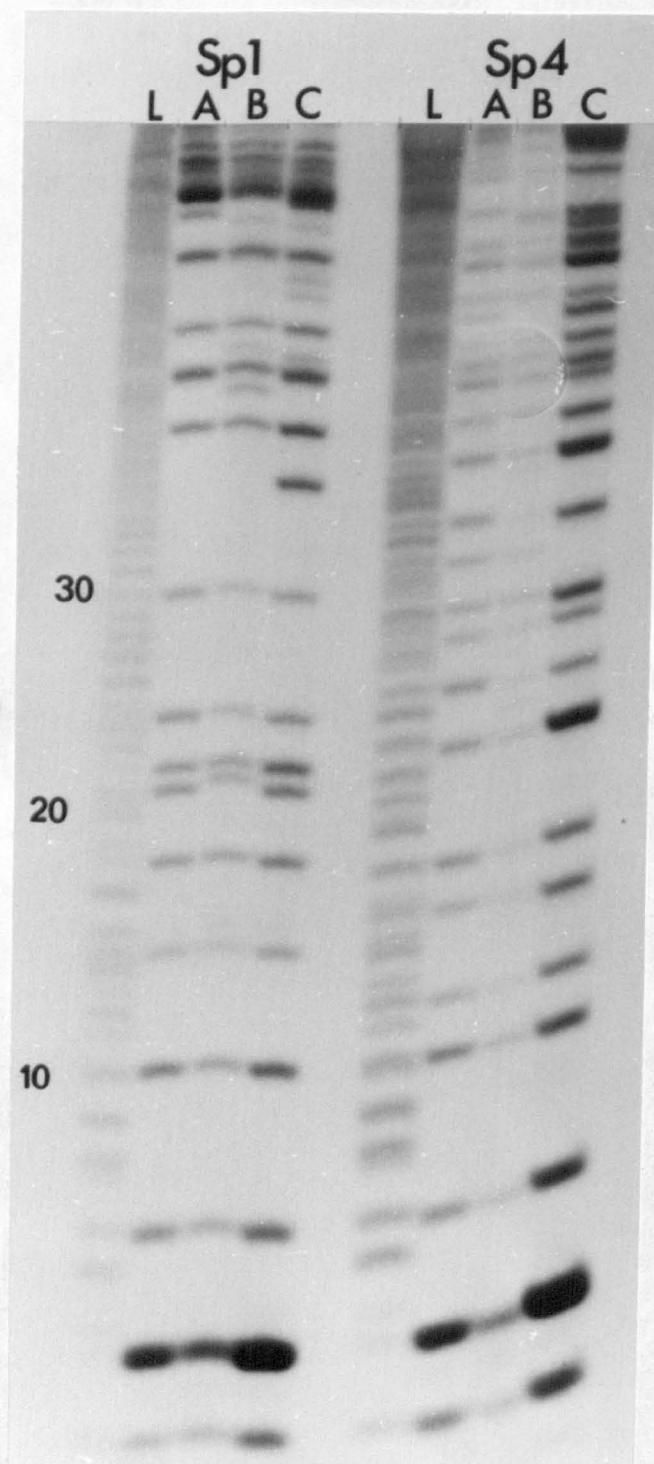


Each isolate was loaded across two adjacent gel tracks labelled A, B and C on this 1.5% preparative agarose gel. Electrophoresis was at 30 mA for 16 hrs at room temperature.

in a similar fashion to that observed for the five bovine isolates in Results Chapter III. The smallest RNA species (Species 11) of the human and pig isolates were lost during electrophoresis preventing their definitive analysis by partial nuclease digestion. Following preparative fractionation of the dsRNA, corresponding genome segments were subjected to partial nuclease digestion analysis. The partial digestion patterns obtained for species 1 and 4 dsRNA's are shown in Figure 33. For the species 1 RNA's it can be seen that identical patterns were obtained for the G positions in the 2-30 terminal nucleotide regions of both RNA strands. Beyond this conserved region minor banding differences appeared. Terminal regions of the species 4 RNA's were also conserved although different from the species 1 RNA's.

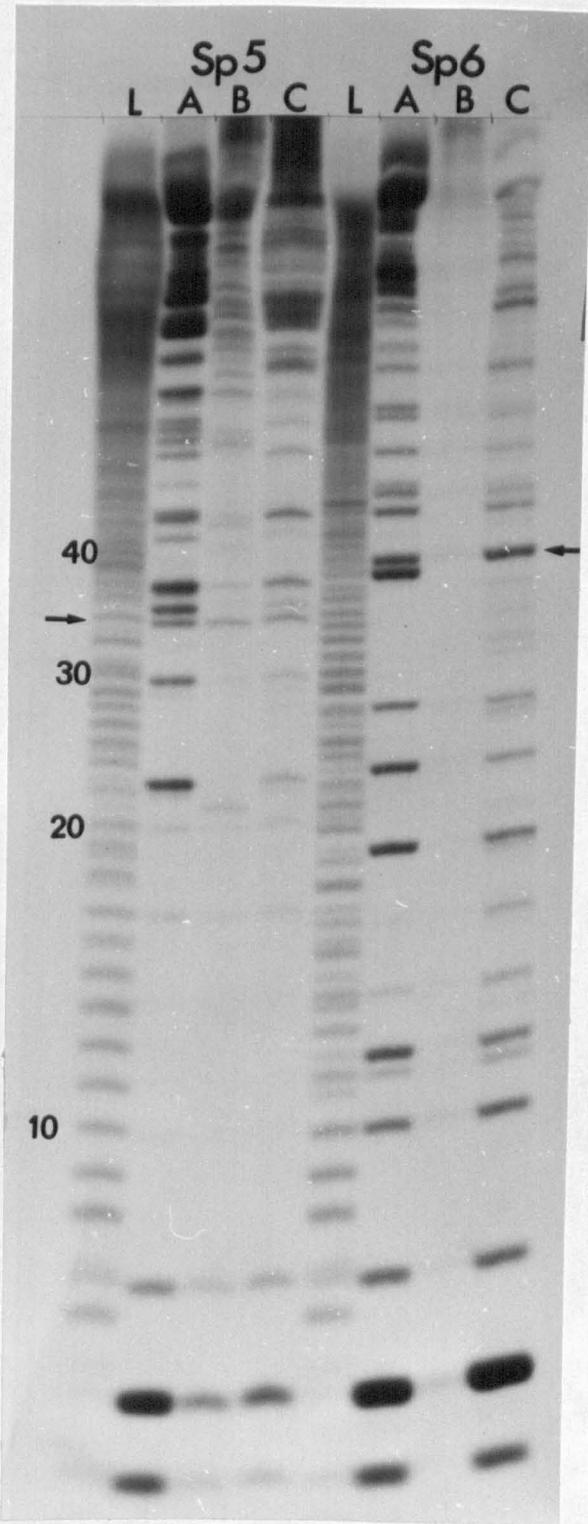
Figure 34 shows the partial nuclease digestion results obtained for the terminal 2-35 nucleotides of RNA species 5 and 6. Regions of terminal conservation with respect to the G positions can be seen for these sets of corresponding RNA species. Closer study of the larger partial digestion fragments of the species 5 RNA's of these rotaviruses isolated from different animal species showed some evidence for variation. These samples were analyzed for a longer gel electrophoresis time to give better resolution of the variation beyond the apparent terminally conserved region of approximately 40 bases. The results of this analysis are shown in Figure 35. The end of the region of terminal similarity is defined by the 'triplet' of G bands arrowed in figure 34 which on better resolution is seen as four bands arrowed in figure 35. Beyond this position the banding patterns of the three species 5 RNA's differ radically. These results were the first evidence from partial nuclease digestion studies that rotavirus isolates can have gross differences in

Figure 33. Partial T₁ Ribonuclease Digestion Fingerprints Obtained
for the Three Species 1 dsRNA's and the Three Species 4 dsRNA's of the
Isolates Under Study.



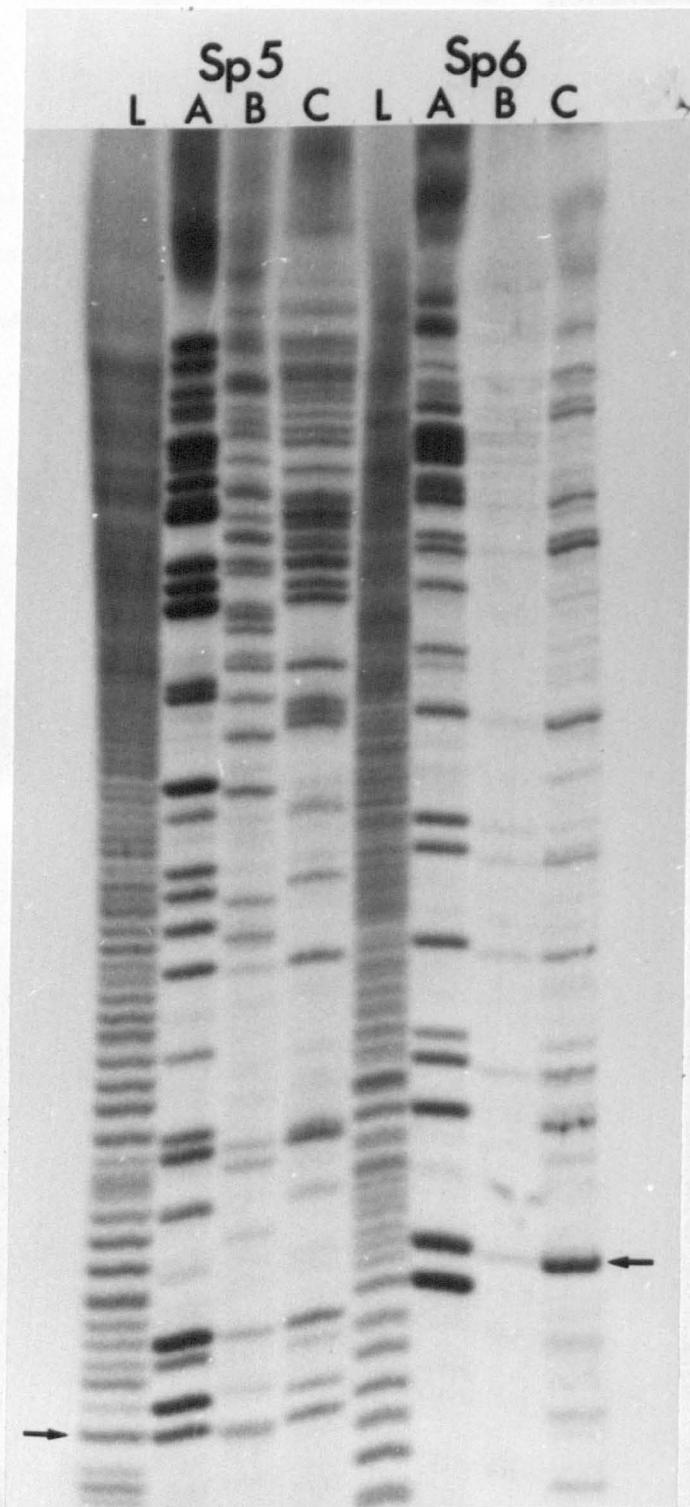
Tracks 'L' are the partial hydrolysis reference ladders for each set of corresponding genome segments, the tracks marked A (calf), B (human), and C (pig) are the partial T_1 ribonuclease digestion patterns for the RNA species 1 or 4 of the isolates A, B and C. Nucleotide positions relative to the terminus are numbered on the left side of the gel adjacent to the ladder track for the Species 1 RNA's. This 18% 20 x 40 cm gel was run at 1.6 KV for 2½ hrs.

Figure 34. Partial T₁ Ribonuclease Digestion Fingerprints Obtained for
the Species 5 dsRNA's and the Species 6 dsRNA's of the Three Isolates
Under Study.



The layout and running conditions of the sample is as described in the legend to Figure 33.

Figure 35. Partial Nuclease Digestion Fingerprints Obtained
for the Near Terminal Regions of the Species 5 and the Species 6
dsRNA's of the Three Isolates Under Study.



This figure shows the same samples in Figure 34 electrophoresed for 6 hrs at 1.6 KV to expand the region beyond terminal position 35. The arrangement of the samples was exactly as described in the legend to Figure 33. The arrow to the left of the ladder track for the species 5 RNA's marks the end of the genome segment-specific region of terminal banding pattern conservation for these RNA species.

primary structure for their corresponding genome segments.

The partial nuclease digestion patterns obtained for genome segments 10 are shown in Figure 36. This figure again shows the unique high level of terminal conservation for each set of corresponding genome segments. Species 11 of the human and pig isolates were not analyzed because of the problem encountered with their recovery from the preparative gel (see Figure 32).

Genome segments 2 and 3, and 7, 8, 9 were analyzed as a combined pair and triplet, the results of these partial digestions are shown in Figure 37. The tracks for species 2 and 3 were over-run therefore this analysis started at terminal nucleotide position 7. Both sets of digestion patterns show 'conserved' regions of approximately 30 terminal nucleotides. Beyond this point many differences in banding patterns are evident these appear to be similar in magnitude to those seen for species 5 RNA in Figure 34 providing more evidence that corresponding genome segments can have primary sequence differences.

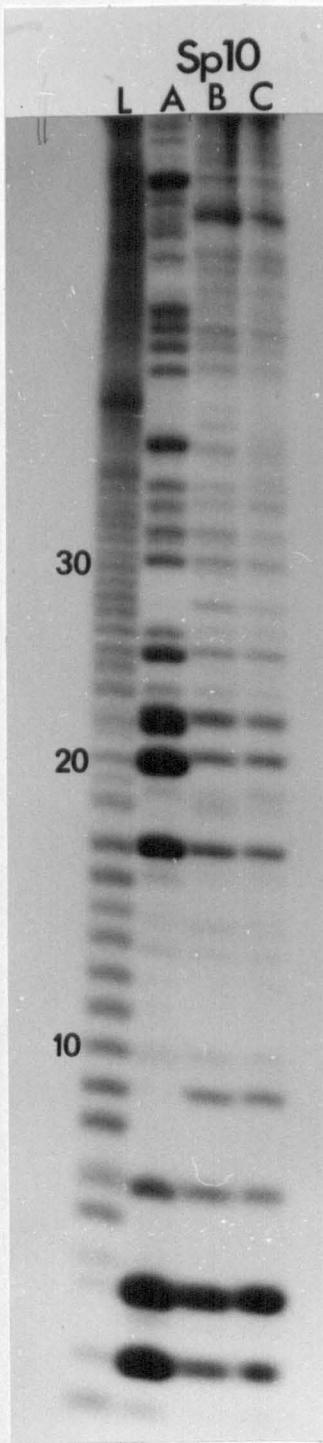
Discussion

This experiment showed that corresponding genome segments of rotaviruses isolated from different animal species and therefore assumed to have antigenic differences share regions of terminal similarity, at least in respect of their G residues, for approximately 35 nucleotides. Each set of corresponding genome segments had a unique and hence characteristic partial nuclease digestion pattern for this region which allowed it to be distinguished from any of the other genome segments. These terminal patterns were entirely consistent with similar results obtained

Figure 36. Partial T₁ Ribonuclease Digestion Fingerprints

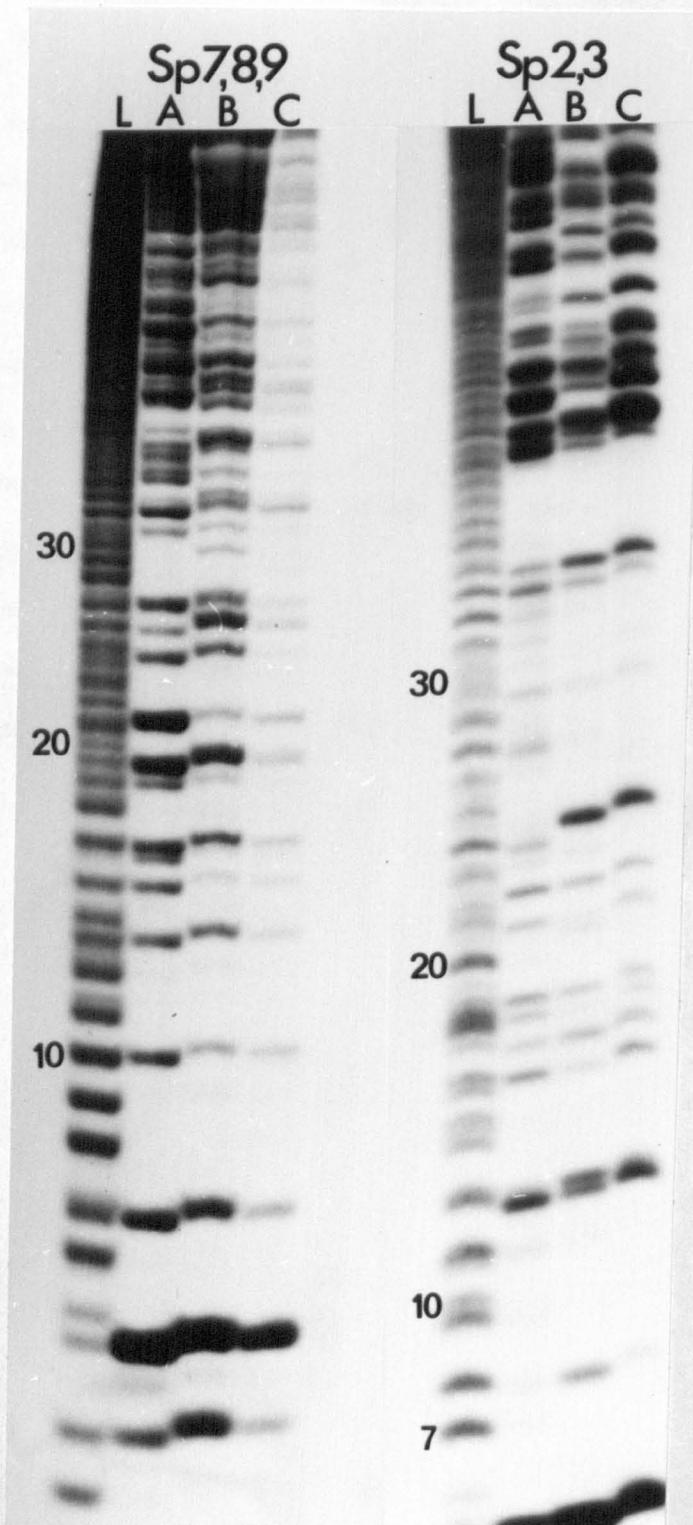
Obtained for the Species 10 dsRNA's From the Three Isolates

Under Study.



The layout and running conditions of the samples is as described in the legend to Figure 33. Minor banding pattern differences are evident for isolate A (calf) at nucleotide positions 9 and 28, despite these two small differences the overall digestion patterns of these corresponding genome segments are highly similar to approximately 40 terminal nucleotides. Beyond this point many differences in banding patterns are apparent.

Figure 37. Partial Digestion Fingerprints Obtained for the
Species 7, 8 and 9 'Triplets' and the Species 2 and 3 'Doublets'
of the Three Isolates Under Study.



The layout and running conditions were as described in the legend to Figure 33 except that the partial digestion of the species 2 and 3 'doublet' was electrophoresed for 3 hrs at 1.6 KV and the six terminal bases were lost.

for the five bovine isolates analyzed in Results Chapter III.

Several genome segments, species 5, the 2, 3 doublet and 7, 8, 9 triplet showed differences in banding patterns beyond the 35 terminal nucleotide position. Further internal analysis of the species 5 RNA's showed that the banding patterns of these corresponding genome segments were radically different. These species 5 RNA's did not co-migrate on simple genome profile analysis as shown by Figure 31, yet co-migrated in the preparative gel system indicating that lack of dissimilarity in this gel system did not reflect similarity in primary structure for corresponding genome segments as was initially thought in Results Chapter III.

These results suggest that future studies of rotavirus variation by the partial nuclease digestion method of genome segment characterization should be applied beyond the regions of apparent terminal similarity which have been evident for all corresponding genome segments so far studied.

GENOMIC VARIATION IN ROTAVIRUSES

By

Ian N. Clarke, B.Sc. (Leeds)

Vol II

**This thesis is presented for the degree of Doctor of Philosophy
in the Department of Biological Sciences, University of Warwick**

October 1982

RESULTS CHAPTER 5

Much of the work in this Chapter has appeared in
press (*Infect. Immun.* 36, (1982) 492-497), or has been
submitted for publication *Virology* (1982)

Results - Chapter 5

Partial T₁ Ribonuclease Digestion Analysis of Five Bovine Rotavirus Isolates

Introduction

The results of partial nuclease digestion analysis described for the five bovine isolates in Results Chapter 3 and the human, bovine and porcine isolates in Results Chapter 4 indicated that each rotavirus genome segment has a region of terminal conservation at least in terms of the G positions of both strands, beyond which variations in banding patterns can occur. Therefore the application of partial nuclease digestion analysis for only the terminal 2-40 bases will not detect significant variation that may exist. Partial nuclease digestion analysis has been shown to be possible for defining up to at least 100 terminal bases (Results Chapter 2). Therefore five bovine isolates different on the basis of genome profiles, and on time and place of isolation were chosen for a detailed study of both terminal (2-40 nucleotides) and near terminal (40-100 nucleotides) regions of their genome segments. Included among these isolates for reference purposes was the U.K. tissue culture adapted bovine rotavirus (Compton strain). In addition to partial nuclease digestion analysis selected genome segments were also characterized by T₁ oligonucleotide finger-printing making use of in vitro labelling following total digestion of the RNA.

Results

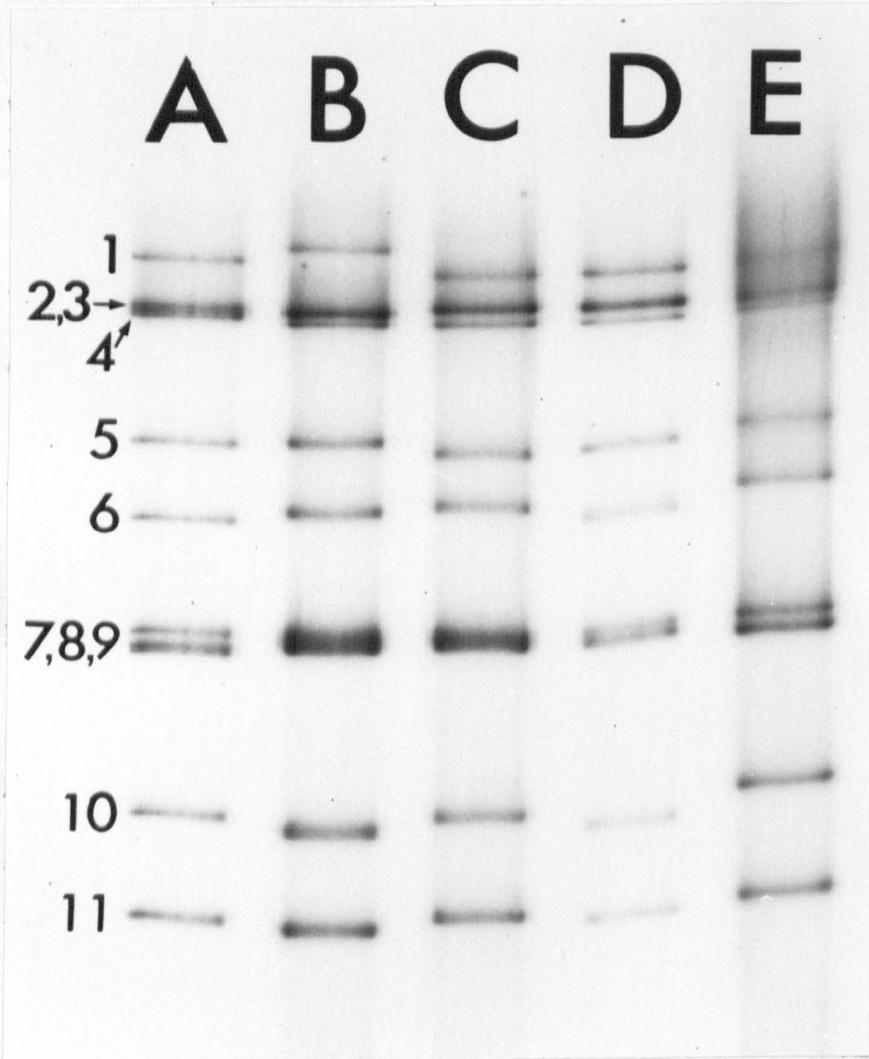
A one dimensional simple genome profile analysis of the 3' end labelled

RNA extracted from these five isolates is shown in Figure 38.

Comparison of the profiles shows clear electrophoretic variation among these isolates for segments 1, 5, 6, 10 and 11.

Figure 39 shows the fractionation of terminally labelled partial digestion products of all five species 1 RNA's. Marker tracks making use of DNA restriction fragments were run on each gel to enable the direct cross comparison of autoradiographs. The smallest DNA fragment evident at the bottom of the marker track in Figure 39(b) runs as a number of bands at RNA fragment sizes of approximately 40 nucleotides. This feature can be clearly found in all the partial nuclease digestion fingerprint figures and is useful as a reference point. The species 1 RNA's in Figure 39 have very similar banding patterns with only a few minor changes in the position of G residues being observed - all beyond the 40 nucleotide marker. This sequence conservation should be contrasted with the large variation in electrophoretic mobility on simple genome profile analysis (see Figure 38). When partial nuclease digestion analysis was performed on two of the other sets of dsRNA species showing mobility variation on simple genome profile analysis, species 5 and 6 (Figures 40, 41 and 42) the results obtained were essentially similar to those for RNA species 1 with a high level of overall band pattern conservation and only minor differences in G positions being observed beyond the 40 nucleotide markers. Partial nuclease digestion analysis of the species 10 RNA's (Figure 43a and b) showed that all these corresponding genome segments had very similar banding patterns except for the U.K. tissue culture adapted isolate which had some banding pattern differences within the 40 terminal nucleotide region, this phenomenon was investigated in

Figure 38. Polyacrylamide Gel to Show a Comparative Genome Analysis of the Five Bovine Isolates Used in This Study.



Viral RNA was 3' end labelled as described in Materials and Methods. Samples were run on a 20 cm 7.5% polyacrylamide gel at 20 mA for 16 hrs. The four field isolates were from geographically distinct locations given in parentheses.

Track A = Isolate A Tissue culture adapted calf rotavirus (U.K.)

Track B = Isolate B 2855 (Carmarthen)

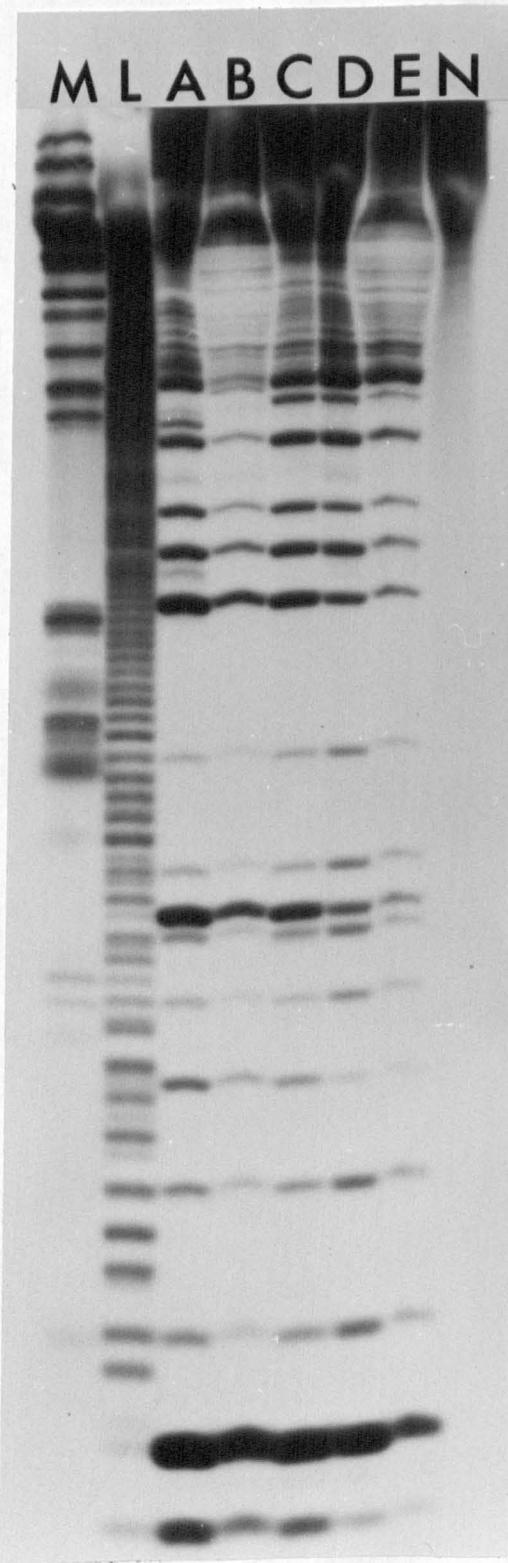
Track C = Isolate C 4329 (Shrewsbury)

Track D = Isolate D 6597 (V.I. Centre Weybridge)

Track E = Isolate E 117 (Penrith)

Migration was from top to bottom, the genome segments of the tissue culture adapted calf rotavirus are numbered 1 to 11.

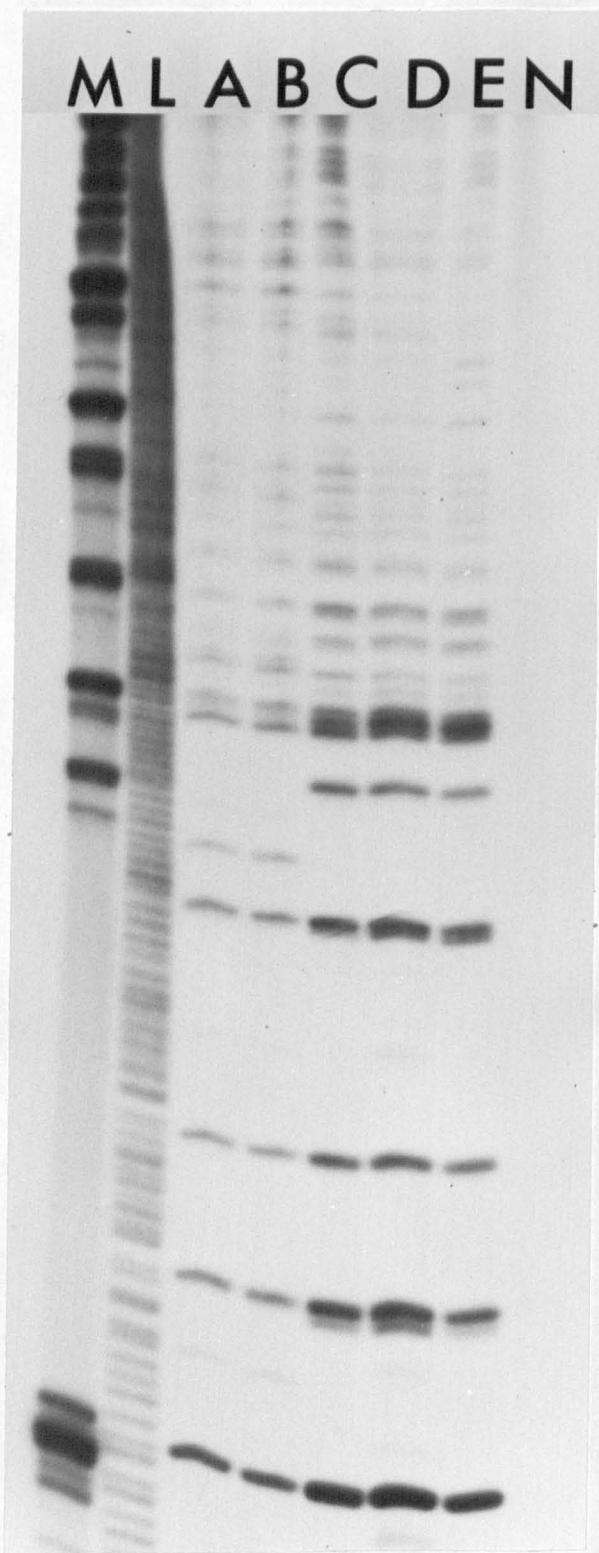
Figure 39(a). Partial T₁ Ribonuclease Digestion Fingerprints
Obtained for Terminal Region of the Five Species 1 dsRNAs from
the Isolates Under Study.



Track M is the marker track showing radiolabelled Hpa II DNA digestion fragments of the plasmid PBR322. Track L is the partial hydrolysis reference 'ladder'. The five individually isolated species 1 RNAs are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 38. A control of native, double-strand denatured undigested RNA of isolate A is in track N. This 16% gel (20 x 40 cm) was run at 1.6 KV for 2½ hrs.

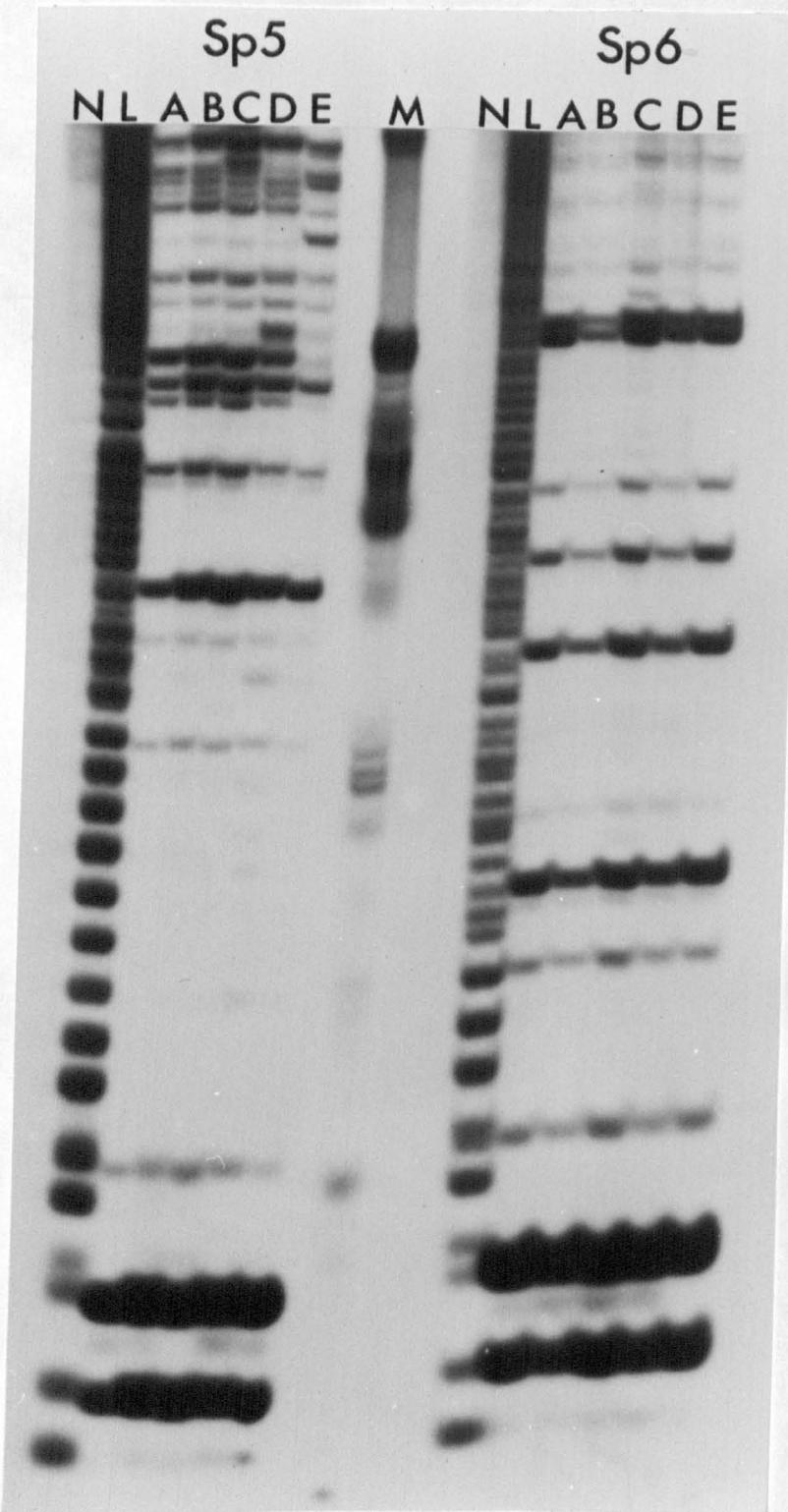
Figure 39(b). Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Near Terminal (> 40 Nucleotides) Region of the
Five Species 1 dsRNA's From the Isolates Under Study.



The arrangement of the samples is exactly as described in the legend to Figure 39(a). The DNA fragment evident at the bottom of the marker track M, as several tight bands runs at approximately nucleotide position 40 according to the partial hydrolysis reference ladder and marks the end of the region of genome segment specific terminal similarity. This 14% gel (20 x 40 cm) was run at 1.6 KV for 6 hrs. Several minor banding pattern differences can be seen between these corresponding genome segments.

Figure 40. Partial T₁ Ribonuclease Digestion Fingerprints
Obtained for the Terminal Regions of the Species 5 and the
Species 6 dsRNA's From the Five Isolates Under Study.

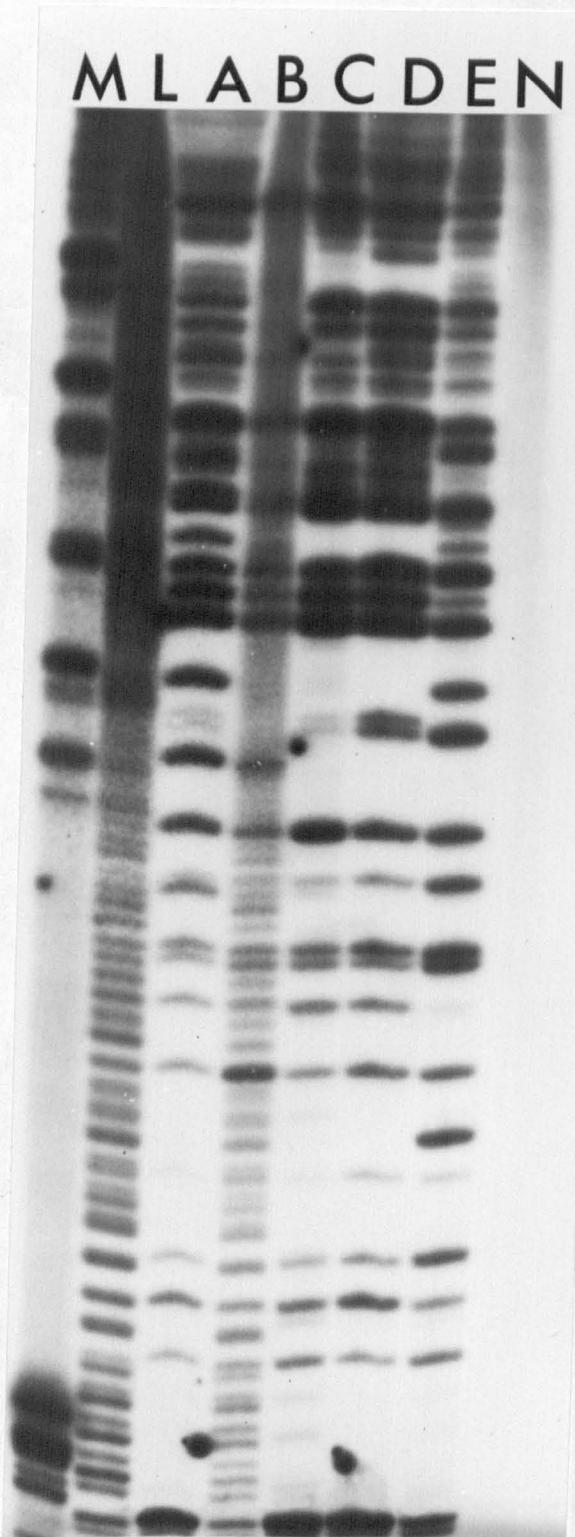


Track M is the marker track showing the Hpa II DNA digestion fragments of the plasmid PBR322. Tracks L are the partial hydrolysis reference 'ladders'. The five individually isolated species 5 and species 6 RNA's are labelled A to E and arranged in the same order as the genome profiles illustrated in Figure 39(a). Controls of native, double-strand denatured undigested RNA of species 5 and 6 from isolate A are in tracks N. This 16% gel (20 x 40 cm) was run at 1.6 KV for 2½ hrs. This figure illustrates the unique genome segment specific regions of terminal similarity for RNA species 5 and 6.

Figure 41. Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Near Terminal Region of the Species 5 dsRNA's

From the Five Isolates Under Study.

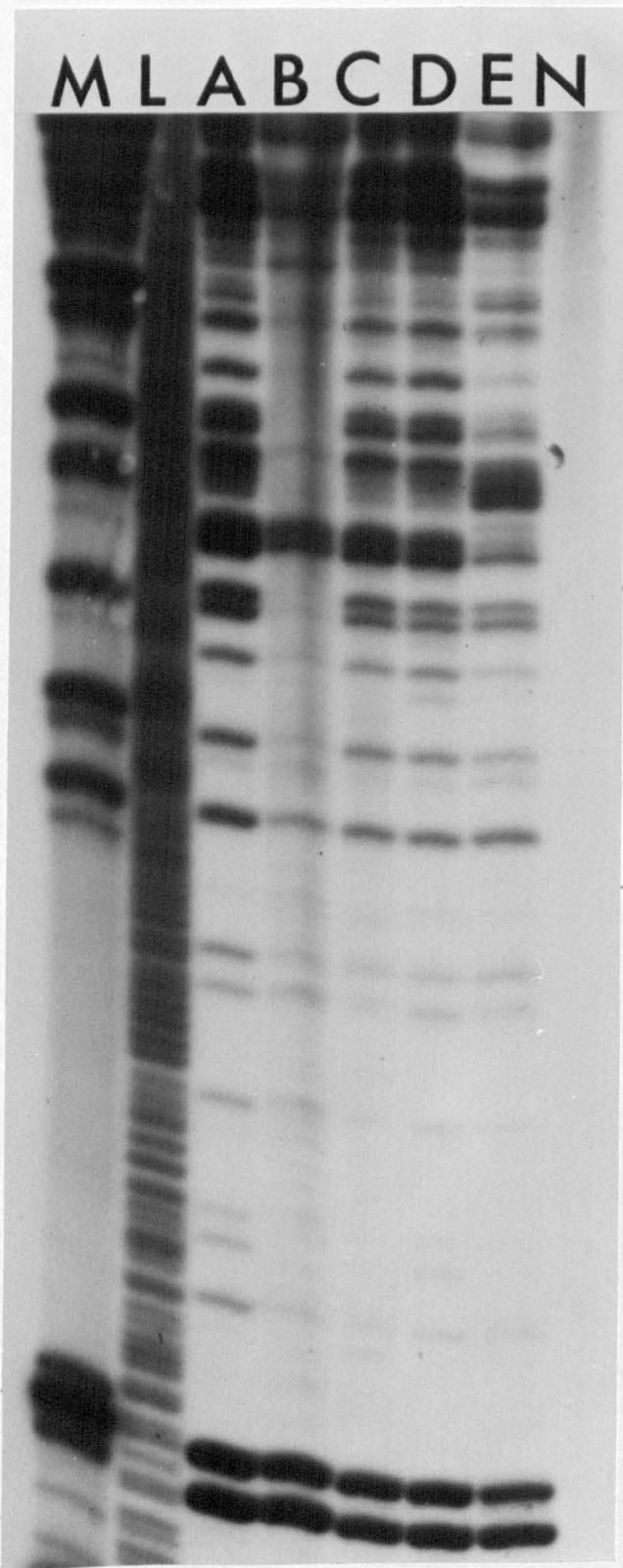


The arrangement and running conditions of the samples is as described in the legend to Figure 39(b). The species 5 RNAs which have different mobilities on simple genome profile analysis share an overall similarity in banding patterns. Minor differences in G positions can be seen for all the samples.

Figure 42. Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Near Terminal Region of the Species 6 dsRNA's

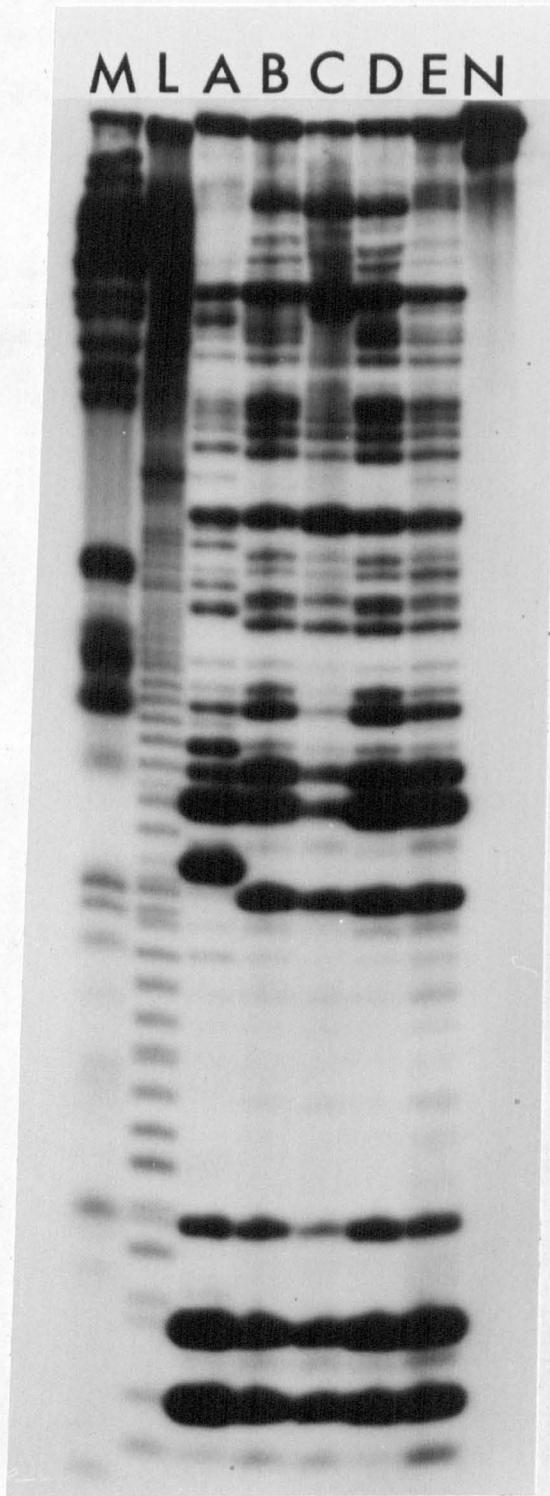
From the Five Isolates Under Study.



The layout and running conditions of the samples is as described in the legend to Figure 39(b). A clear distinguishing feature of species 6 RNA's by partial nuclease digestion analysis is a strong 'doublet' at nucleotide position 40. This 'doublet' migrates approximately with the 40 nucleotide position DNA fragment in the marker track. Beyond this feature the banding patterns for all five RNA species remain very similar, the sample in track B has been underdigested, hydrolysis of the RNA is also apparent by the faint background ladder of nucleotides in the gel track.

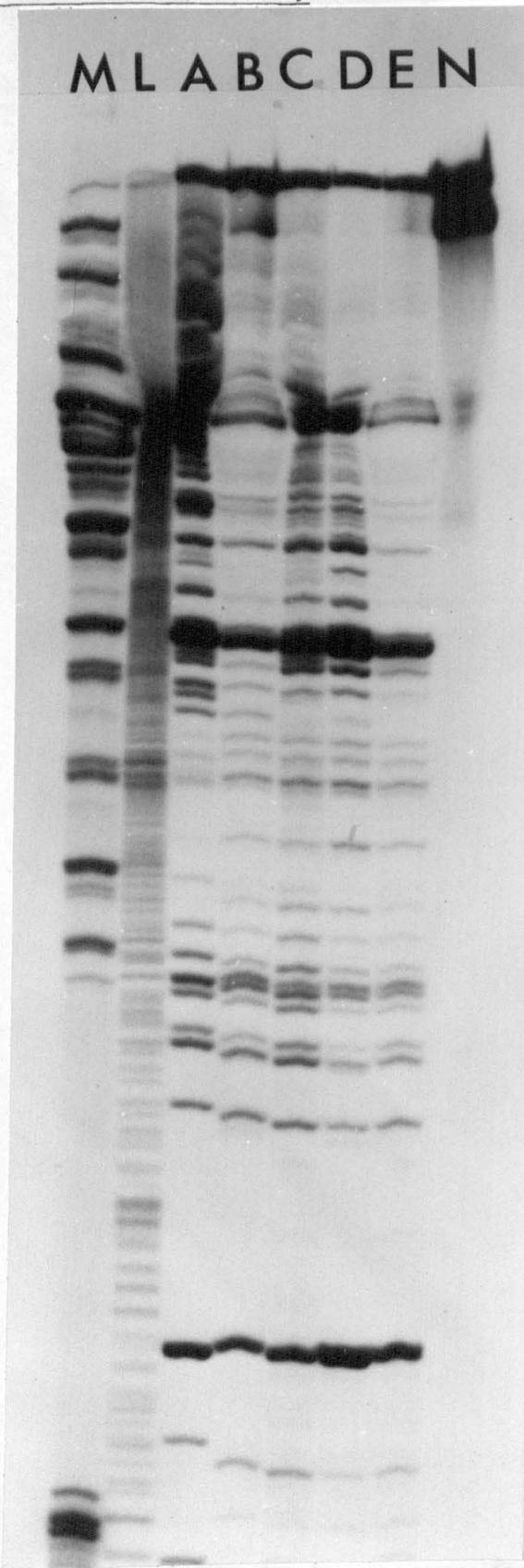
Figure 43(a). Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Terminal Regions of the Species 10 RNA's of the
Five Isolates Under Study.



The layout and running conditions of the samples is exactly as described in the legend to Figure 39(a). Species 10 RNAs from isolates B, C, D and E show complete similarity in banding patterns for their terminal 40 nucleotides.

Figure 43(b). Partial T₁ Ribonuclease Digestion Fingerprints
Obtained for the Near Terminal Regions of the Species 10 RNAs
of the Five Isolates Under Study.



The arrangement of the samples is exactly as described in the legend to Figure 39(a). This 14% gel was run at 1.6 KV for 6 hrs. The characteristic DNA fragment that runs at nucleotide position 40 can be seen at the bottom of the marker track M.

more detail in the Results Chapter 8.

To confirm that the similarities of sequence observed for corresponding genome segments that do not co-migrate were not artefacts generated by analyzing near terminal regions of the dsRNA, complete two dimensional oligonucleotide fingerprints for two of the species 10 RNA's were prepared (isolates C and D). These RNA species had almost identical partial nuclease digestion patterns yet had a clear mobility difference as shown by Figure 38. The results of this analysis (Figure 44) confirmed the structural similarities observed with the terminal partial nuclease digestion analysis procedure. These results have established that detectable variation in electrophoretic mobility can result from minor changes in primary sequence.

Genome segments from the isolates which showed no detectable electrophoretic variations were also subjected to partial nuclease digestion. Species 2 and 3, and 7, 8 and 9 were analyzed as a combined pair and a 'triplet' respectively and species 4 was analyzed as an individual segment. All species 4 RNA's co-migrate except that of the U.K. tissue culture adapted calf virus which migrates very slightly slower than the others (Figure 38). Autoradiographs of partial nuclease digestion fragments for the near terminal regions of the 2, 3 doublet (Figure 45), for the 7, 8 and 9 triplet (Figure 46) and for species 4 (Figure 47b) are shown. From Figures 45 and 46 it is clear that the banding patterns of RNA species in both these figures are highly conserved, with only one area (indicated by arrows in Figure 46) where any clear differences exist. In this particular case these differences probably represent a minimal estimate of the variation present since a

Figure 44.

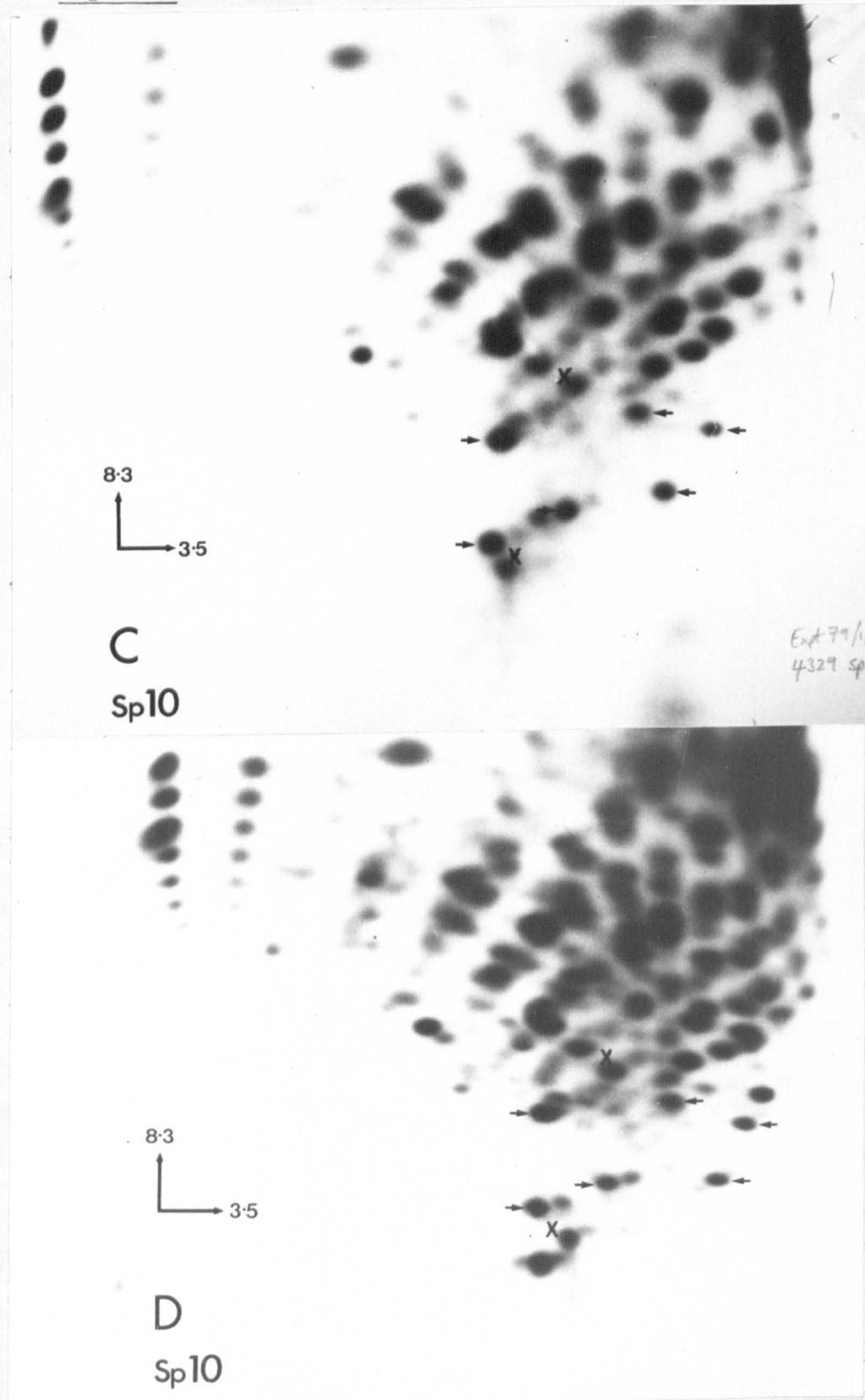
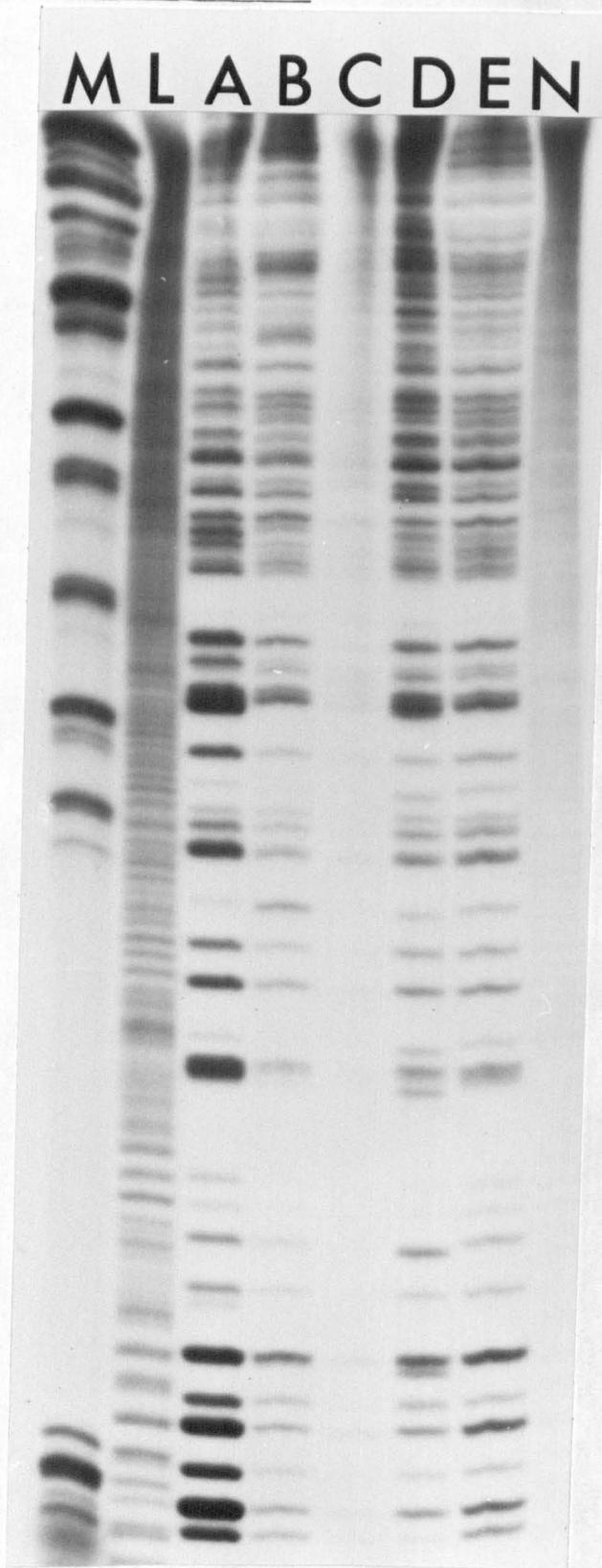


Figure 44. Two Dimensional T₁ Oligonucleotide Fingerprint

Analysis of the Species 10 RNAs From Isolate C (Top Panel) and
Isolate D (Bottom Panel).

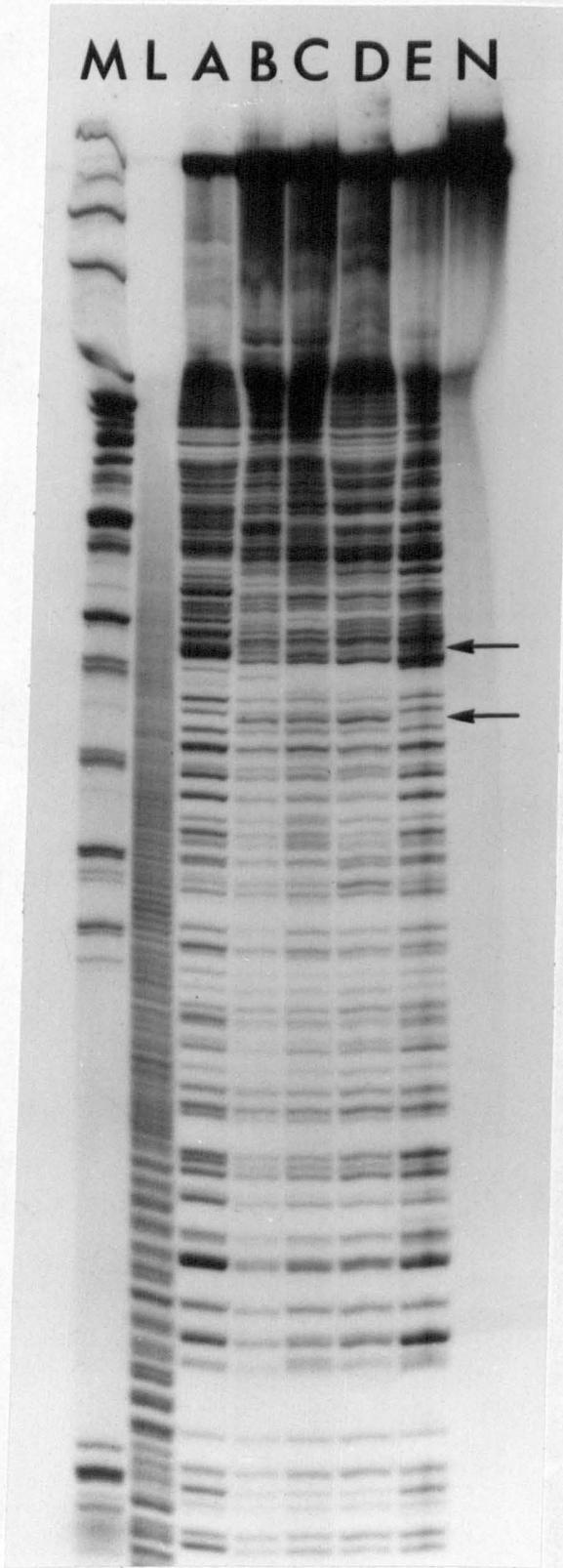
These RNA species have almost identical partial nuclease digestion patterns (Figure 43) yet do not co-migrate on simple genome profile analysis (Figure 38). Cross comparison of the two fingerprints shows there to be only a few spots different between them. The arrows mark diagnostic oligonucleotides common to both RNA samples. The positions of the two dye markers are indicated (x, lower left xylene cyanol, and x, centre of fingerprint bromophenol blue). Two triangles of oligonucleotides, indicated by horizontal arrows are shared by both fingerprints. The first dimension gel was run from left to right, while the second dimension was from bottom to top. Gel running conditions are described in Materials and Methods.

Figure 45. Partial Nuclease Digestion Fingerprints Obtained
for the Near Terminal Region of Species 2 and 3 dsRNA "Doublets"
of the Five Isolates Under Study.



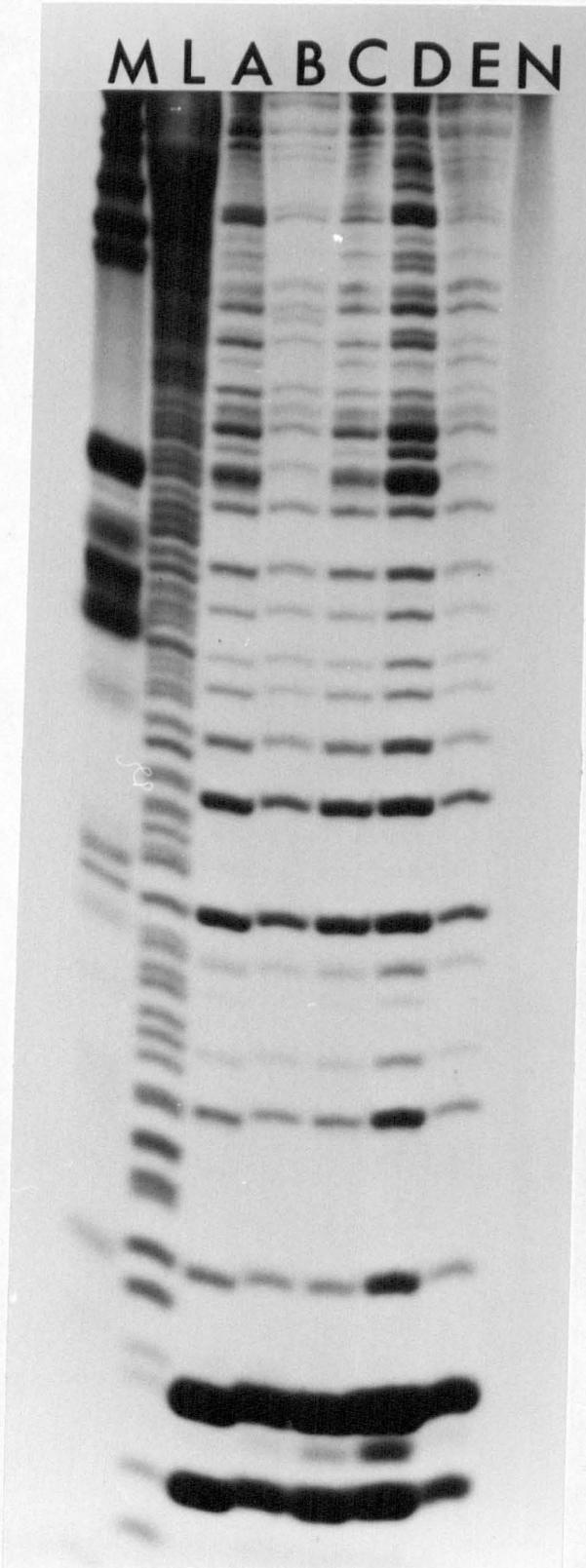
Track M is the marker track showing radiolabelled DNA size markers. Track L is the partial hydrolysis reference ladder. The partial nuclease digestion products of the five doublet RNAs of the isolates A to E are arranged as described in the legend to Figure 38. Track N contains a control of native double strand denatured undigested RNA from the doublet of isolate A. The partial digestion of isolate C has not worked very effectively giving only faint bands. This 20 x 40 cm 14% gel was run at 1.6 V for 6 hrs.

Figure 46. Partial Nuclease Digestion Fingerprints Obtained
For the Near Terminal Region of Species 7, 8 and 9 dsRNA
"Triplets" of the Five Isolates Under Study.



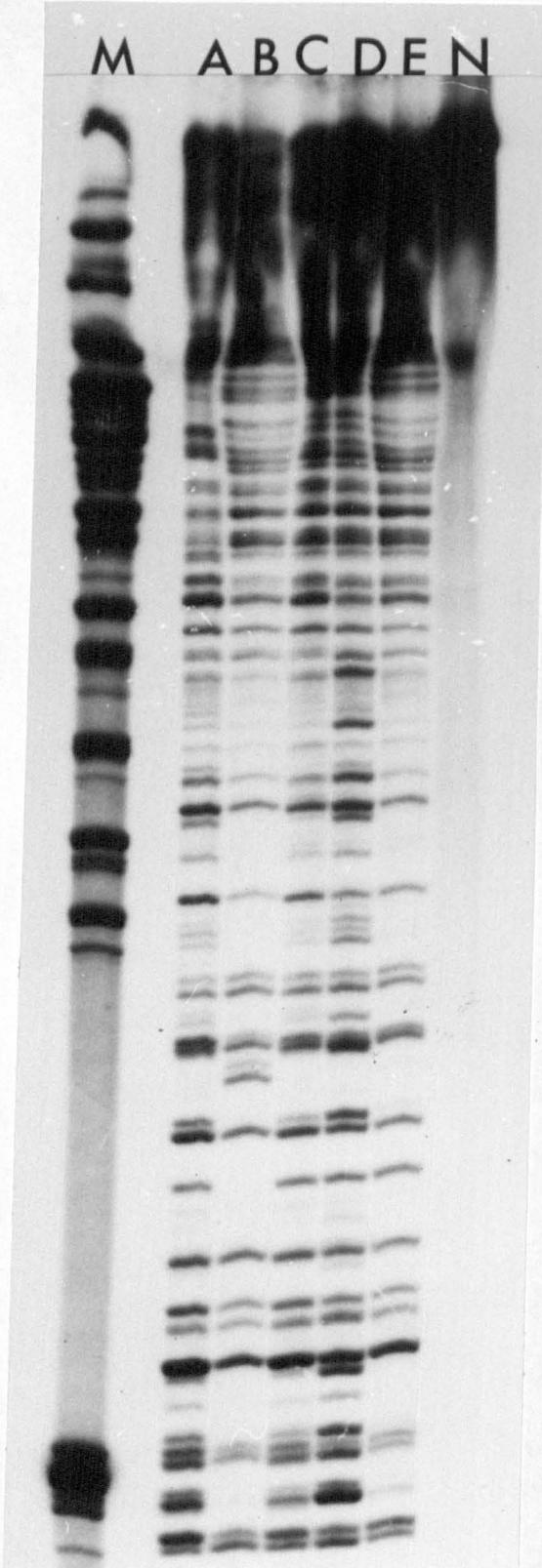
The arrangement of samples and gel running conditions are exactly as described in the legend to Figure 39(b). This figure shows very high similarity in banding patterns for the 'triplets' up to the region marked by the arrows. Minor differences in banding patterns are clearly apparent in the region indicated between the two arrows. Banding pattern differences are also evident internally beyond this region.

Figure 47(a). Partial T₁ Ribonuclease Digestion Fingerprints
Obtained for the Terminal Region of the Five Species 4 RNAs
From the Isolates Under Study.



The layout of samples and the gel running conditions are exactly as described in the legend to Figure 39(a). The banding patterns obtained for the first 40 nucleotides of all five species 4 RNAs are absolutely conserved. Beyond this genome segment-specific region of terminal conservation some differences are apparent, these are more clearly illustrated in Figure 47(b).

Figure 47(b). Partial T₁ Ribonuclease Digestion Fingerprints
Obtained for the Near Terminal Region of the Species 4 RNA's
From the Five Isolates Under Study.



The arrangement of samples and the gel running conditions are exactly as described in the legend to Figure 39(b) except that no ladder track was loaded for this analysis. This figure indicates that beyond 40 terminal nucleotides many banding pattern differences are apparent.

G band at a given position may result from one or more of the six strands under analysis. The variation in band intensities at given G positions which were more evident in these samples may be indicative of minor variation in sequence among the three dsRNA species under test. Figure 45 shows some minor banding pattern differences beyond the 40 nucleotide position DNA marker for the combined species 2 and 3 doublet. Partial nuclease digestion analysis of the species 4 RNA's (Figure 47b) revealed greater differences than those seen for either the 2, 3 doublet or the 7, 8, 9 triplet. On the basis of the partial nuclease digestion patterns the species 4 RNA's of these five isolates could be divided into two distinct groups made up of isolates A, C and D, and isolates B and E. Within each group slight variations in banding patterns are evident but these are less than the differences that existed between the two groups. Despite these differences in banding patterns between the two groups of species 4 RNA's, there is an underlying conservation in the banding patterns among all five of them. To investigate whether these banding pattern differences reflected major structural differences, the species 4 RNA's from isolates C, D and E were subjected to two dimensional T_1 oligonucleotide fingerprint analysis (see Figure 48). These results indicated that all three of these RNA species were radically different from each other despite the very similar terminal region as indicated by Figure 47(a). The results show that even where there is no detectable variation in mobility of corresponding genome segments, differences in their primary structure can exist which are as great or even greater than those observed between corresponding genome segments that do not co-migrate.

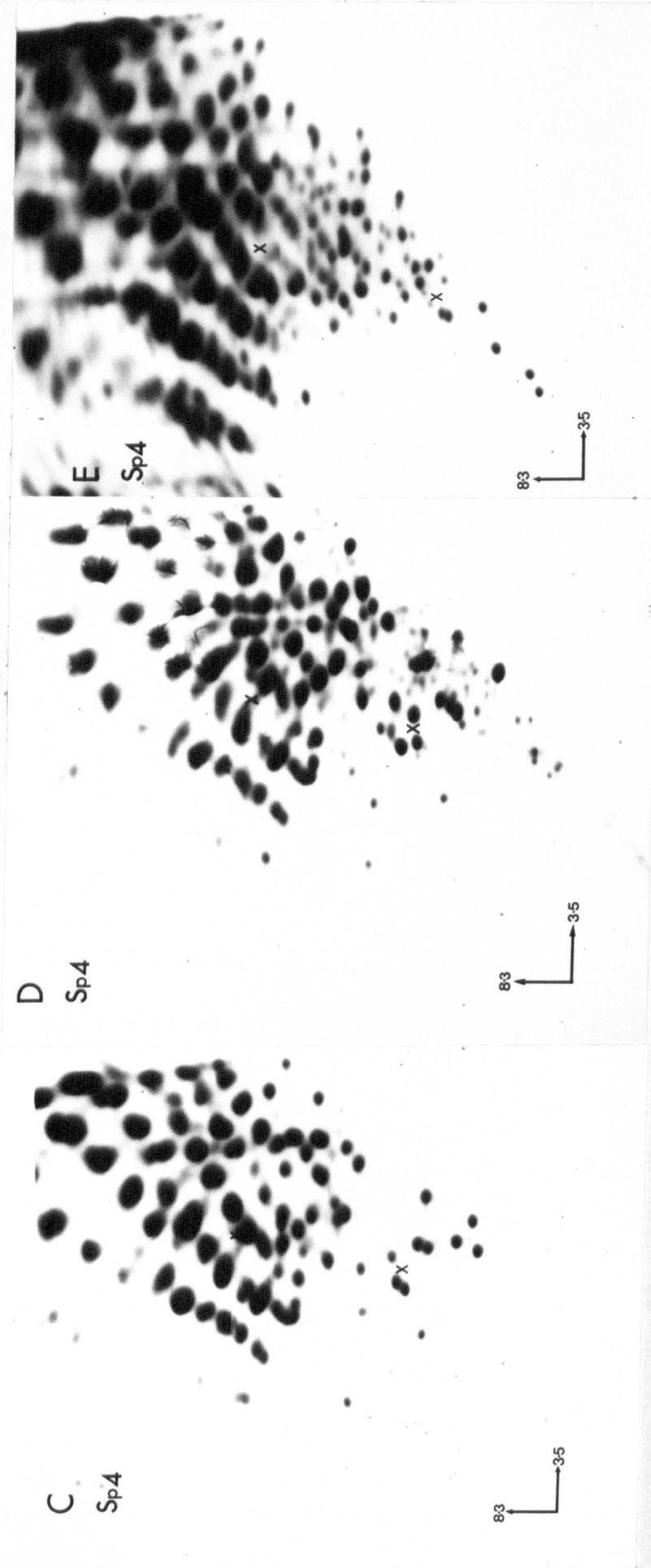


Figure 48.

Figure 48. Two Dimensional T₁ Oligonucleotide Fingerprint Analysis of the Species 4 RNAs from Isolate C (Left Panel), Isolate D (Middle Panel) and Isolate E (Right Panel).

The fingerprint analysis of these three RNA species shows them to be totally unrelated despite having similar partial nuclease digestion patterns for their terminal regions (Figure 47(a)).

The results of partial nuclease digestion analysis of the species 11 RNA's from the five isolates are shown in Figure 49. The patterns obtained beyond the region of terminal conservation (Figure 49(b)) could be divided into two very distinct groups, isolates A, C and E and isolates B and D, within these groups slight variations in banding patterns can be distinguished. The almost total difference in primary structure between the two groups was confirmed for isolates C and D by two dimensional T_1 oligonucleotide fingerprinting (see Figure 50).

Discussion

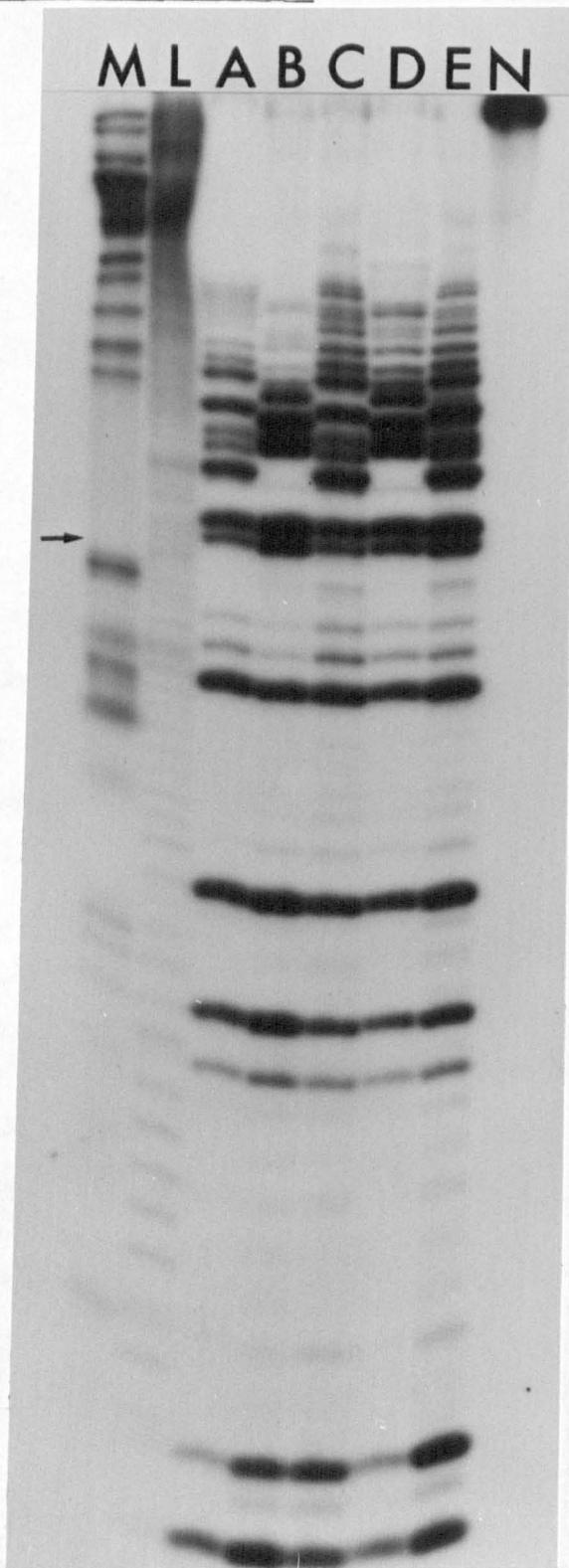
These results showed conclusively that variation in electrophoretic mobility on simple genome profile analysis does not necessarily reflect gross variations in primary sequence of the genome segments concerned. This point is clearly illustrated by the structural analysis of genome segments 10 and 11 from isolates C and D. The species 10 RNA's have mobility differences on simple genome profile analysis which reflect only minor differences in primary structure. Similar differences in migration seen for the species 11 RNA's reflect radical differences in primary structure between them.

Substantial nucleotide sequence heterogeneity is not restricted solely to those genome segments with different electrophoretic mobilities as is shown by the analysis of the species 4 RNA's.

This work showed conclusively that the use of simple single dimension genome profile analysis as a method for surveying and grouping rotaviruses could be very misleading.

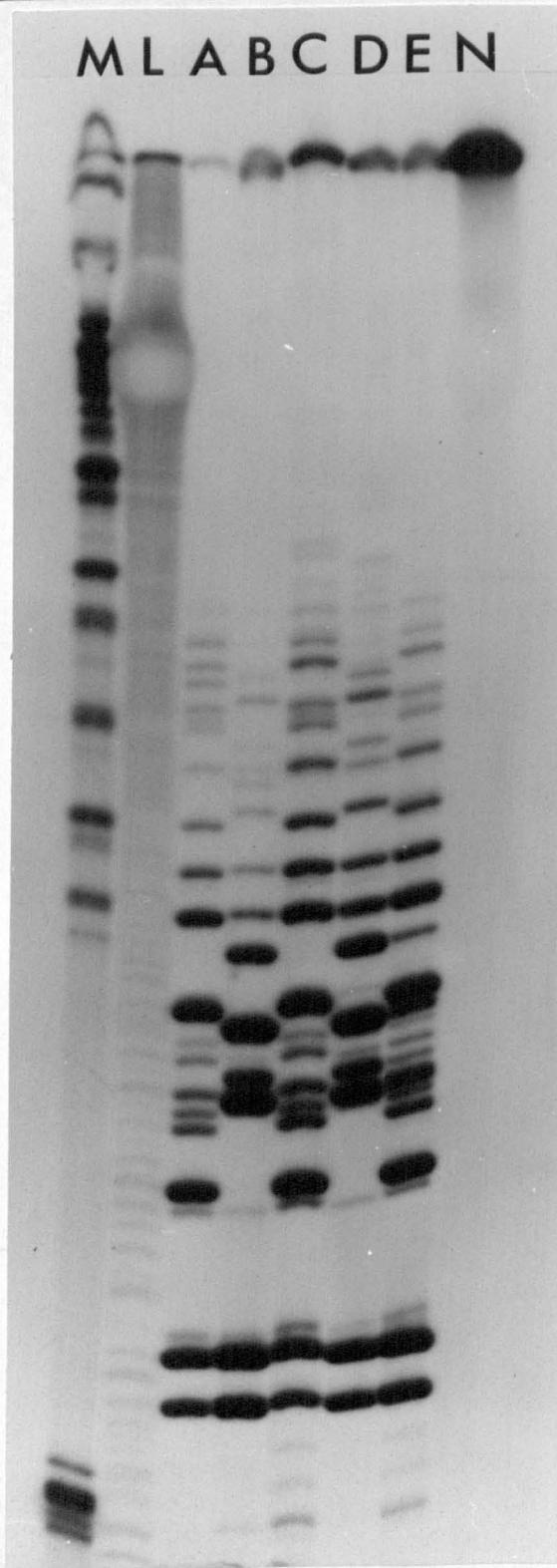
Figure 49(a). Partial T₁ Ribonuclease Digestion Fingerprints

Obtained for the Terminal Regions of the Species 11 dsRNAs From
the Five Isolates Under Study.



The arrangement of samples and the gel running conditions are exactly as described in the legend to Figure 39(a). The arrow to the left of the marker track indicates the end of the region of banding pattern conservation just beyond the DNA fragment marking the 40 nucleotide position.

Figure 49(b). Partial T₁ Ribonuclease Digestion Fingerprints
Obtained for the Near Terminal Regions of the Species 11 dsRNAs
From the Five Isolates.



The arrangement of the samples and the gel running conditions are exactly as described for Figure 39(b). The doublet of bands shared by all genome segments and indicated by an arrow in Figure 49(a) is seen at the bottom of this figure, beyond this point the banding patterns differ greatly.

Figure 50.

C

Sp11

8.3
↑
3.5



D

Sp11

8.3
↑
3.5



Figure 50. Two Dimensional T₁ Oligonucleotide Fingerprint
Analysis of the Species 11 RNAs From Isolate C (Top Panel) and
Isolate D (Bottom Panel).

This figure shows these two species 11 RNAs to be totally unrelated with no diagnostic oligonucleotide spots in common.

The results also provide strong evidence suggesting that rotaviruses undergo gene reassortment in nature; this aspect is considered in the general discussion.

RESULTS CHAPTER 6

Chapter 6

Partial T₁ Ribonuclease Digestion Analysis of Four Porcine Rotavirus Isolates

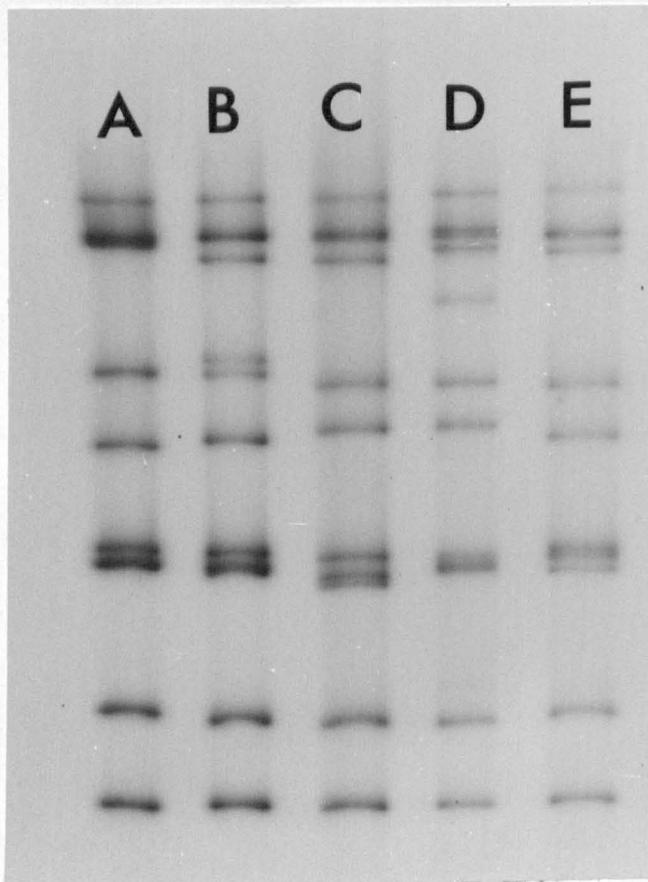
Introduction

The results of analyzing the three rotavirus isolates isolated from different animal species (Chapter 4) indicated their corresponding genome segments shared regions of terminal similarity for both RNA strands. The purpose of this study was to investigate the internal structure of pig rotavirus RNA species beyond these regions of terminal homology. Results of this analysis would show whether near-terminal regions of pig rotavirus genome segments had similar banding patterns to their calf rotavirus counterparts analyzed in the previous Chapter. Three wild porcine isolates were selected for analysis together with a calf isolate that had been passaged six times in piglets. This isolate (isolate 50) was antigenically distinct from calf rotavirus as it was neutralized by antiserum to pig rotavirus (J. Bridger, personal communication).

Results

Figure 51 shows a one dimensional genome profile analysis of the 3' end labelled dsRNA of the four porcine isolates together with a genome profile of the U.K. tissue culture adapted calf rotavirus; two of the isolates had 'extra' genomic bands. Isolate B has a doublet at the position of RNA species 5 indicating that this isolate may represent a mixed infection. Isolate D is a wild isolate which has an extra band midway between RNA species 4 and 5. The 'extra' genomic bands

Figure 51. Polyacrylamide Gel to Show a Comparative Genome Profile Analysis of the Four Porcine Isolates Used in This Study.



Viral RNA was 3' end labelled as described in Materials and Methods. Samples were run on a 20 cm 7.5% polyacrylamide gel at 20 mA for 16 hrs.

Track A = isolate A, U.K. tissue culture adapted bovine rotavirus

Track B = isolate B (50 - Compton)

Track C = isolate C (33 - Compton)

Track D = isolate D (2756 - Liverpool)

Track E = isolate E (21180 - Lincoln)

Migrational differences can be seen for species 4, 5, 6, 7, 8, 9, 10 and 11. Isolate B has an extra band at the position of RNA species 5, isolate D also has an extra band midway between RNA species 4 and 5.

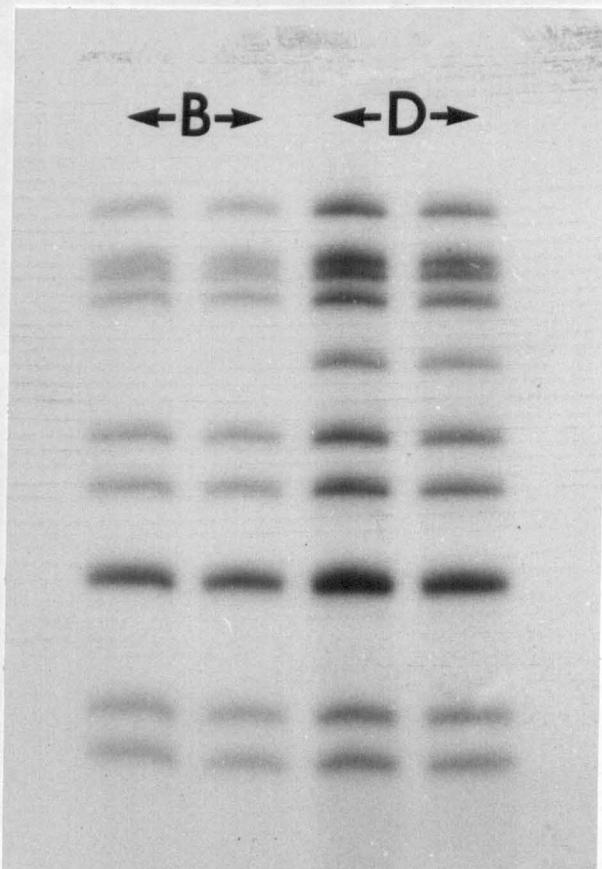
from both isolates were of approximately equal intensity to the other individual RNA species in their respective genome profiles indicating them to have been present in equimolar amounts.

Preparative gel fractionation (Figure 52) shows disappearance of the 'extra' band for isolate B. The 'extra' band observed for isolate D remained on this analysis and was recovered as a discreet fraction for further study. Preparative fractionation of these four isolates allowed recovery of their dsRNA as nine fractions with individual resolution of RNA species 2 and 3. All nine fractions were subjected to partial nuclease digestion analysis for their near-terminal (40-100 nucleotide regions).

Figure 53 shows the banding patterns produced following partial T_1 ribonuclease digestion of the four porcine species 1 RNAs. The characteristic DNA marker fragment running at nucleotide position 40 is arrowed in this figure and can be seen in all other near terminal analyses in this Chapter. All four porcine species 1 RNAs had similar partial digestion fingerprints with only a few minor banding pattern differences between them.

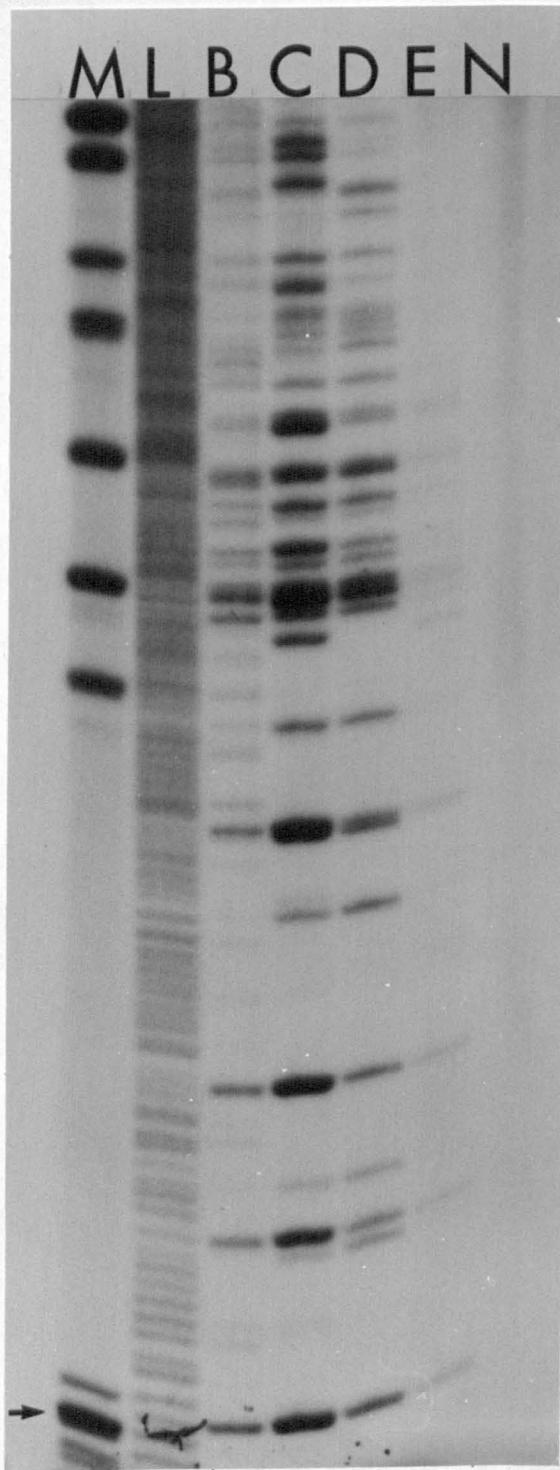
The partial nuclease digestion patterns obtained for the individually isolated species 2 and 3 dsRNAs are not presented. These results showed very similar patterns for both RNA species indicating these two very closely migrating bands may have been cross-contaminated therefore meaningful interpretation of this data was not possible. The species 4 RNAs showed mobility differences on simple genome profile analysis (see Figure 51). The partial nuclease digestion products of these RNA species (Figure 54) had a high level of

Figure 52. Preparative Fractionation of Viral dsRNA From Isolates B and D.



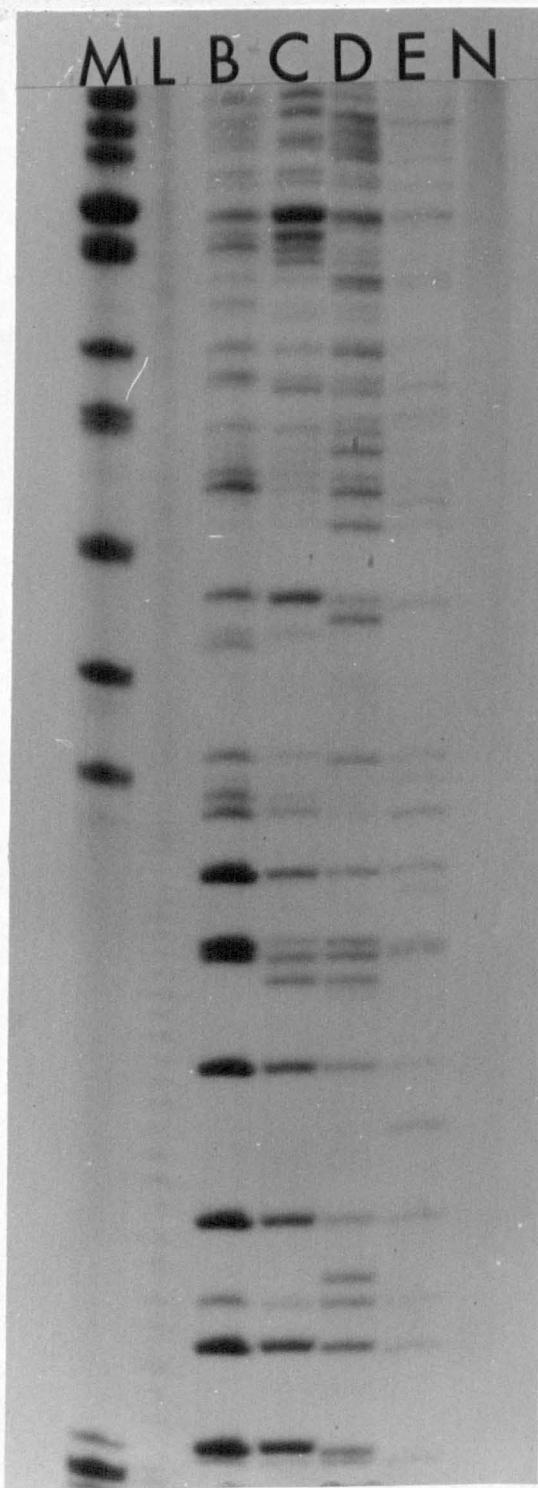
3' end labelled viral RNA of isolate B and isolate D were each loaded across two wells of this 1.5% agarose gel. Electrophoresis was conducted at 30 mA for 16 hrs.

Figure 53. Partial T₁ Ribonuclease Digestion Analysis of the Near Terminal Region of the Species 1 RNAs From the Four Isolates Under Study.



Track L is the partial hydrolysis reference ladder track, track M is the marker track containing radiolabelled Hpa II digestion fragments of the plasmid PBR 322. The DNA fragment at the bottom of this track that runs at nucleotide position 40 and defines the approximate end of the region of genome segment specific homology is arrowed. Tracks B-E are the partial T_1 ribonuclease digestion patterns for RNA species 1 from the four porcine isolates shown in Fig. 51. Track N is native undigested RNA from isolate B. Throughout this experiment problems were encountered with digestions of RNA species from isolate E due to the initial poor dsRNA recovery from the preparative gels. Electrophoresis was conducted at 1.6 Kv for 6 hrs.

Figure 54. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 4 RNA's From the Isolates
Under Study.



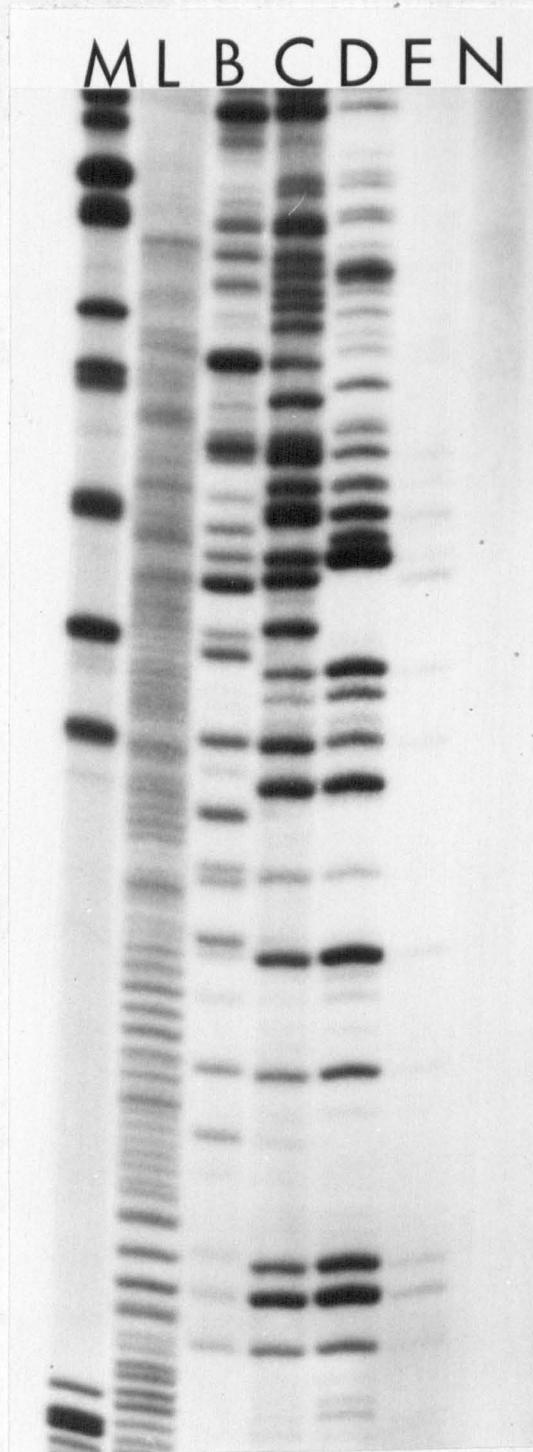
The arrangement of samples and the gel running conditions were as described in the legend to Figure 53. Many banding pattern differences can be seen between these corresponding genome segments.

heterogeneity in the 35-100 nucleotide near-terminal regions with only approximately 50% of bands co-migrating. A similar level of heterogeneity in banding patterns was also observed for the species 4 RNAs of the five bovine isolates in Results, Chapter 5 (see Figure 47(b)). However, comparison of the banding patterns obtained for these porcine isolates showed them to be entirely different from those of the bovine isolates. Variation in this genome segment may be expected as it is believed to code for an outer shell protein in the U.K. tissue culture adapted calf virus (McCrae and McCorquodale, 1982).

Genome profile analysis indicated the presence of two bands at the position of RNA species 5 in isolate B. This 'doublet' was obtained as a single band on preparative fractionation and was subjected to partial nuclease digestion analysis together with the other species 5 RNAs (Figure 55). The banding pattern of species 5 RNA from isolate B appeared to be totally different from the digestion patterns of the species 5 RNAs from isolates C and D. Cross comparison of these partial digestion patterns with those of the bovine isolates indicated that the pattern obtained for isolates C and D were very different. However the species 5 RNA of isolate B had a very similar pattern (see Figure 41) to bovine isolate D.

The partial nuclease digestion patterns of the species 5 RNA's for porcine isolates C and D were identical for a region of approximately 35 nucleotides from the bottom of the gel (Figure 55). Beyond this point radical differences appeared between them; both RNA species had similar mobilities on genome profile analysis.

Figure 55. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 5 RNAs From the Isolates
Under Study.



The arrangement of the samples and the gel running conditions were as described in the legend to Figure 53. The partial nuclease digestion fingerprint for isolate B is entirely different from the patterns obtained for isolates C and D. The patterns of isolate C and D are identical for approximately 35 nucleotides from the 40 nucleotide DNA size marker and then become radically different.

An hypothesis to account for these major differences (beyond the region of further homology from 40-85 nucleotides) might be that one of these RNA species had undergone a minor deletion or addition giving rise to the apparently major change in digestion patterns. Under these circumstances the overall primary structure of both corresponding genome segments would remain similar.

To test the sequence relatedness of these two RNA species they were subjected to two dimensional oligonucleotide fingerprint analysis. The results (Figure 56) shows completely different patterns of spots indicating these genome segments had substantially different primary sequences.

Partial nuclease digestion analysis of the species 6 RNAs is shown in Figure 57. Some minor differences between the isolates are apparent although the banding patterns are highly conserved. Cross comparison with the species 6 RNAs of the bovine isolates (see Figure 42) revealed that the banding patterns of these genome segments have some similarities. Figure 58 illustrates the partial nuclease digestion analysis of the species 7, 8, 9 complex, the digestions have worked well for isolates B and C these tracks show a high level of banding pattern similarity. Comparison with the results for the bovine isolates showed these patterns to be entirely different.

Analysis of the species 10 RNAs is shown in Figure 59; many minor differences in banding patterns were evident although their overall banding patterns had an underlying conservation. Cross comparison with the corresponding bovine genome segments indicated these partial nuclease digestion products to be completely different. Figure 60

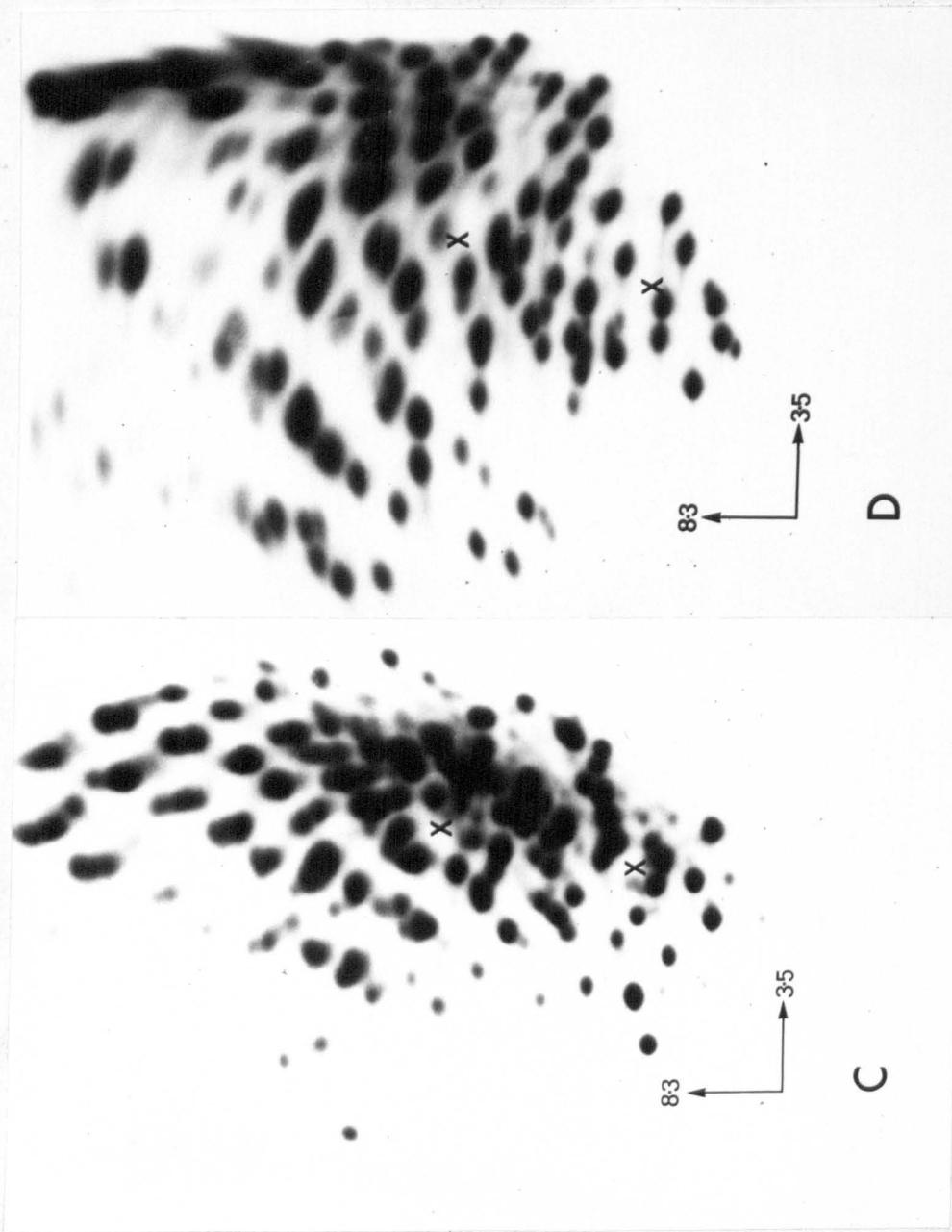


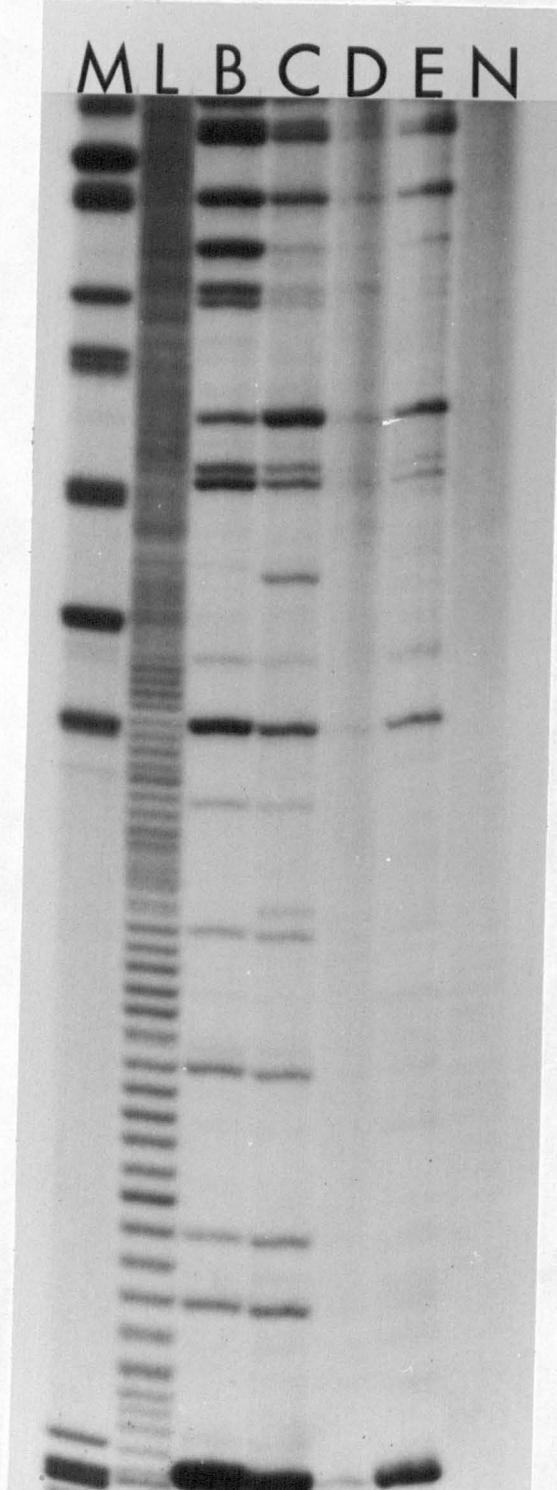
Figure 56.

Figure 56. Two Dimensional T₁ Oligonucleotide Fingerprint

Analysis of RNA Species 5 From Porcine Isolates C and D.

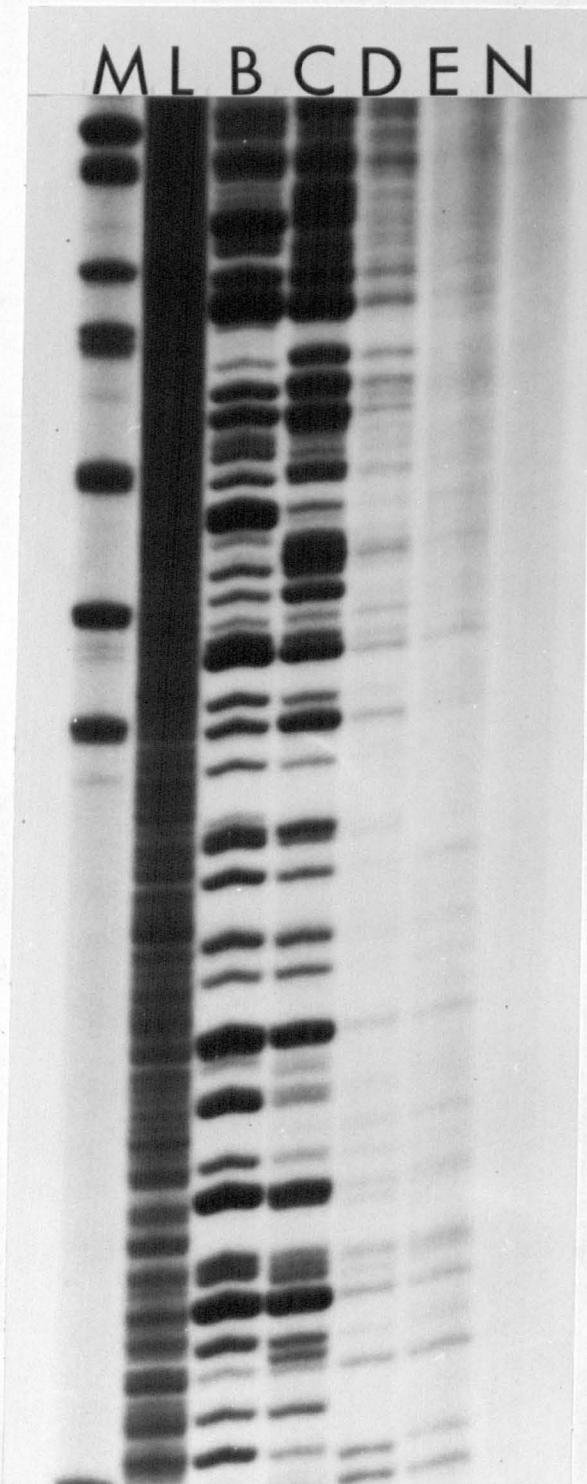
The oligonucleotide fingerprints are presented in two panels, the left panel is of isolate C and the right panel is of isolate D. The positions of two dye markers are indicated (x, lower centre, xylene cyanol, and x, centre, bromophenol blue). The first dimension gel was run from left to right, while the second dimension was run from bottom to top.

Figure 57. Partial T₁ Ribonuclease Digestion of the Near Terminal Region of the Species 6 RNAs From the Isolates Under Study.



The arrangement of the samples and the gel running conditions were as described in the legend to Figure 53.

Figure 58. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 7, 8, 9 'Triplets' of the
Isolates Under Study.



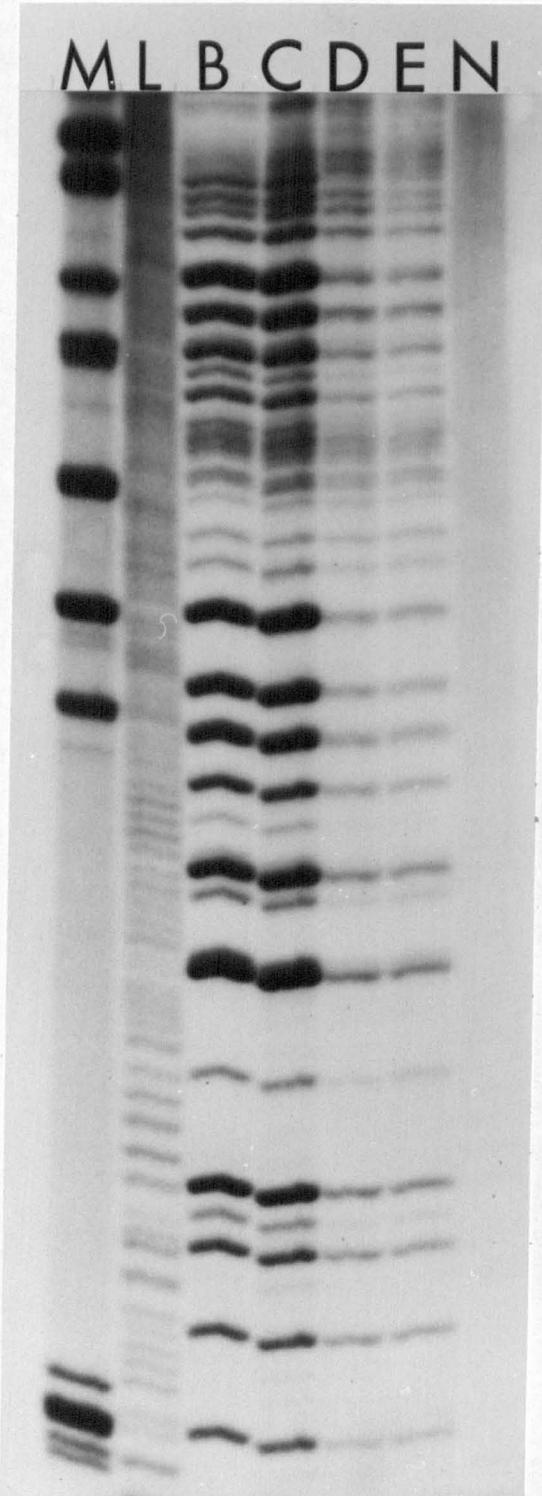
The arrangement of the samples and the gel running conditions
were as described in the legend to Figure 53.

Figure 59. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 10 RNAs From the Isolates
Under Study.



The arrangement of the samples and the gel running conditions
were as described in the legend to Figure 53.

Figure 60. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 11 RNAs From the Isolates
Under Study.



The arrangement of the samples and the gel running conditions
were as described in the legend to Figure 53.

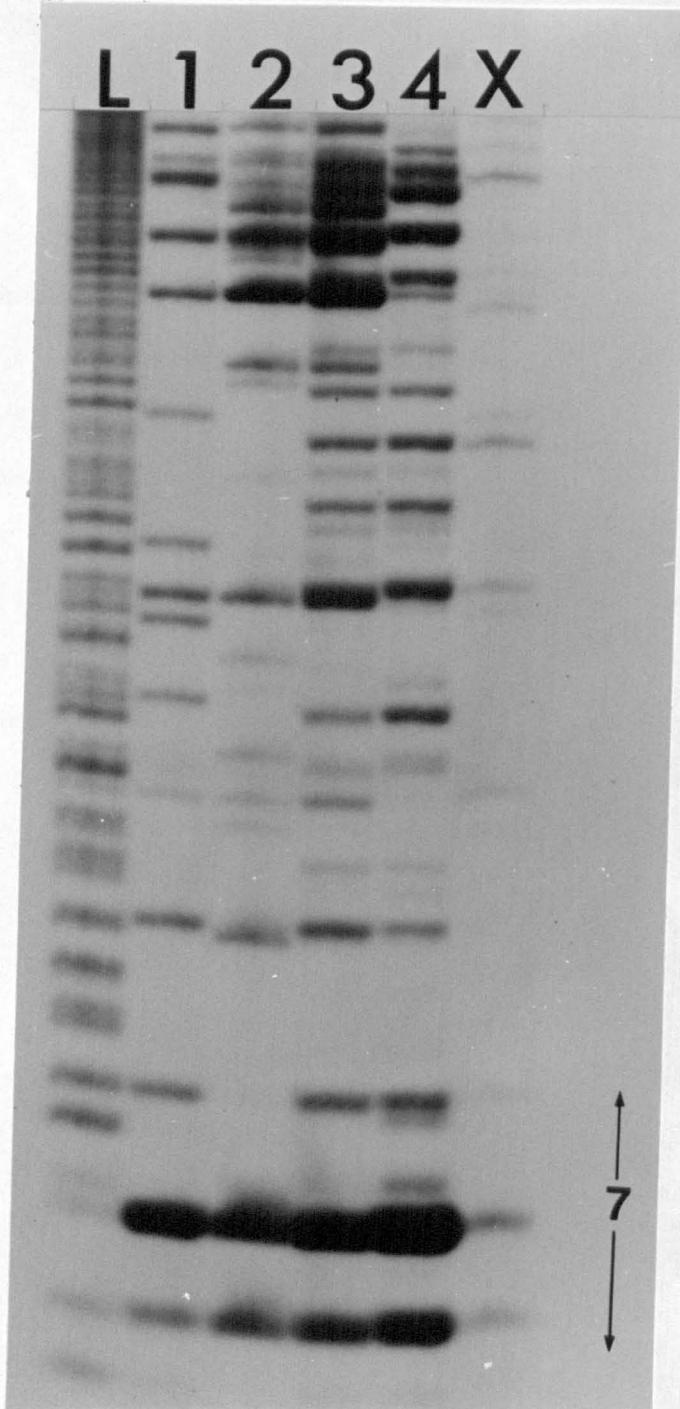
shows the partial nuclease digestion patterns obtained for the species 11 RNAs. These fingerprints were all very similar; comparison with the two distinct groups of patterns produced for the corresponding bovine RNA species indicated that the pig sp 11 patterns were unrelated to either.

The extra band of isolate D was further analyzed to determine the nature of its structure. The native individually isolated radio-labelled band was treated with high concentrations of S1 nuclease and T₁ ribonuclease. There was no significant decrease in TCA insoluble counts following the 30 minute incubation periods with either treatment. These results together with the very tight migration of the band in the high concentration polyacrylamide gel (Figure 51) indicated that this band was probably of a double-stranded nature. Following treatment with 90% DMSO at 45°C for 15 mins the extra band became sensitive to digestion with T₁ ribonuclease indicating that it was composed of RNA. It was possible that this band was derived from the larger RNA species. Therefore together with genome segments 1-4 it was subjected to partial nuclease digestion analysis (see Figure 61). These results indicated that although the extra band had a rotavirus-like terminal pattern, it was completely unrelated to the near terminal regions of sp 1-4. Further investigation was prevented by lack of material for analysis.

Discussion

The results of analyzing the four porcine rotavirus isolates provided several interesting conclusions. Like the bovine isolates co-migrating

Figure 61. Partial T₁ Ribonuclease Digestion Analysis of the Terminal Region of the Species 1-4 and the Extra Band of Isolate D.



Track L is the partial hydrolysis reference ladder track,
tracks 1-4 are the terminal partial nuclease digestion
products of species 1-4 RNAs of isolate D. Track X is a
partial nuclease digestion analysis of the extra band. The
seven absolutely conserved terminal nucleotides are arrowed
at the bottom of the gel. This 16% gel was run at 1.6 KV for
2½ hrs.

corresponding genome segments had as many banding pattern differences as corresponding genome segments that did not co-migrate.

Corresponding RNA species 6's from the porcine isolates showed some similarity with their bovine counterparts. In the U.K. tissue culture adapted calf virus RNA species 6 codes for VP6 the common or group antigen of rotaviruses (McCrae & McCorquodale, 1982) therefore these genome segments may be expected to have some structural similarity for all rotaviruses. Cross comparison of the banding patterns obtained for all the other genome segments showed no similarity with the bovine isolates.

However the species 5 RNA of isolate B had a very similar banding pattern to the bovine species 5 RNA's. The double band observed for species 5 of isolate B on genome profile analysis indicated that this isolate may be composed of a mixture of two rotaviruses. The partial nuclease digestion results for the other genome segments of isolate B showed them to be very similar to pig rotavirus genome segments. There was no evidence for mixed pig/calf partial nuclease digestion patterns strongly suggesting that this isolate was not a mixed infection but a genuine pig isolate as characterized by the serological analysis. Isolate B was originally from a calf. During the passaging through piglets it may have undergone gene reassortment with a pig rotavirus so that only one bovine genome segment remained. The unnatural passage history and the uncertainty regarding the double band for RNA species 5 has precluded any conclusions regarding the 'natural' variation of isolate B.

RESULTS CHAPTER 7

Chapter 7

Partial T₁ Ribonuclease Digestion Analysis of Human Rotavirus

Isolates

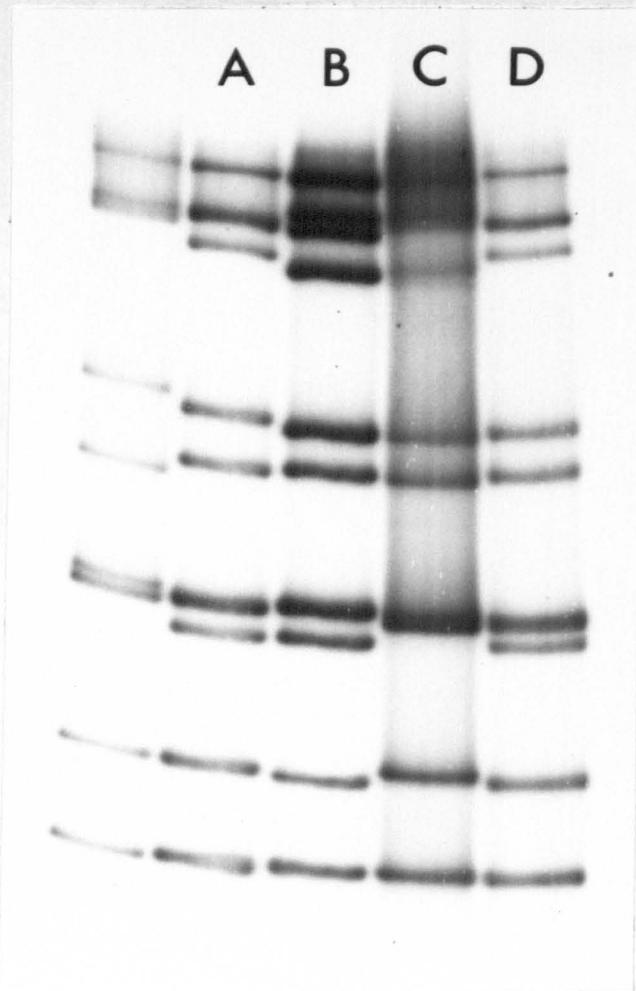
Introduction

To conclude this investigation of the nature and extent of rotavirus genome segment variation by partial nuclease digestion analysis four human isolates were selected for study. Results of these experiments would show whether near-terminal regions (40-100 nucleotides) of human rotavirus genome segments shared similar banding patterns with those of the calf and pig isolates already analyzed and may provide evidence for interspecies gene re-assortment as a mechanism by which these viruses can vary.

Results

A one dimensional genome profile analysis of the 3' end labelled dsRNA of the four human isolates used in this study is shown in Figure 62. Each isolate was collected as 8 fractions of dsRNA following preparative agarose gel fractionation. Because of the problems encountered in Chapter 6 no attempt was made to resolve genome segments 2 and 3 in this experiment. The near-terminal partial nuclease digestion results of all eight fractions are shown: Figure 63 (species 1 and 4); Figure 64 (species 5 and 6); Figure 65 (species '2-3' and '7-8-9'); Figure 66 (species 10 and 11). Small differences in banding patterns of the type that may be expected from variation by antigenic drift were apparent within all sets of corresponding genome segments.

Figure 62. Polyacrylamide Gel to Show a Comparative Genome Profile Analysis of the Four Human Isolates Used in This Study.



Viral RNA was 3' end labelled as described in Materials and Methods. Samples were run on a 20 cm 7.5% polyacrylamide gel at 20 mA for 16 hrs.

Track A = isolate A Holcomber London March 81

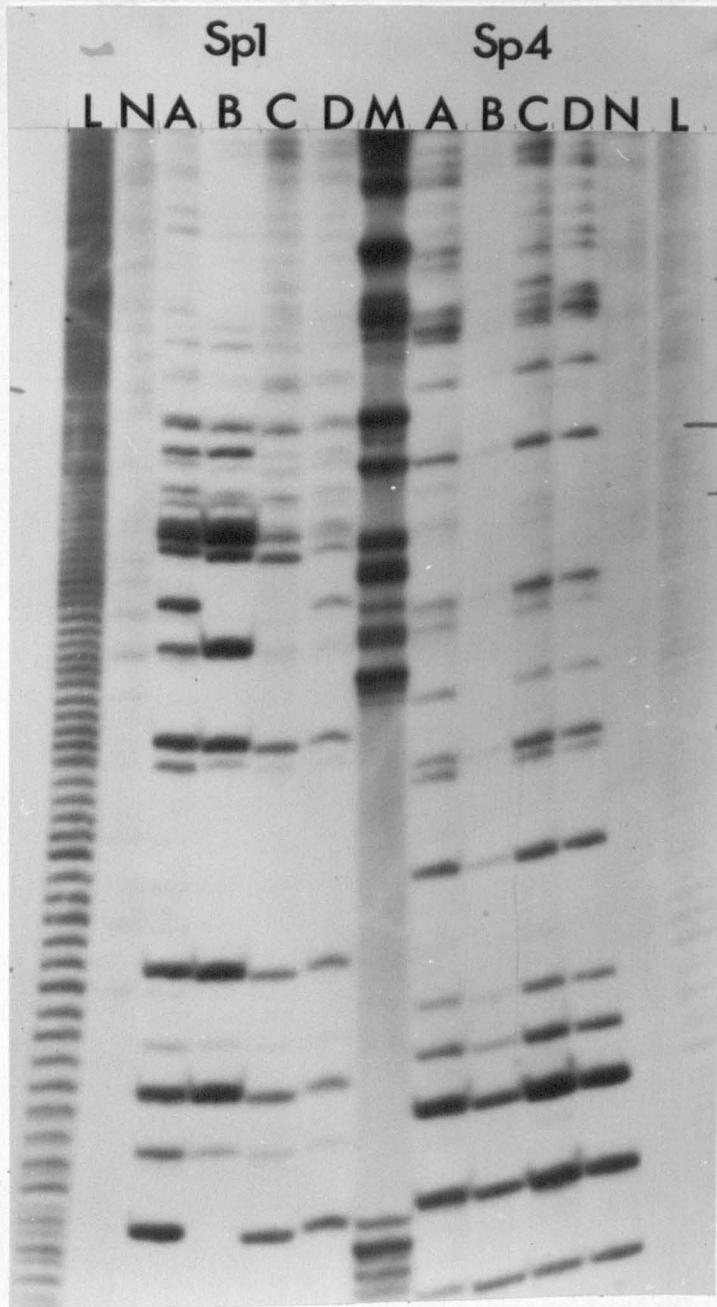
Track B = isolate B Kenny London August 81

Track C = isolate C Mallin Birmingham February 78

Track D = isolate D Lam London March 81

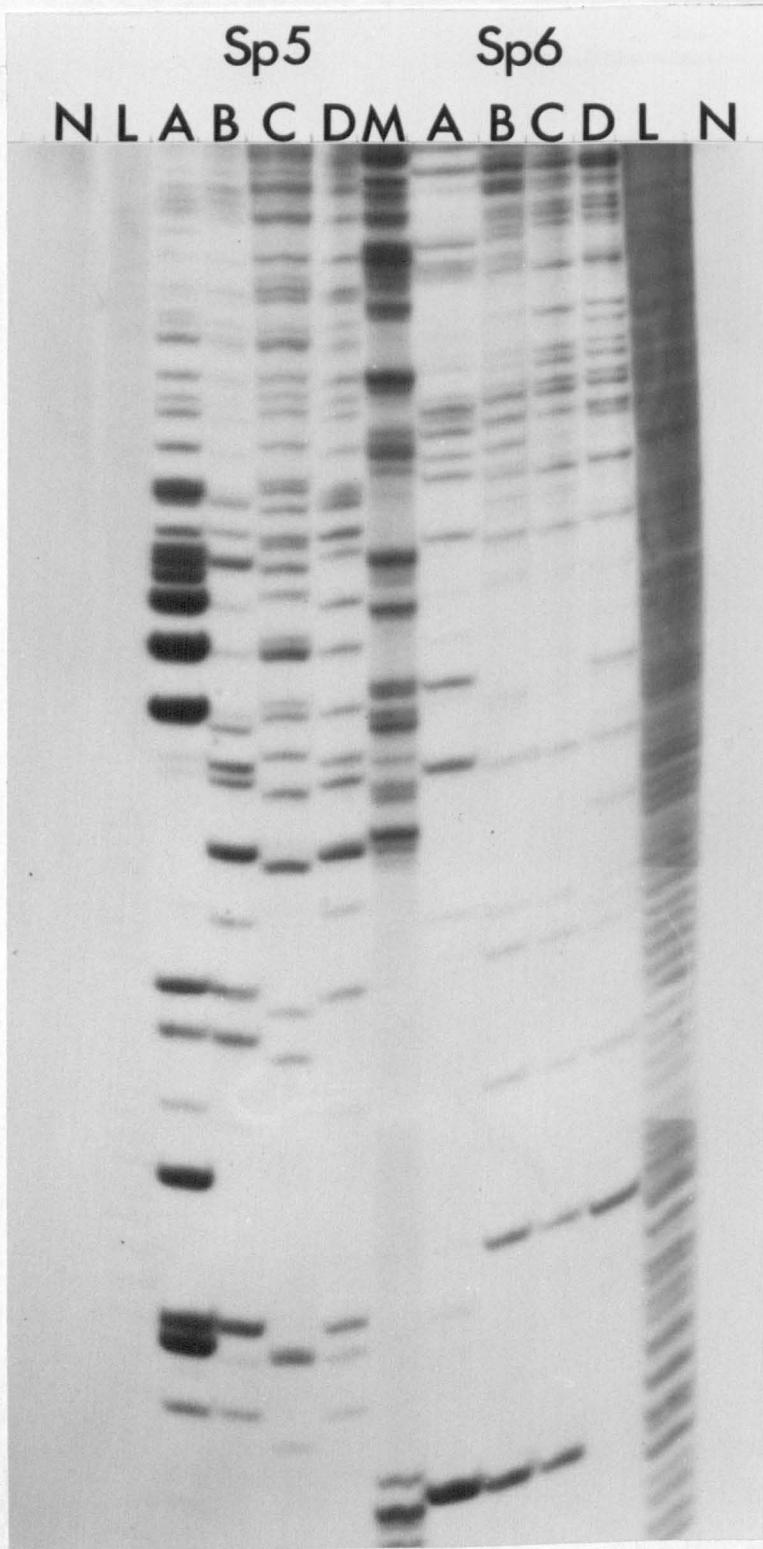
The unmarked track to the left of isolate A shows the genome profile of the U.K. tissue culture adapted calf virus which was included for reference.

Figure 63. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 1 and Species 4 RNAs of the
Four Isolates Under Study.



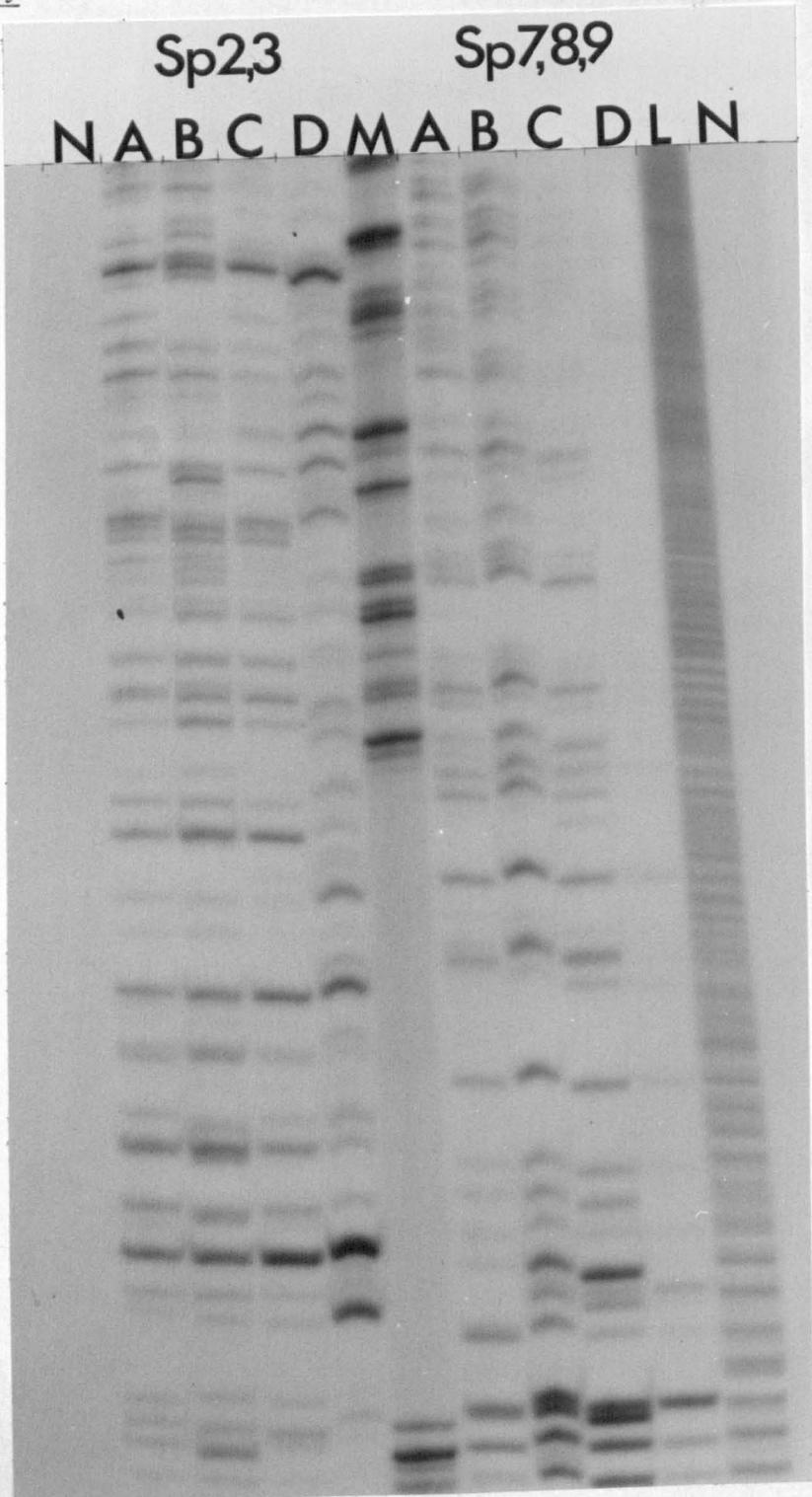
Tracks L are the partial hydrolysis reference ladder tracks, track M is the marker track containing radiolabelled Hpa II digestion fragments of the plasmid PBR 322. The DNA fragment at the bottom of this gel track runs at nucleotide position 40 and is also seen in Figures 64, 65 and 66. Tracks A-D are the partial T₁ ribonuclease digestion products from RNA species 1 or RNA species 4 of the four human isolates A-D shown in Figure 62. Tracks N are native undigested RNA samples from isolate A for RNA species 1 and 4. This 14% gel was run at 1.6 KV for 6 hrs.

Figure 64. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 5 and Species 6 RNA of the
Four Human Isolates Under Study.



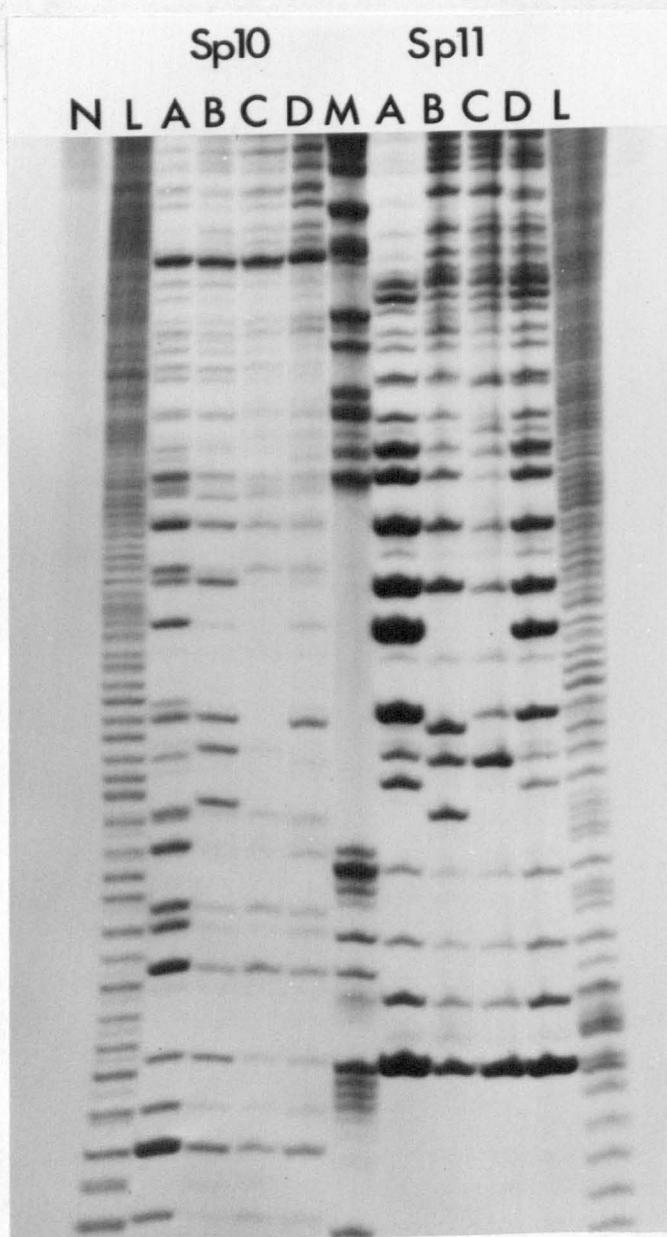
The arrangement of samples and the gel running conditions were as described in the legend to Figure 63.

Figure 65. Partial T₁ Ribonuclease Digestion Analysis of the Near Terminal Region of the Species 2, 3 'Doublet' and the Species 7, 8, 9 'Triplet' of the Four Human Isolates Under Study.



The arrangement of samples and the gel running conditions were as described in the legend to Figure 63. Species 7, 8, and 9 of isolate D were overdigested during the sample preparation.

Figure 66. Partial T₁ Ribonuclease Digestion Analysis of the
Near Terminal Region of the Species 10 and Species 11 RNA of
the Isolates Under Study.



The arrangement of samples and the gel running conditions were as described in the legend to Figure 63.

Major pattern differences consistent with radical structural differences observed in Results Chapters 5 and 6 were apparent only for the corresponding human species 5 RNA's. Cross comparison of these results with those obtained for corresponding sets of genome segments from the calf and pig isolates showed all the human genome segments to be entirely different.

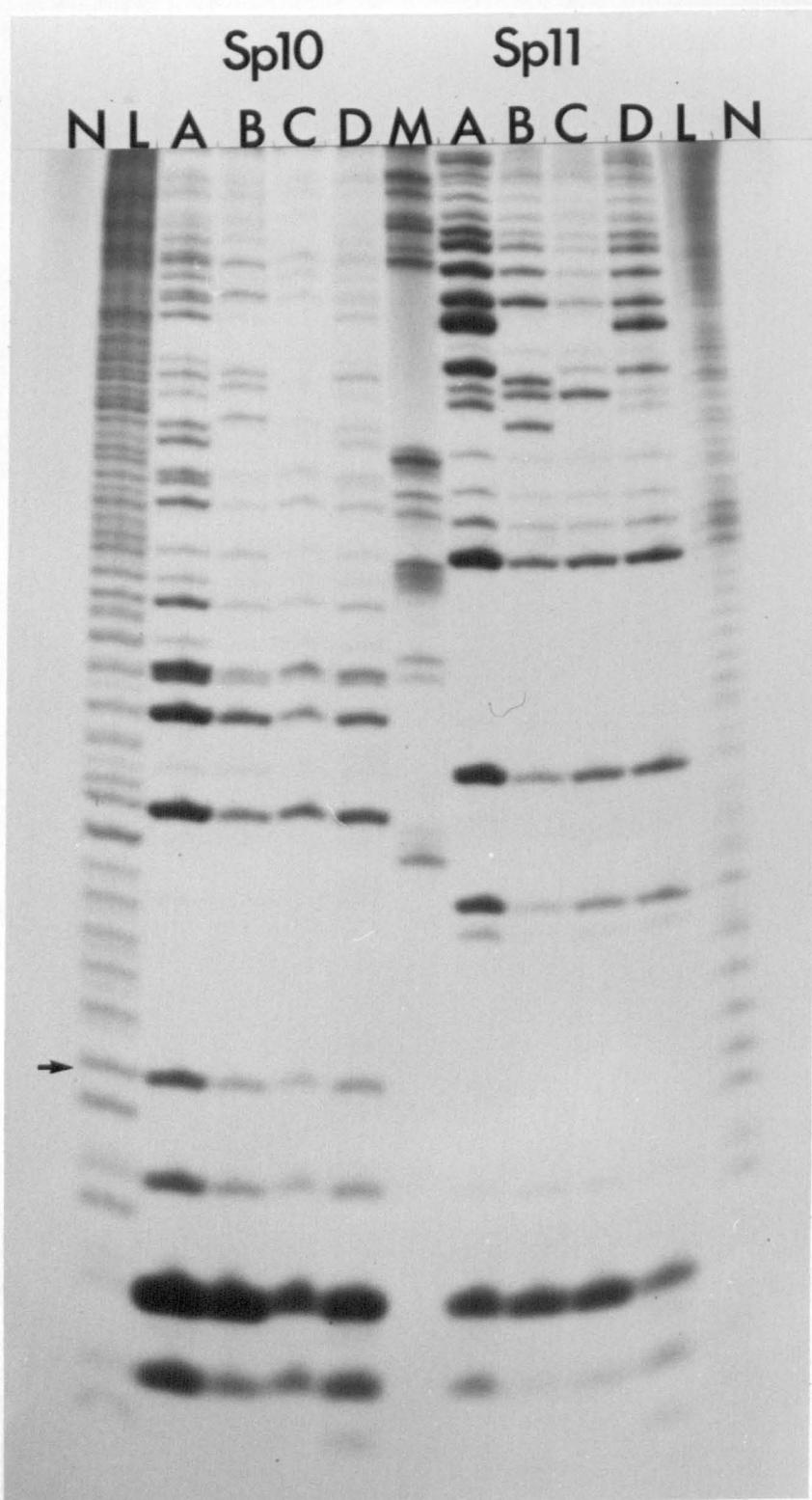
Figure 67 shows the results of partial nuclease digestion analysis of the terminal region (2-40 nucleotides) of species 10 and 11 RNAs. These results indicated that the human isolates shared conserved terminal regions for their corresponding genome segments. The arrow against the species 10 RNA partial digestion indicates an extra band present only in human and pig isolates (see Figure 36 Results Chapter 4).

Discussion

These human isolates although sharing common terminal regions with both calf and pig isolates were unique with respect to near-terminal partial nuclease digestion patterns for all their corresponding genome segments. Like both calf and pig isolates, co-migrating corresponding RNA species had as many banding pattern differences as corresponding RNA species that did not co-migrate.

In this study the digestion patterns obtained for the human isolate species 6 RNAs (Figure 64) were completely different from the patterns obtained for the species 6 RNAs of calf and pig isolates indicating that this genome segment which is believed to code for the group or common antigen can have significant differences in primary structure between isolates from different animal species.

Figure 67. Partial T₁ Ribonuclease Digestion Analysis of the Terminal Region of the Species 10 and Species 11 RNA of the Four Human Isolates Under Study.



The arrangement of samples was as described in the legend to Figure 63. This 16% gel was run at 1.6 KV for 2½ hrs. The arrow marks the position of an extra band at nucleotide position 9 characteristic of species 10 RNA's from human isolates.

However nucleic acid heterology need not necessarily indicate antigenic dissimilarity; only minor sequences in common may be necessary for antigenic similarity since antigenic sites of proteins may only involve relatively short regions of particular genes as shown by variants of the influenza H3 gene (Wiley et al., 1981).

An important point is clearly made by the analysis of the two isolates 'Holcomber' (isolate A) and 'Lam' (isolate B) which were both isolated in the same place, at the same time and had identical genome profiles. Partial nuclease digestion analysis showed banding pattern differences for their corresponding species 1 and species 5 RNAs. These results show unequivocally that isolates with identical genome profiles have primary structure differences for their corresponding genome segments. Therefore the implicit assumption that isolates with identical genome profiles have identical primary structures for their corresponding genome segments (Rodger & Holmes, 1979) is not correct.

RESULTS CHAPTER 8

Chapter 8

Terminal RNA Sequence Analysis of Selected Rotavirus Genome Segments

Introduction

The results of partial T_1 ribonuclease digestion analysis have established that the banding patterns obtained for the terminal 35 nucleotides of corresponding rotavirus genome segments are very similar with respect to their 'G' positions for both plus and minus strands. This high level of positional homology for one particular nucleotide strongly suggests that the nucleotide sequence of these genome-segment-specific terminal regions may be conserved. To investigate this possibility the species 11 RNAs of the O.S.U. tissue culture adapted porcine rotavirus and three calf isolates (U.K. tissue culture adapted calf virus, isolate 4329, and isolate 6597), known to have different near-terminal partial nuclease digestion patterns (Results, Chapter 5) were chosen for RNA sequence analysis of their 3' termini.

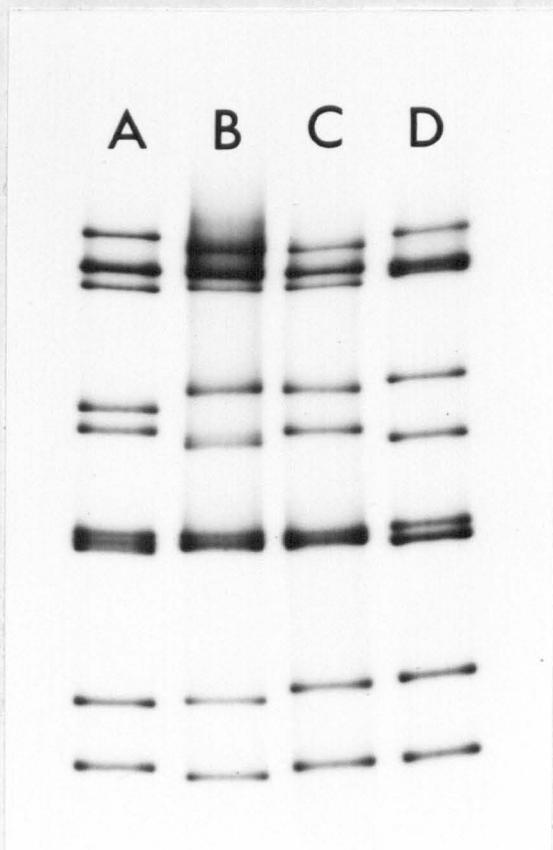
In the same study the species 10 RNAs from these isolates were also analyzed. Genome segment 10 of the U.K. tissue culture adapted calf virus was of particular interest as this was the only RNA species to show clear differences in the otherwise apparently very similar genome segment specific 8-35 terminal nucleotide region. Sequence determination would indicate whether these differences were due to changes in only one or in both RNA strands.

Results

Figure 68 shows an autoradiograph of the 3' terminally labelled genome segments of the four isolates used in this study. Preliminary developmental work was performed using O.S.U. dsRNA as this was available in abundance. The strategy employed for the preparation of single-stranded samples for RNA sequence analysis was as described by McCrae (1981). In summary, 3' terminally labelled dsRNA was fractionated on long (20 x 40 cm) 6% polyacrylamide gels, the RNA bands were located by autoradiography, excised as gel slices and eluted electrophoretically. Following strand separation the genome segments were hybridized to a 100 fold excess of unfractionated plus strand mRNA synthesized in vitro by rotavirus 'cores'. After hybridization the displaced terminally labelled plus-strands were separated from the labelled minus-strands (now in double-stranded form) by CF11 column chromatography.

However, using double-stranded RNA from the O.S.U. rotavirus isolate this method of strand separation which was originally developed for reovirus (Ito & Joklik, 1972) did not give the expected two equal peaks of radioactively labelled RNA following elution from the CF11 columns. Instead a large single stranded peak was observed with a very small double-stranded RNA peak; many incorporated counts also remained stuck to the cellulose matrix. Control samples of rotavirus mRNA and dsRNA eluted perfectly from these columns. Assuming that both 3' ends of the dsRNA were equally labelled these results indicated that the strand displacement reaction was not completely successful: it was thought that unlabelled mRNA-labelled minus strand dsRNA hybrids

Figure 68. Polyacrylamide Gel to Show a Comparative Genome Profile Analysis of the Four Rotavirus Isolates Used in This Study.



Viral RNA was 3' end labelled as described in Materials and Methods. Samples were run on a 20 cm 7.5% polyacrylamide gel at 20 mA for 16 hrs.

Track A = Ohio State University (O.S.U.) tissue culture adapted porcine virus

Track B = Bovine isolate 6597

Track C = Bovine isolate 4329

Track D = U.K. tissue culture adapted calf virus

may not have been forming. To investigate this possibility the stringency of the hybridization conditions were relaxed thereby increasing the chances of forming bona fide homologous unlabelled mRNA labelled minus strand dsRNA hybrids. The results of this experiment using O.S.U. species 1 as a test sample are shown in Table 3. At 25°C and in 0.204 M NaCl for 48 hrs the value closest to a 1:1 ratio of 'single-strand' to 'double-strand' fractions was obtained. In an attempt to verify whether the distribution of the peaks reflected the fractionation of pure plus and pure minus RNA strands equal counts from both peaks for all five hybridization reactions were subjected to partial digestion with T_1 ribonuclease. The resulting fragments were analyzed on a sequencing gel (see Figure 69). These results showed that the two peaks obtained for each set of reaction conditions gave distinctly different digestion patterns. However, all five 'single-strand' fractions gave the same unique digestion pattern as did all five 'double-strand' fractions. It was hoped to positively differentiate the two strands on the basis of differences in their absolutely conserved termini (2-8 nucleotides) but it later appeared that both plus and minus strands can have G residues at terminal positions 3 and 4. Therefore although these results did not prove the fractions to be pure sets of plus or minus strands it was reasonable to assume that this was the case.

On this basis hybridization at 25°C for 48 hrs in 0.204 M NaCl was used for all future experiments to achieve strand displacement. Following elution from the Franklin columns 3' end labelled plus strands were ready for immediate sequence analysis. However, the 3' end labelled minus strands were purified in double-stranded form

Table 3. The Effect of Changing the Hybridization Conditions on the Strand Displacement Reaction

Hybridization conditions	Total incorporated counts recovered in plus (+) and minus sense (-) fractions + $\times 10^2$ -	Counts remaining on column
1. 0.102 M NaCl: 37°C conditions for reovirus (Ito & Joklik, 1972)	60	14
2. 0.102 M NaCl: 25°C	80	2.2 (few)
3. 0.204 M NaCl: 25°C	49	39 very few
4. 0.6 M NaCl: 25°C	60	20
5. 1.02 M NaCl: 25°C	12	12

The stringency of the hybridization reactions were arbitrarily relaxed by increasing the salt concentrations and lowering the temperature. Hybridization was for 48 hrs in 6.59 mM EDTA and 34 mM Tris-HCl pH 7.4.

Figure 69. Fractionation of Partial T₁ Ribonuclease Digestion
Products of the Plus and Minus Fractions From the Hybridization
Reactions Described in Table 3.



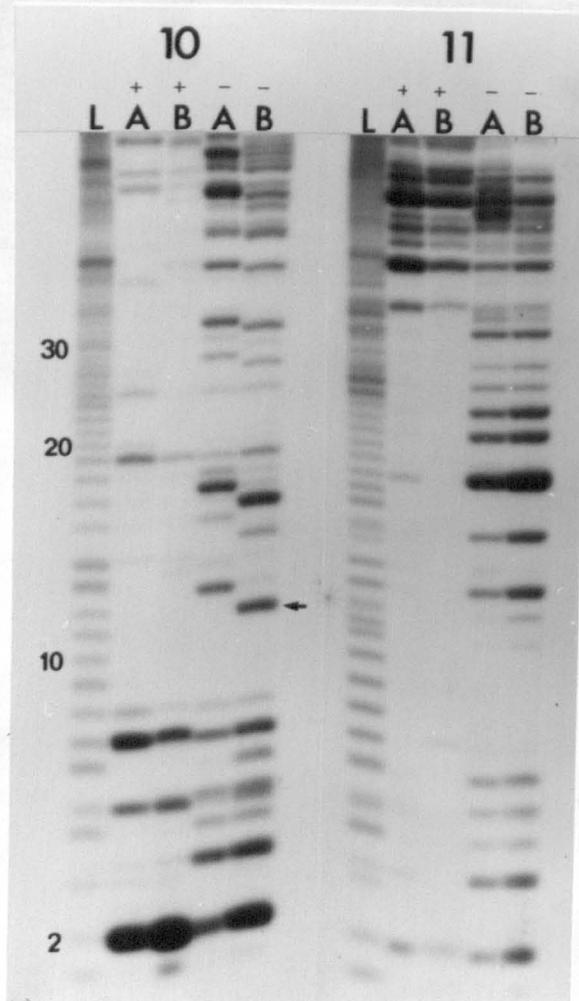
Equal incorporated counts of plus (+) and minus (-) fractions from the hybridization reactions numbered 1 → 5 described in Table 3 were run on this 16% gel for 2½ hrs at 1.6 KV. Partial digestion of the samples was performed as described in Materials and Methods. Track L is the reference ladder track.

which was resistant to the ribonucleases used in the sequencing reactions. To overcome this difficulty these molecules were denatured in 90% DMSO at 45° for 20 minutes; single-stranded RNA was then precipitated directly from the DMSO with ethanol. The opportunities for rehybridization were minimized by resuspending the dried RNA pellet directly in the nuclease solutions used for sequence analysis.

No homologous plus strand mRNA was available to give strand displacement for the two bovine wild isolates (isolate 4329 and isolate 6597) therefore mRNA from the U.K. tissue culture adapted calf virus was used as a substitute. These hybridization reactions worked well for the species 10 and 11 RNAs of isolate 4329 and also for the species 10 RNA of isolate 6597. However the species 11 RNA of isolate 6597 which was demonstrably different from the species 11 RNA of the U.K. tissue culture adapted calf virus by partial nuclease digestion analysis gave only a 1:6 ratio of dsRNA to SS RNA following CF11 chromatography and many incorporated counts remained stuck on the column. Despite the uneven distribution enough dsRNA (minus-strand fraction) was recovered to allow an attempt at terminal sequence determination.

Unfortunately isolate 4329 did not label efficiently enough in the ligase reaction to allow terminal sequence analysis. Therefore both RNA strands from this isolate together with those of the U.K. tissue culture adapted calf virus were digested only with U_2 ribonuclease (see Figure 70).

Figure 70. Partial U₂ Ribonuclease Digestion Analysis of Sp 10
and 11 Genome Segments From Wild Isolate 4329 and the U.K.
Tissue Culture Adapted Calf Virus.



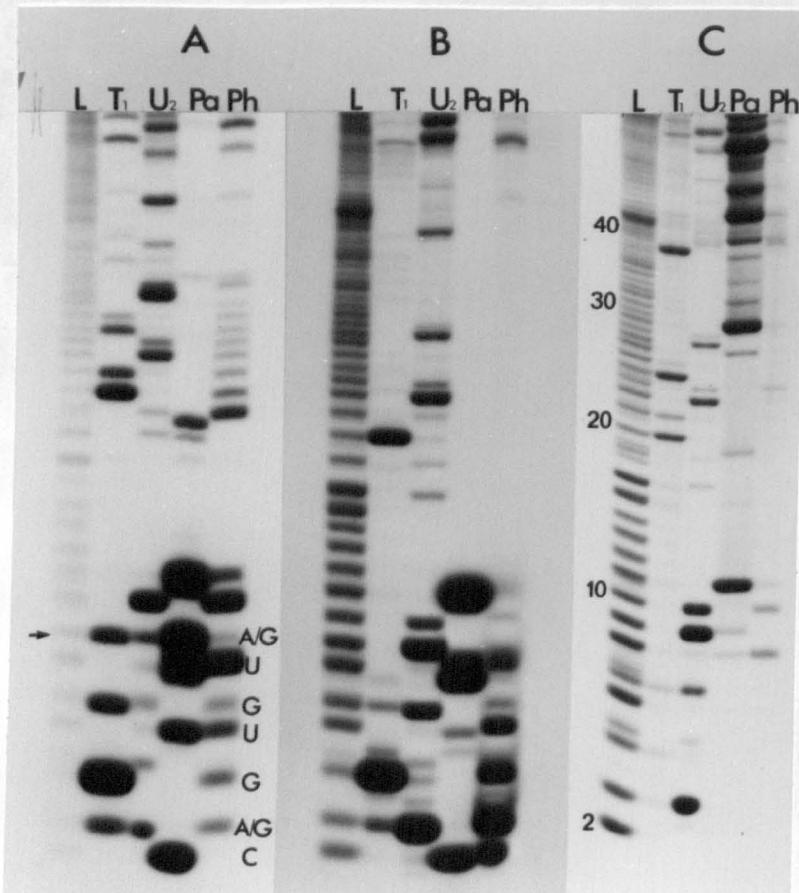
Tracks marked L are the partial nuclease digestion reference ladders, U₂ digestion was performed as described in Materials and Methods. U₂ partial digestion products of plus (+) and minus (-) strands of the U.K. tissue culture calf (A) and isolate 4329 (B) species 10 RNAs are in the left panel,

similar digestions of their species 11 RNAs are in the right panel.
The arrow at nucleotide position 12 marks the first differences
between the minus strands of RNA species 10 from these two
isolates.

Figure 71 shows comparative sequence analysis of the plus sense strand of species 10 RNAs from O.S.U., the U.K. tissue culture adapted calf virus and isolate 6597. This figure illustrates the two major difficulties that were encountered with the partial nuclease digestion method of RNA sequence analysis.

The first problem involved finding the correct enzyme concentrations for each of the four ribonuclease partial digestions; these were calculated from the amount of RNA to be digested which was determined spectrophotometrically. If this determination was not exactly correct samples were either over-digested (for example the pancreatic ribonuclease digest of isolate B) or were under-digested (pancreatic ribonuclease digestion of isolate C). It appeared that the optical density readings may have been affected by slight contaminants. The same problem was also encountered with the species 11 positive strand sequence analysis, see Figure 72. These differing levels of digestion made unequivocal sequence determination for these plus strands extremely difficult. The results also indicated that the specificity of the digestion reactions of the enzymes ribonuclease U₂, Pancreatic ribonuclease A, and polycephalum physarum ribonuclease were affected by their concentrations. An example of this is clearly illustrated at nucleotide position 8 (arrowed in Figure 71) of O.S.U. RNA species 10. Partial nuclease digestion analysis has revealed pig isolates as having a G residue at this position which is absent for calf isolates (see Results, Chapter 4, Figure 36). This figure shows that O.S.U. species 10 plus strand nucleotide position 8 may be G, A or U. Many other similar discrepancies were also present.

Figure 71. Partial Nuclease Digestion Sequence Analysis at the
3' End of Genomic Plus Strands for RNA Species 10 of Isolates
O.S.U. (A); U.K. Calf (B) and 6597 (C).



Partial alkali and nuclease digestions were performed as described in Materials and Methods, the digestion products were fractionated on 16% gels run at 1.6 KV for 2½ hrs. The following track designations were used in this and all other nuclease digestion sequencing figures.

L = partial alkali hydrolysis reference ladder.

T₁ = partial T₁ (G-specific) nuclease digestion products.

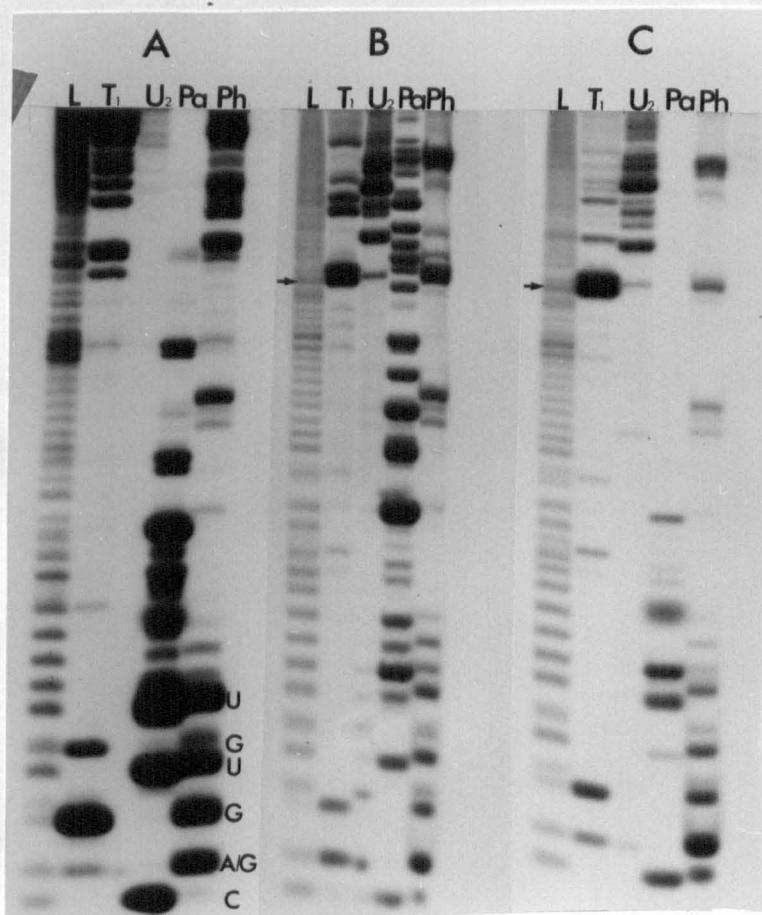
U₂ = partial U₂ (A-specific) nuclease digestion products

Pa = partial pancreatic (pyrimidine specific C & U).nuclease
digestion product

Ph = partial physarum (G, A, U specific) nuclease digestion
products

The absolutely conserved 3' terminal sequence is marked for the
porcine isolate. The arrow indicates a 'G position' not present
for the calf isolates. Nucleotide positions are numbered from
2 to 40 in panel C.

Figure 72. Partial Nuclease Digestion Sequence Analysis at the 3' End of Genomic Plus Strands for^{*} RNA Species 11 of Isolates O.S.U. (A); U.K. calf (B) and 6597 (C).



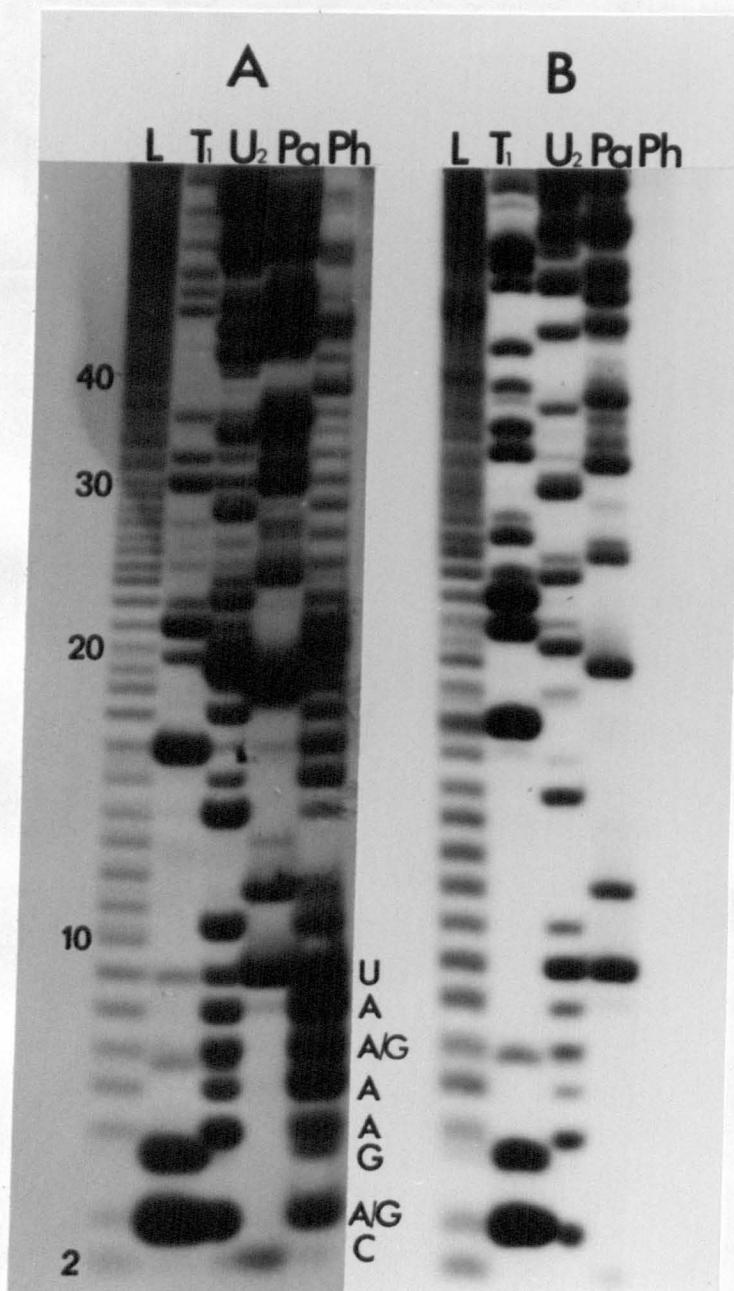
The layout and running conditions are as described in the legend to Figure 71. The arrows in panels B and C mark the position beyond which major differences can be seen in the T₁ digestion tracks for these two bovine isolates.

The second problem concerned regions of the gel where no bands were present for any of the four ribonuclease digestions, for example between terminal positions 11 to 14 of the species 10 RNA plus strands (Figure 71). A possible explanation is that this phenomenon may represent a region of double-stranded secondary structure resistant to ribonuclease attack. Despite these difficulties cross comparison of the sequencing gels for the species 10 (Figure 71) and the species 11 plus strands (Figure 72) show some underlying similarity for each set of results in their terminal 2-40 nucleotide regions.

Sequence analysis of the species 10 and 11 minus strands of the O.S.U. strain and the wild isolate 6597 are shown in Figures 73 and 74. Comparison of the two sequencing gels of the species 10 RNAs (Figure 73) showed them to be almost identical in the 2-40 nucleotide region. Unfortunately the physarum digested sample was lost in preparation and this prevented their absolute sequence comparison.

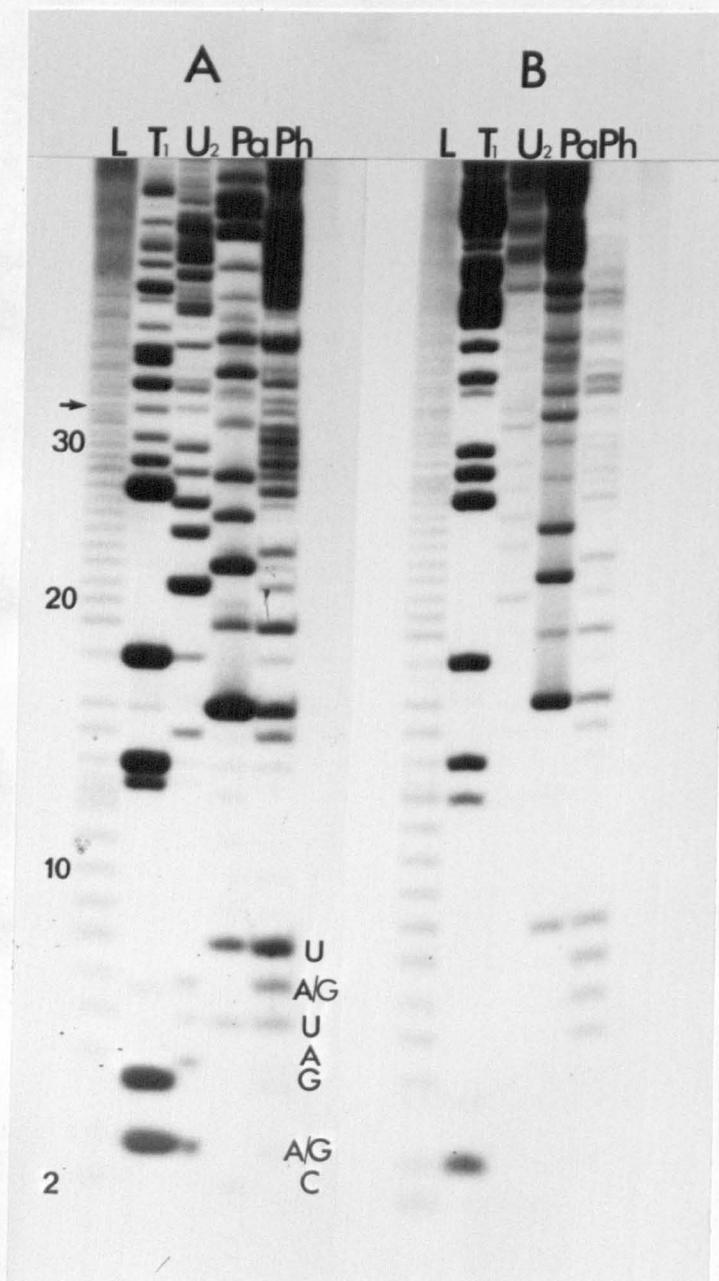
Although the species 11 RNA minus strand of isolate 6597 has been slightly under-digested, definitive comparison to the sequence of the O.S.U. minus strand was possible. Like the plus strands of the species 10 RNAs (Figure 71) a region where no bands were present for all four digestion tracks was also apparent between nucleotides 9 and 12 for these RNA strands. Figure 74 shows that both gels appear to have an identical terminal region of 35 nucleotides with major differences only beginning at the point arrowed for isolate A. The data for the minus strands of these species 10 and species 11 RNAs provided much stronger evidence for the existence of regions of genome segment specific terminal sequence homology, the sequences

Figure 73. Partial Nuclease Digestion Sequence Analysis at the
3' End of Genomic Minus Strands for RNA Species 10 of Isolates
O.S.U. (A) and 6597 (B).



Partial nuclease digestion arrangement and gel condition are as described in the legend to Figure 71. The physarum sample was lost in preparation, however the patterns of the other digestions for 6597 are very similar with those of O.S.U. for approximately 35 nucleotides from the terminus. The conserved 3' terminal sequence is marked for the porcine isolate.

Figure 74. Partial Nuclease Digestion Sequence Determination at
the 3' End of Genomic Minus Strands of RNA Species 11 From
Isolates O.S.U. (A) and 6597 (B).



Partial nuclease digestion arrangement and gel conditions are as described in the legend to Figure 71. The arrow marks the point at which these sequences begin to differ. The terminal nucleotide sequences are given:-

		3'				
2	C		25	C	C	
3	A/G	↑ Common terminus	26	A	A	
4	G		27	G	G	
5	A		28	A/U	A	
6	U		29	G	G	
7	A/G		30	A	A	
8	U	↓	31	G	G	
9	X		32	U	U	
10	X		33	G	A	← differences begin
11	X			A	U	
12	X			5'	5'	
13	C	3'				
14	G	G				
15	G	G		A	= Adenine	
16	A	X		C	= Cytosine	
17	U	U		G	= Guanine	
18	G	G		U	= Uracil	
19	U	U		X	= Unknown	
20	C	X				
21	A	A				
22	C	C				
23	X	X				
24	A	A				

determined for these species 11 minus strands are also presented in Figure 74.

Discussion

The T_1 partial nuclease digestion patterns obtained on the sequencing gels for the individual plus and minus strands of the samples presented are consistent with their partial T_1 ribonuclease digestion fingerprints.

The sequencing data presented confirmed the 3' termini of the minus strands of species 11 RNAs from isolates 6597 and O.S.U. to be identical for approximately 35 nucleotides. Comparison of the sequencing results for the minus strands of the species 10 RNAs from these isolates also confirmed them to have a unique conserved terminal region of 35 nucleotides. Analysis of the common sequence determined for the species 11 minus strand has not revealed the presence of the nucleotide triplet $^{3'}\text{UAC}^{5'}$ in any reading frame. By transposition this would be an initiation codon in the complementary mRNA which is known to be a full length copy of the genomic minus strand (McCrae & McCorquodale, 1982b).

The species 10 RNA of the U.K. tissue cultured adapted calf virus was selected for analysis as this RNA species showed some significant variation in the terminal regions by partial nuclease digestion analysis. Several differences from the other bovine species 10 RNAs were caused by changes in the 3' terminal region of the minus strand of this isolate as is clearly shown in Figure 70. An additional single nucleotide between terminal bases 8 and 12 would account for the first difference (arrowed in Figure 70). Deletion of a single

nucleotide at position 19 and an extra base between nucleotides 25 and 26 could account for the other differences. The significance of these changes for the species 10 RNA of this virus is unknown.

Sequence analysis of the plus strand of species 10 and 11 did not give clear results. Because of this uncertainty no attempt has been made to interpret this data in detail. However similarity in digestion patterns can be discerned up to approximately 35 nucleotides for both RNA species.

The absolute conservation of G residue positions together with these preliminary sequencing results strongly suggests the existence of genome segment specific regions of terminal conservation. The functional role of these regions is unknown; however the fact that they are found adjoining the absolutely conserved terminal sequences common to all rotavirus genome segments allows speculation that they may have regulatory functions. Control of RNA transcription and/or replication, involvement in the quantitative regulation of translation or a function in genome segment selection during virion assembly all lend themselves as possible functions.

A first step towards defining these possible functions requires more definitive comparative nucleotide sequence analysis from cDNA clones of corresponding genome segments followed by the subsequent development of an experimental system whereby the biological effect of synthetically introduced changes in these regions could be assessed.

GENERAL DISCUSSION

General Discussion

At the inception of this thesis there were two main aims:- to determine the nature and extent of genome segment diversity amongst various rotavirus isolates; and then to undertake a detailed structural characterization of the genome segments involved in such variation to ascertain whether gene reassortment (recombination) occurs in nature.

This discussion will be concerned with the main conclusions from the study of rotavirus genome segment variation by partial nucleic acid digestion analysis. Evidence obtained for gene re-assortment as a mechanism by which rotavirus isolates can exchange genetic information in nature will also be considered. The final section gives a future perspective for rotavirus research.

(i) Variation in Rotavirus Genome Profiles

Following suggestions that genome profile analysis may prove to be a valuable method for classifying rotavirus isolates, the first objective of this thesis was to develop a simple, rapid and reproducible method allowing genome profile analysis to be performed on a large range of field isolates. The method developed to fulfill these requirements is described in detail in the Results, Chapter 1. Application of this technique has revealed heterogeneity of genome segment mobility for rotaviruses isolated at the same and different times, from the same and different geographical locations and from the same and different animal species. Similar findings have also been demonstrated by other groups studying rotavirus genomic profile variation (Schnagl

et al., 1981; Espejo et al., 1980; Kalica et al., 1978). It was apparent from this work that no conclusions regarding the relatedness of isolates could be made until the structural basis of genome segment variation was understood.

This consideration led to the second objective of this thesis which was to develop a sensitive and reproducible method to allow a detailed structural analysis of individual genome segments. Several different approaches could have been adopted to achieve this aim; the final method and the results and rationale behind the successful development of this technique are described and discussed in the Results, Chapter 2.

Initial findings making use of the partial T_1 ribonuclease digestion method of genome segment analysis suggested corresponding sets of genome segments shared unique, conserved terminal regions (2-40 nucleotides) for both of their RNA strands. Application of partial nuclease digestion analysis beyond these apparently conserved regions permitted several important conclusions.

Analysis of the five bovine isolates in Results, Chapter 5 showed that co-migrating corresponding genome segments could have very similar or radically different primary structures. Conversely, corresponding genome segments with different mobilities on simple genome profile analysis could have very similar or radically different primary structures. These conclusions from partial nuclease digestion analysis were also confirmed by T_1 oligonucleotide fingerprinting. Partial nuclease digestion analysis of porcine and human isolates allowed similar conclusions regarding their corresponding genome

segments. This work showed that interpretation of simple genome profile analysis for molecular epidemiological purposes can be very misleading. Two isolates with identical genome profiles isolated in the same place, at the same time can have structural differences for their corresponding genome segments as shown by comparative analysis of the isolates Holcomber and Lam in Results, Chapter 7. Therefore definitive conclusions regarding the relatedness of rotavirus isolates should only be drawn after detailed structural analysis of their genome segments.

(ii) Rotavirus Gene Reassortment

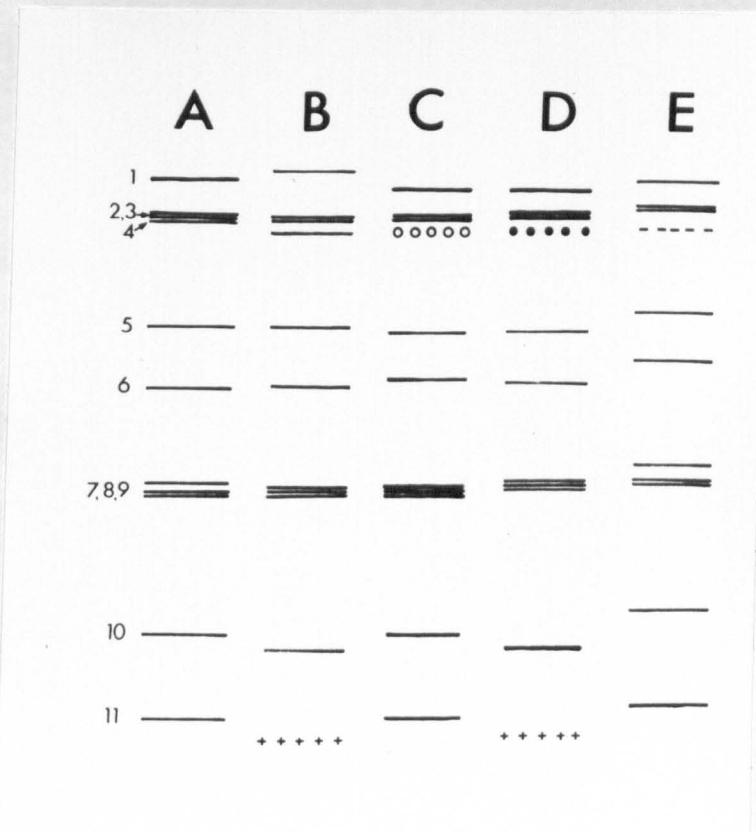
The common morphology (Holmes, 1975), shared or group antigen (Flewett, 1978) and ability to cross species barriers (Mebus et al., 1976) all indicate a common evolutionary origin for the rotaviruses. Therefore, the high level of structural heterogeneity observed by partial nuclease digestion analysis is quite surprising considering the small geographical area (U.K.), short time period (3 years) and limited number of isolates analyzed (13).

Progressive evolutionary divergence together with gene re-assortment could account for these large differences of sequence relationship between rotavirus isolates from the same and from different animal species. The results obtained for the five bovine isolates in Chapter 5 have provided evidence supporting gene re-assortment as a mechanism by which 'new' rotaviruses may emerge in nature.

Partial nuclease digestion analysis suggested that all corresponding genome segments were very similar except the species 4 and species 11 RNAs. Despite these differences the species 4 RNAs showed an underlying similarity by partial nuclease digestion. However no criteria have been established by which differences in partial nuclease digestion patterns are known to indicate radical sequence differences. Two corresponding genome segments may have very different primary structures yet the regions analyzed by partial nuclease digestion may indicate them to be similar. Further studies of genome segments known to code for the surface antigen(s) from serologically distinct isolates will be required to determine when differences in partial nuclease digestion patterns define major or minor sequence variation. Therefore, in important cases RNA species have also been analyzed by T_1 oligonucleotide fingerprinting. On this basis the species 4 RNAs of isolates C, D and E were shown to be unrelated (Figure 48). The species 11 RNAs of wild isolates B and D were also demonstrated to be radically different from those of isolates C and E. These results are shown schematically in Figure 75.

Pairwise comparison of corresponding genome segments from isolates C and D indicates that they share nine common genome segments with species 4 and species 11 RNAs being radically different. Similar analysis of isolates C and E show that they have ten common genome segments with differences only for their species 4s. Cross comparison of isolates B and D show them to have ten common genome segments with possible variation only between their species 4s (species 4 of isolate B was not subject to T_1 oligonucleotide fingerprinting) - see Figure 75.

Figure 75. Schematic Representation of the Partial T₁ Digestion
and Oligonucleotide Fingerprinting Results for the Five Bovine
Isolates Analyzed in Chapter 5.



Tracks A = U.K. tissue culture adapted calf virus

Tracks B = isolate 2855 Carmarthen

Tracks C = isolate 4329 Shrewsbury

Tracks D = isolate 6597 V.I. Centre Weybridge

Tracks E = isolate 117 Penrith

} Wild isolates

Genome segments are numbered 1-11 for isolate A.

The symbols were used to denote differences in primary structure for corresponding genome segments. For example there are two types of species 11 RNA isolates B and D (++++) and isolates A, C and E (—).

The results indicated that these five bovine isolates share a common gene pool. The virtual identity of RNA species 1, '2-3', 5, 6, '7-8-9' and 10 together with the radical independent variation of RNA species 4 and 11 can be best explained by gene re-assortment. From the extreme difference between the two sets of species 11 RNAs it is reasonable to assume that the gene re-assortment of the species 11's occurred between bovine rotavirus and a parental virus from a different animal species, most likely a domestic animal sharing the same environment.

The partial nuclease digestion patterns from corresponding genome segments of porcine and human isolates established that these viruses had not been involved in genome segment exchange with the five calf isolates studied here. However, the independent radical variation of the species 5 RNAs of the two wild porcine isolates, and of the human isolates, provided more evidence supporting gene reassortment as a mechanism for generating rotavirus diversity in nature.

Further circumstantial evidence for gene reassortment is provided by two observations. Gene reassortment has been demonstrated under selective conditions in the laboratory following mixed infections of cultured cells confirming that this event can occur between rotaviruses from the same and different animal species (Faulkner-Valle et al., 1982; Matsuno et al., 1980; Grønberg et al., 1981). Secondly, during the initial genome profile analysis approximately 10% of isolates had a mixture of profiles indicating that single animals were simultaneously infected with at least two viruses. Mixed infection of a single host is a necessary pre-requisite for gene reassortment to occur.

Experimental evidence supporting gene reassortment as a mechanism by which new influenza viruses may arise in nature has been presented using influenza virus genome segment specific hybridization probes (Scholtissek et al., 1978a) and by the technique of T_1 oligonucleotide fingerprinting (Desselberger et al., 1978). The rationale behind these approaches was similar to that used for the five bovine rotaviruses; however the virus isolates selected for analysis had been characterized serologically and had both antigenic similarities and major antigenic differences. By pairwise analysis of corresponding genome segments from the different isolates, deductions regarding their origin were made.

Scholtissek et al. (1978a) chose to analyze the pandemic H1N1, H2N2 and H3N2 human influenza A subtypes. ^{32}P labelled Singapore H2N2 vRNA was hybridized to the cold cRNA of all three subtypes; the results of this analysis are shown in Table 4. The table shows that segments 1, 5, 7 and 8 of the H2N2 subtype have a sequence homology of nearly 100% to those of the H1N1 virus while the homology of the other genome segments is significantly lower. These two subtypes have four genes that are almost identical and four genes different. These data were compatible with the explanation that four genes of the H2N2 subtype were derived from the H1N1 subtype by reassortment. Comparison of the base homologies between the H3N2 and the H2N2 subtypes revealed differences for only one of their genome segments, that coding for the haemagglutinin. This independent radical variation for individual genome segments provided very strong evidence for gene reassortment as the major mechanism responsible for antigenic shift in influenza.

Table 4. Base Sequence Homologies (%) Between ^{32}P -Labelled vRNA Segments of Singapore (H2N2) and Influenza Virus Prototypes PR8 (H1N1) and Hong Kong (H3N2)

^{32}P labelled segments of Singapore strain	cRNA of:	Singapore H2N2	Hong Kong H3N2
PR8 H1N1			
1	96	100	98
2	72	100	96
3	75	100	97
4	24	100	24
5	92	100	97
6	26	100	96
7	94	100	98
8	95	100	98

From Scholtissek et al (1978a).

The evidence for rotavirus gene reassortment is surprising considering the isolates analyzed were selected on arbitrary criteria and unlike influenza no serological data was available to suggest the presence of any shared or different antigenic properties. Therefore genome segment exchange is probably a common event for rotaviruses in nature.

Confirmation of the high level of rotavirus genome segment sequence polymorphism demonstrated by partial nuclease digestion has also recently been shown by hybridization studies of human and animal isolates (Flores et al., 1982; Street et al., 1982; Schroeder et al., 1982).

Rotaviruses - A Future Perspective

The major goal for research into rotaviruses is the development of an effective vaccine. An understanding of the natural variation of rotaviruses is essential before any control measures can be introduced. The reluctance of wild isolates to grow routinely in tissue culture has greatly restricted epidemiological studies. The present state of rotavirus serology is extremely poor and requires a great deal of improvement. Tests such as ELISA, complement fixation, and immune adherence haemagglutination are of little epidemiological value as they do not identify the antigen involved in virus neutralization. The use of conflicting terminology has also caused considerable confusion concerning the serological nomenclature of rotaviruses.

Inhibition of virus infectivity by neutralizing antibody is the most useful and definitive method for classifying rotaviruses. Recent advances in the routine adaptation of wild isolates to growth in tissue culture should facilitate more detailed epidemiological studies by serum neutralization. The production of monoclonal antibodies specific for the virus neutralizing antigen will also increase the precision and sensitivity of these analyses. The already demonstrated large number of human serotypes (Flewett et al., 1978; Sato et al., 1982) together with the possibilities that many more human and animal serotypes may exist will make the task of producing an effective vaccine increasingly difficult.

The development of genome segment specific cDNA clones for use as hybridization probes should facilitate a detailed understanding of sequence relationships between the genome segments of human and animal rotavirus isolates. Such analyses should establish the extent and hence the importance of 'interspecies' gene re-assortment as a mechanism for generating 'new' pathogenic strains of rotavirus.

If genome segment exchange occurs frequently across human-animal species barriers as well as across animal-animal species barriers then prospects for controlling rotavirus infections by vaccination will be reduced. Better prospects to control human rotavirus infections may come from improved hygiene measures and the use of oral rehydration to dramatically increase survival chances and therefore allow protection by natural immunity.

On the farm, especially under conditions of intensive modern agriculture, it is very difficult to control rotavirus by hygiene measures alone. This fact coupled to huge economic losses caused by viral gastroenteritis makes a very strong commercial argument in favour of prophylactic control. However many important questions still remain unanswered in the quest to produce an effective rotavirus vaccine. Besides the need for more understanding of natural variation a detailed knowledge of the mechanisms of immunity is required, therefore at present the prospects for an effective rotavirus vaccine remain distant.

APPENDIX

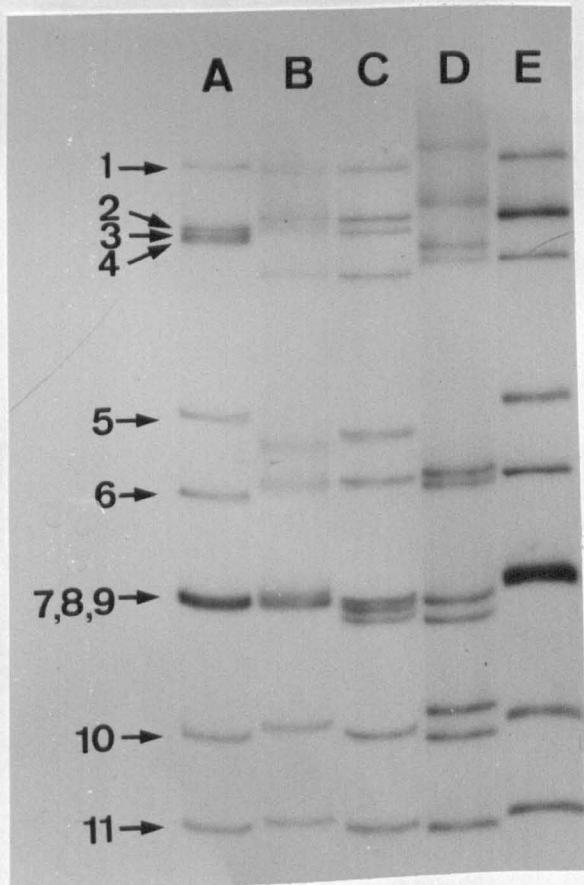
Appendix

During the course of this thesis several reports of atypical rotavirus isolates lacking the common or group antigen have appeared (Bohl et al., 1982; McNulty et al., 1981 and Saif et al., 1980). These isolates from both birds and pigs were morphologically indistinguishable from previously-described rotaviruses and had 11 dsRNA genome segments with a similar overall size range although their genome profiles were markedly different. Figure 76 shows a comparative genome profile analysis of the atypical 'porcine rotavirus-like-agent' from Compton, U.K., together with several other rotavirus isolates.

Corresponding genome segments from the Compton porcine rotavirus-like-agent, the U.S.A. atypical porcine rotavirus (Saif et al., 1980) and the O.S.U. porcine rotavirus have been analyzed by partial nuclelease digestion (Pedley et al., 1982). The results indicated that all 'Corresponding' genome segments were completely different for their terminal regions (2-40 nucleotides) and on this basis these isolates could be considered as representing different groups.

The importance of these atypical isolates in causing acute gastroenteritis has not been established. However, the fact that they have only very recently been discovered and then from just two different animal species indicates that they do not represent a major epidemiological problem. Bridger et al. (1982) have shown the Compton atypical porcine isolate to be pathogenic for piglets, ^{and} immunity to it does not afford protection against rotavirus infection. Further studies are

Figure 76. Comparative Genome Profile Analysis of the Compton Atypical Porcine-Rotavirus-Like Agent with Previously Described Rotaviruses from Several Animal Species.



Electrophoresis on this 7.5% polyacrylamide gel was conducted at 20 mA for 16 hrs.

Track A = U.K. tissue culture adapted bovine rotavirus

Track B = Human rotavirus

Track C = Porcine rotavirus

Track D = Atypical porcine-rotavirus-like-agent

Track E = Bovine rotavirus

required to establish the frequency with which these isolates occur.

No evidence for atypical isolates has been obtained by the genome profile analyses from the fairly extensive range of field isolates studied in this thesis.

PUBLICATIONS

PUBLICATIONS

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