STUDIES ON CALF ROTAVIRUS IN A CALF KIDNEY
DIPLOID CELL LINE

Thesis presented to the University of Surrey
for the Degree of Doctor of Philosophy in the
Department of Microbiology

by

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SUMMARY

Besides primary cell cultures, few cell lines support the growth of the bovine rotaviruses with development of cytopathic effect. So a calf kidney diploid cell line was developed which has predominantly fibroblastic morphology. The chromosomal number is 2n = 60 with 29 pairs of autosomes and two "X" chromosomes.

The cell line supported good replication of bovine rotaviruses with development of cytopathic effect. In the absence of trypsin the cells did not support the productive replication of bovine rotaviruses present in 12 separate samples of calf diarrhoea, although development of cell-associated viral antigen was recorded up to seventh passage in 3 samples. The cells also supported the replication of bovine rhinotracheitis virus, a bovine enterovirus and poliovirus (type 1 vaccine strain). In each case there was development of cytopathic effect.

A plaque assay for bovine rotaviruses was developed which worked well within narrowly defined limits. The reproducibility of the plaque test has made it possible to examine the kinetics of heat and serum inactivation. The virus was neutralised readily by homologous antirotavirus serum than heterologous serum and similar observations were also made between two bovine rotaviruses (the U.K. and the Northern Ireland strains). The two strains are being differentiated on the basis of their plaque morphology and serum neutralisation kinetics by homologous and heterologous serum.

The presence of higher salt concentrations in the medium during virus adsorption was found to shorten the replication cycle of virus. However, when the cells were grown in Earle's rather than Hank's balance salt solution in the base medium, the replicative cycle of virus was shortest (20 hours) with virtual destruction of cell monolayer in 24
hours. The role of calcium in rotavirus replication was emphasized and the isolation of rotaviruses in primary monkey kidney cells with higher concentrations of Ca\(^{2+}\) in the medium is proposed.
ACKNOWLEDGEMENTS

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<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 'O'</td>
<td></td>
</tr>
<tr>
<td>Acute epidemic viral enteritis of man and animals.</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 1</td>
<td></td>
</tr>
<tr>
<td>Development and characterisation of a calf kidney diploid cell line.</td>
<td>45</td>
</tr>
<tr>
<td>Chapter 2</td>
<td></td>
</tr>
<tr>
<td>The enhancement of virus replication by salts and chemicals. Virus isolation.</td>
<td>85</td>
</tr>
<tr>
<td>Chapter 3</td>
<td></td>
</tr>
<tr>
<td>Development of a plaque assay and differentiation of rotaviruses.</td>
<td>113</td>
</tr>
<tr>
<td>Concluding Remarks</td>
<td>176</td>
</tr>
<tr>
<td>Appendices</td>
<td>178</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>189</td>
</tr>
<tr>
<td>References</td>
<td>191</td>
</tr>
</tbody>
</table>
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Bacterial enteropathogen.</td>
<td>7</td>
</tr>
<tr>
<td>0.2</td>
<td>Characteristics of acute infectious viral agents of man and animals.</td>
<td>11</td>
</tr>
<tr>
<td>0.3</td>
<td>Detection of rotaviruses in animals.</td>
<td>13</td>
</tr>
<tr>
<td>0.4</td>
<td>Coronaviruses associated with gastroenteritis of man and animals.</td>
<td>33</td>
</tr>
<tr>
<td>0.5</td>
<td>Parvovirus-like agent associated with human acute gastroenteritis.</td>
<td>36</td>
</tr>
<tr>
<td>1.1</td>
<td>Viruses used in the study.</td>
<td>56</td>
</tr>
<tr>
<td>1.2</td>
<td>Different media and sera used for the growth of the cells.</td>
<td>60</td>
</tr>
<tr>
<td>1.3</td>
<td>Infectivity titer of viruses grown and titrated in calf kidney diploid cell line.</td>
<td>77</td>
</tr>
<tr>
<td>2.1</td>
<td>Calf faecal specimens.</td>
<td>93</td>
</tr>
<tr>
<td>2.2</td>
<td>Effect of different salts on virus adsorption.</td>
<td>98</td>
</tr>
<tr>
<td>2.3</td>
<td>Effect of temperature on virus replication.</td>
<td>101</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect IUdR on virus replication.</td>
<td>104</td>
</tr>
<tr>
<td>2.5</td>
<td>Influence of hypotonic treatment on cells.</td>
<td>106</td>
</tr>
<tr>
<td>2.6</td>
<td>Virus release from persistently infected cells.</td>
<td>111</td>
</tr>
<tr>
<td>2.7</td>
<td>Serial passaging of the rotavirus in the calf kidney cells.</td>
<td>114</td>
</tr>
<tr>
<td>3.1</td>
<td>Cell lines used for development of plaque assay.</td>
<td>140</td>
</tr>
<tr>
<td>3.2</td>
<td>The effect of the concentration of DEAE-dextran in the overlay medium on plaque count.</td>
<td>150</td>
</tr>
<tr>
<td>3.3</td>
<td>The effect of the time of incubation on the plaque count.</td>
<td>150</td>
</tr>
<tr>
<td>3.4</td>
<td>The interrelationships between the time and the concentration of magnesium ions on the plaque count.</td>
<td>150</td>
</tr>
</tbody>
</table>
3.5 The effect of the size of inoculum on the plaque count. 157
3.6 Infectivity titer of rotaviruses by plaque and microtiter assay. 157
3.7 Heat inactivation kinetics of the U.K. strain of rotavirus. 157
3.8 The inactivation of rotavirus (U.K. strain) by homologous serum at 20°C. 158
3.9 The inactivation of rotavirus (U.K. strain) by homologous serum at 4°C. 158
3.10 The inactivation of rotavirus (U.K. strain) by human antirotavirus serum (Pig 111). 164
3.11 The inactivation of two strains of bovine rotavirus (U.K. and N.I. strains) by heterologous sera. 164
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>CHAPTER 1</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Rapidly growing cells amongst cellular debris.</td>
<td>61</td>
</tr>
<tr>
<td>1.2 Growth of epithelial and fibroblastic cells together.</td>
<td>61</td>
</tr>
<tr>
<td>1.3 Fibroblast-like cells.</td>
<td>62</td>
</tr>
<tr>
<td>1.4 Epithelial cells.</td>
<td>62</td>
</tr>
<tr>
<td>1.5 Diagramatic representation of behaviour of calf kidney diploid cell line in culture.</td>
<td>64</td>
</tr>
<tr>
<td>1.6 Absence of mycoplasma at the cellular interjunction.</td>
<td>66</td>
</tr>
<tr>
<td>1.7 Monolayer of calf kidney cells.</td>
<td>66</td>
</tr>
<tr>
<td>1.8 Chromosomal spread of calf kidney diploid cell line.</td>
<td>67</td>
</tr>
<tr>
<td>1.9 Arbitrary arrangement of chromosome of calf kidney diploid cell line.</td>
<td>68</td>
</tr>
<tr>
<td>1.10 Chromosomal spread showing isochromosome.</td>
<td>67</td>
</tr>
<tr>
<td>1.11 Rotavirus infected cells at 48 hours.</td>
<td>70</td>
</tr>
<tr>
<td>1.12 Rotavirus infected cells at 48 hours at higher magnification.</td>
<td>70</td>
</tr>
<tr>
<td>1.13 Healthy, living monolayer of calf kidney cells. Infected at 96 hours.</td>
<td>72</td>
</tr>
<tr>
<td>1.14 Rotavirus infected cells showing bright granulated intracytoplasmic fluorescence.</td>
<td>73</td>
</tr>
<tr>
<td>1.15 Cells infected with infectious bovine rhinotracheitis virus.</td>
<td>74</td>
</tr>
<tr>
<td>1.16 Cells infected with bovine enterovirus.</td>
<td>74</td>
</tr>
<tr>
<td>1.17 Cells infected with poliovirus type 1.</td>
<td>75</td>
</tr>
<tr>
<td>1.18 Electronmicrograph of infectious bovine rhinotracheitis virus.</td>
<td>78</td>
</tr>
<tr>
<td>1.19 Electronmicrograph of bovine rotavirus complete particle showing hexagonal sub-units.</td>
<td>79</td>
</tr>
</tbody>
</table>
1.20 Electronmicrograph of complete particles of bovine rotaviruses showing penetration of stains to internal structure. 79

1.21 Electronmicrograph of bovine enterovirus. 78

CHAPTER 2

2.1 Effect of salts on virus replication. 99
2.2 Effect of incubation temperature on virus replication. 102
2.3 Effect of two different growth media on virus replication 107
2.4 Uninfected calf kidney diploid cells. Cell infected with bovine rotavirus after 24 hours. 108
2.5 Extensive cytopathic effect after 96 hours of infection with bovine rotavirus. 109
2.6 Uninfected calf kidney diploid cells. Cells infected with bovine rotavirus at 24 hours. 112
2.7 Electronmicrograph of human reovirus-like agent. 115
2.8 Live, uninfected calf kidney diploid cells. Cells infected with human reovirus-like agent. 117
2.9 Uninfected live vero cells. Vero cells infected with human reovirus-like agent. 118

CHAPTER 3

3.1 (a) Plaque morphology of bovine rotavirus (U.K. strain) with incorporation of neutral red in second overlay medium. 147
(b) Plaque morphology of bovine rotavirus (U.K. strain) without incorporation of neutral red in second overlay medium. 147
(c) Plaque morphology of bovine rotavirus (Northern Ireland strain). 148
3.2 The effect of time of incubation on the plaque count. 151
| 3.3 | The influence of the concentrations of magnesium ions on the plaque count. | 152 |
| 3.4 | The interrelationship between the time of virus adsorption and the concentrations of magnesium ions on the plaque count. | 153 |
| 3.5 | Actual and theoretical plaque count using different volumes of inocula. | 155 |
| 3.6 | Heat inactivation kinetics of U.K. strain of rotavirus. | 159 |
| 3.7 | Inactivation of rotavirus (U.K. strain) by two different homologous sera at 20°C. | 160 |
| 3.8 | Inactivation of rotavirus (U.K. strain) by one homologous serum at 4°C and 20°C. | 161 |
| 3.9 | Inactivation of rotavirus (U.K. strain) by human antirotavirus serum (Pig 111). | 162 |
| 3.10 | Inactivation of two strains of bovine rotaviruses (U.K. and N.I. strains) by heterologous sera. | 165 |
CHAPTER 'O'

ACUTE EPIDEMIC VIRAL ENTERITIS OF MAN AND ANIMALS
CONTENTS OF CHAPTER 'O'

0.1 Introductory remarks 3
0.2 Historical background 8
0.3 Reovirus-like (Rotavirus) agents of epidemic enteritis of man animals 10
  0.3.1 Morphology of virion. 12
  0.3.2 Incidence of rotavirus 12
  0.3.3 Epidemiology
      (A) Animals 16
      (B) Man 16
  0.3.4 Clinical features
      (A) Animals 20
      (B) Infants and children 21
  0.3.5 Pathology 23
  0.3.6 Transmission studies 24
  0.3.7 Laboratory diagnosis of rotavirus 27
  0.3.8 Immunology 30
0.4 Coronavirus 32
0.5 Parvovirus-like agent 35
0.6 Parvovirus 39
0.7 Astrovirus 40
0.8 Calicivirus 42
0.9 Adenovirus 43
0.10 Miscellaneous small enteric virus-like agents associated with acute gastro-enteritis of humans. 43
Acute undifferentiated diarrhoea is one of the most common causes of illness in children and young animals. Neonatal calf diarrhoea or calf scour is a relentless problem of the dairy and beef industry claiming great financial toll each year (White et al., 1970). In the U.S.A. the loss of about two million dairy calves annually is largely attributed to neonatal diarrhoea (Daniels et al., 1977). The economic loss is not only due to mortality (0 - 50%) but to morbidity which may vary from 90 - 100 percent (Burgess and Simpson, 1976; Mebus et al., 1971b; Morin et al., 1974; White et al., 1970) and may involve decreased weight at weaning, loss of genetic potential and high risk to exposure to other disease. In spite of most modern husbandry practices, neonatal calf diarrhoea remains the most important disease problem in calves (Schlafer and Scott, 1979) and it is estimated that the annual loss in the U.S.A. due to the condition approaches one hundred million dollars (House, 1978).

Acute diarrhoeal disease has also long been recognised as one of the major causes of morbidity and mortality in infants and young children especially in developing countries, where the current situation can be compared to that prevailing in the industrialised countries at the beginning of the century. For example, in 1900 in New York City, there were 5,603 deaths per 100,000 infants and for preschool children 398 per 100,000, levels that are matched in few regions of the world today (Gordon, 1971). During the 20th century there has been a dramatic reversal in infant mortality due to acute infectious enteritis in developed countries, for instance, the mortality rates from acute gastroenteritis in infants under one year of age have decreased in the U.S.A. and the U.K. from more than 30 during 1900 to less than 0.5 per 1,000 live births in recent years (Gordon et al., 1963).
But death, mainly associated with rotaviral gastroenteritis continues to be recorded in Australia (Davidson et al., 1975), Canada (Carlson et al., 1978; Middleton et al., 1974), Japan (Konno et al., 1977) and the United Kingdom (Report, Public Health Laboratory, U.K. 1976) and the U.S.A. (Moffett et al., 1968; Palmer et al., 1977a). Although there is a lack of reliable information on the incidence and associated mortality from the diarrhoeal disease in other areas where the problem is most serious, it has been estimated that there are about 500 million episodes per year of diarrhoea in children below 5 years of age in Asia, Africa and Latin America. Three to four percent of them usually end in death and in endemic areas 30% or more beds in hospitals are occupied by cases of severe diarrhoea, many receiving expensive drugs and intravenous fluid (Anon, 1979). The severity of the diarrhoeal disease can be well judged from the fact that every year nearly 1.4 million children below 5 years of age die in India (Registrar General and Census Commissioner, India, 1972, cited by Maiya et al., 1977). The diarrhoeal disease not only takes the lives of millions of children in the developing world each year but also results in retardation of physical growth and contributes to malnutrition which results from malabsorption of food by the survivors.

The involvement of an infectious agent in causation of neonatal calf diarrhoea was recognised as early as 1917 by Hagan and later Smith and Orcutt (1925). For many years Escherichia coli was considered to be the main cause (Smith and Hall, 1967) but it is now generally accepted that certain pathogenic strains of E. coli are important in septicaemic and enterotoxaemic forms of the disease but there is doubt concerning the role of the organism in all outbreaks of typical calf scour (Smith and Hall, 1967). The viruses, chlamydia, mycoplasma, bacteria, fungi, protozoa as well as nutritional and chemical factors have all at one time or other been incriminated as the causative factor (Fernelius, 1973).
A number of bovine viruses possess the potential to cause neonatal calf diarrhoea including bovine virus diarrhoea virus, BVDV (Lambert and Fernelius, 1968; Paterson, 1962), bovine herpes virus, BHV (Lambert and Fernelius, 1968; Paterson, 1962), bovine parvovirus (Storz and Bates, 1973), bovine adenovirus (Lambert and Fernelius, 1968; Mattson, 1973) and bovine enterovirus (Lambert and Fernelius, 1968). However, incrimination of all or any one of these viruses in the epidemic form of calf scour is unlikely. However, the viral aetiology of the epidemic form of calf scour was suspected for some time although most workers were reluctant to deviate from the generally accepted concept that the calf scour was essentially due to collibacillosis. The definitive work of Mebus and his co-workers in 1969, who repeatedly isolated virus from calf scour put an end to the controversy and established the viral aetiology of some forms of calf diarrhoea. After this successful isolation of virus some others were found, particularly, coronavirus (Stair et al., 1972) and small round viruses (Woode and Bridger, 1978b). This emphasized the importance of viral aetiology of neonatal calf diarrhoea. In the most publications the virus was considered the primary pathogen but sometimes associated with *Escherichia coli* or *Salmonella* infections (Casaro et al., 1971; Lambert et al., 1974; Mebus et al., 1978). There are few publications, reporting multiple mixed viral infections, mixed bacterial and viral infections in calves (Acres et al., 1975; Morin, 1975; Storz, 1973; Waldhalm et al., 1974) and recently the simultaneous presence of rotavirus, coronavirus and BVD like virus in gastro-enteritis of calves has been described (Almeida et al., 1978a; Marsolais et al., 1978; Van Opdenbosch et al., 1979) and also the simultaneous presence of rotavirus, calicivirus and a 23 nm small round virus in gastroenteritis of a pig has been reported (Saif et al., 1980).

A survey of the published literature reveals that the aetiological agent in sporadic outbreaks of enteritis in children was recognised in only 20% of cases, the remaining
80% was due to unspecified causes, presumably some at least due to a virus (Davidson et al., 1975). Enteropathogenic bacteria like E. coli, Salmonella and Shigella are implicated in only about 10 - 20% of the cases of acute infectious enteritis in young children (Bishop et al., 1976; Du Pont, 1975; Middleton et al., 1974) with E. coli accounting for a large proportion of endemic gastroenteritis (Du Pont et al., 1971). However, recent investigation suggest that in developing countries such organism account for a significant proportion of endemic diarrhoea (20 - 50%) of native cases (Albert et al., 1978; Guerrant et al., 1975; Jonas et al., 1979; Nalin et al., 1975; Sack et al., 1976) and also in diarrhoea of travellers to such areas (Gorbach et al., 1975; Marson et al., 1976; Shore et al., 1974). However, even in areas where enterotoxigenic organisms are common, they are numerically more important in adults than in children (Sack et al., 1976). In North America and Europe enterotoxigenic or enteroinvasive organisms have not been shown to be numerically important cause of diarrhoea in children (Gurwith and Williams, 1977). The bacteria associated with acute diarrhoea and their mode of action in man is summarized in Table 0.1.

A similar survey of infantile enteritis in different parts of the world revealed that a multiplicity of serotypes of known enteric viruses were involved including echoviruses, coxsackie viruses, polioviruses, adenoviruses. Such virus were found in 20 - 50% of ill infants but also 20% in control groups, which made interpretation of the data difficult (Behbehani and Wenner, 1966; Ramos-Alvarez and Clarke, 1964; Ramos-Alvarez and Sabin, 1958). Viruses were isolated and identified from up to 80% of the infant with diarrhoea (Parks et al., 1967; Pelon, 1965) and up to 95% of the control group (Abraham and Damin, 1977). In fact, a strain of echovirus 22 isolated from many symptomatic and asymptomatic babies was considered to be part of the normal flora since a 3 year surveillance has shown that 86% of the babies acquired this virus within two weeks of admission to the
<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>SITE OF INFECTION</th>
<th>PATHOGENIC MECHANISM</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholera vibrio</td>
<td>Small intestine</td>
<td>Enterotoxin</td>
<td>Very potent enterotoxin</td>
</tr>
<tr>
<td>E. coli (ETEC)</td>
<td>Small intestine</td>
<td>Heat labile &amp; heat stable enterotoxin</td>
<td>Some strains produced both types of enterotoxin</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>Small intestine</td>
<td>Probably enterotoxin and invasion</td>
<td>Invades epithelial cells in experimental animals. Toxin less potent than cholera toxin.</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>Small intestine</td>
<td>Probably enterotoxin and invasion</td>
<td>Cause uncertain. Perhaps toxin or bacterial.</td>
</tr>
<tr>
<td>Clostridium perfringens Type A</td>
<td>Small intestine</td>
<td>Probable enterotoxin</td>
<td>Type F organisms cause necrotizing enteritis mucosal damage.</td>
</tr>
<tr>
<td>Shigella</td>
<td>Colon</td>
<td>Invasion and multiplication</td>
<td>Probable role for cytotoxin. Ill defined at present.</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Ileum</td>
<td>Invasion and multiplication</td>
<td>? enterotoxin</td>
</tr>
<tr>
<td>E. coli (InvEC)</td>
<td>Colon</td>
<td>Invasion and multiplication</td>
<td>No toxin isolated at present.</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>Small bowl and colon</td>
<td>Invasion</td>
<td>Unique enteric syndromes - mesenteric adenitis, terminal ileitis.</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Small bowl</td>
<td>Probable enterotoxin</td>
<td>Aeromonas shigelloides, Klebsiella pneumoniae; Pseudomonas aeruginosa; Enterobacter cloacae</td>
</tr>
</tbody>
</table>

Detection of adenovirus 9 and 15 were also common in the same study. The evidence that enterovirus infection is an important cause of diarrhoea in children is therefore unconvincing (Abraham and Damin, 1977; Birch et al., 1977; Ferris, 1965), although there was serological evidence for a rise in specific antibody in many cases (Tufvesson and Johnsson, 1976). The field of acute nonbacterial gastro-enteritis remained a hunting ground for virologists for quite some time and it was not until the early 1970s that a break through was made, when Kapikian et al., (1972) and Bishop et al., (1973) isolated two new viruses, Norwalk agent and a reovirus-like virus respectively from cases of acute nonbacterial gastroenteritis of adults and children. Since then these viruses have been isolated from acute infectious gastroenteritis of children and adult from all over the world.

0.2 HISTORICAL BACKGROUND

Rivers (1937) and Huebner (1957) proposed a modification of Koch's postulates that would allow proof of causation of viral illness. They suggested that, for viral disease (i) specific virus must be regularly associated with the disease, (ii) that illness must be transmissible to susceptible host from material known to be free from nonviral agents and (iii) that control and immunologic studies be carried out to exclude that the virus was fortuitously present or picked up from the experimental host. Supporting immunological criteria are also important, including evidence that specific viral antibody is absent before infection and that it is produced by the infection, the absence or presence of antibody should correlate with disease susceptibility and protection, respectively.

Early studies of the aetiology of viral enteritis were based on epidemiological surveys, and the inoculation of bacteria free filtrates to experimental animals and human volunteers. The best known disease is transmissible
gastroenteritis (TGE) of newborn pigs which was first described by Doyle and Hutchings (1946) and is now known to be a coronavirus (Tajima, 1970). The mortality rate of the disease may reach 100% in epizootics amongst neonates born to non-immune herds. At the same time there were several reports concerning viral aetiology of diarrhoea in neonatal calves but also often associated with pneumonia (Baker, 1943; Jennings and Glover, 1952; Moll and Brandly, 1955). However, these workers were reluctant to deviate from the generally accepted concept that neonatal calf pneumo-enteritis was due to collibacillosis but Brandly and McClurkin (1956) characterised the aetiological agent of calf pneumoenteritis as a 17 nm diameter virus and by means of serial transmission in calves using a bacteria free inoculum, they established transmissibility of the agent and which they postulated was transmitted by air. Even earlier Light and Hodes (1943) induced diarrhoea in gnotobiotic calves with a filtrable agent derived from the diarrhoeal stools of human infants but the agent eventually lost its infectiveness for the gnotobiotic calves (Hodes, 1977). Thereafter, several other investigators attempted to induce disease in human volunteers under controlled conditions by the administration of bacteria free faecal filtrate derived from naturally occurring epidemic cases of viral enteritis. The route of inoculation was either respiratory (Reimann et al., 1945) or oral (Gordon et al., 1947). Gordon et al., (1947) and Jordan et al., (1953) isolated two filtrable agents, Marcy and Family study (FS) strains and both induced short term homologous, but not heterologous, immunity, thus two agents were not closely related (Jordan et al., 1953). A Japanese agent, Niigata strain (Kojima et al., 1948) was subjected to multiple passage in human volunteers and the agent conferred protection against infection with the Marcy strain (Fukumi et al., 1957). However, none of the agents could be visualised or cultivated in the laboratory and they were discarded and the work was abandoned. However, in the 1970s when more advanced virological techniques for identification of viruses in stools were available similar studies were renewed.
Since 1974, numerous preliminary reports concerning viral enteritis agents have appeared in various journals. They were interesting but because of any clear experimental data, they were extremely difficult to interpret. Certainly the presence of virus in the faeces was not always associated with any disease syndrome, especially in neonates (Bishop et al., 1976; Chrystie et al., 1978; Madeley et al., 1977; McLean et al., 1977; Murphy et al., 1977; Rodriguez et al., 1977; Van Renterghem et al., 1980). Many virus-like structures have been identified in faecal specimens by electron-microscopy, only a few types have met the criteria set by Rivers (1937) and Huebner (1957). Table 0.2 summarizes the characteristics of the proven and candidate aetiological agents of acute gastroenteritis of man and animals.

0.3 REOVIRUS-LIKE AGENT (ROTAVIRUS) OF EPIDEMIC ENTERITIS OF MAN AND ANIMALS

After the original report by Mebus et al., (1969) several similar agents have been isolated and various names have been proposed, Nebraska calf diarrhoea virus (Mebus et al., 1969), Reovirus-like agent (Kapikian et al., 1976b) Duovirus (Davidson et al., 1975) and infantile gastro-enteritis virus (IGV, Orbivirus group) (Petrie et al., 1975) to a group of virus morphologically indistinguishable from each other (Flewett and Woode, 1978). The name "Rotavirus" was suggested (Flewett et al., 1974a). It was derived from Latin, "rota", a wheel, and was given because the complete virion in electronmicrographs looks like a little wheel having a wide hub with short spokes and a thin, clearly defined circular rim. It has been accepted by the International Committee on Taxonomy of viruses as the official name for a new genus within Reoviridae family (Matthews, 1979).
<table>
<thead>
<tr>
<th>Agents</th>
<th>Host</th>
<th>Etiological relationship established</th>
<th>Method by which identified</th>
<th>Size</th>
<th>Density in cesium chloride gms/cm²</th>
<th>Nuclic acid</th>
<th>Replication in cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Established agent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norwalk agent</td>
<td>Human</td>
<td>Yes</td>
<td>EM*, RIA, IAHA, human volunteer studies</td>
<td>27 nm</td>
<td>1.38-1.40</td>
<td>Presumed DNA</td>
<td>Negative</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>Dog, Cattle</td>
<td>Yes</td>
<td>EM, Transmission to dogs and calves</td>
<td>20 nm</td>
<td>1.38-1.46</td>
<td>Single stranded DNA</td>
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<tr>
<td>Astrovirus</td>
<td>Human, Lamb, Turkey, calves No</td>
<td></td>
<td>EM, transmission to lamb, adult volunteer</td>
<td>28 nm</td>
<td>1.33-1.34</td>
<td>RNA</td>
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<td>Calicivirus</td>
<td>Calves</td>
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<td>EM, IEM, transmission to calves</td>
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<td>1.37-1.38</td>
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<tr>
<td>Coronavirus</td>
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<td>EM, transmission to calves, organ culture</td>
<td>pleomorphic RNA</td>
<td>120 - 230 nm</td>
<td>Enveloped RNA</td>
<td>Yes Limited</td>
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<td><strong>B. Candidate agent</strong></td>
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<td>EM</td>
<td>75 nm</td>
<td>1.35</td>
<td>DNA</td>
<td>Usually Negative</td>
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<tr>
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<td>EM</td>
<td>32 nm</td>
<td>-</td>
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<td>Negative</td>
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<tr>
<td>Otefuke agent</td>
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<td>EM</td>
<td>35-40 nm</td>
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<td>Negative</td>
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</table>

* EM = Electronmicroscopy; IEM = Immunelectronmicroscopy; RIA = Radioimmunoassay; CIDOP = Counter immuno electro osmophoresis; ELISA = Enzyme linked immunosorbent assay; IAHA = Immune adherence haemagglutination assay; FVPT = Fluorescent viral precipitation test.
0.3.1 MORPHOLOGY OF VIRION

Morphologically, rotavirus from humans, calves, piglets, foals, lambs, epizootic diarrhoea of infant mice (EDIM) virus, SA11 (simian agent), 'O' (offal) agents are all indistinguishable from each other (Kapikian et al., 1976a; Woode et al., 1976b). In addition, morphologically, indistinguishable rotaviruses have been reported from various other animals, including wild animals (Table 0.3).

Intact rotavirus particles have a double-layered capsid structure, which resembles reovirus. However, rotaviruses are distinguishable from them by their sharply defined outer edge (McNulty, 1978). The intact rotavirus particles have been described as double-shelled (Holmes et al., 1975) or smooth (Woode et al., 1976b) particles measuring approximately 70 nm in diameter. The particle has a 38 nm diameter electron dense centre and is hexagonal in outline surrounded by an electron lucent layer (Palmer et al., 1977b). The outer capsid layer of the intact particle is sometimes absent and leaves a single shelled or rough particle which is morphologically similar to an orbivirus, but is 10 nm smaller than intact particle (McNulty, 1978). The faecal material from infected man and animals contains both types of particles and in addition, there may be elongated forms or tubular structures (Flewett et al., 1974c; Holmes et al., 1975; Kimura and Murakami, 1977; Woode et al., 1976b).

0.3.2 INCIDENCE OF ROTAVIRUS

The rotaviruses have been recorded from almost all parts of the world both from domesticated as well as wild animals (Table 0.3). Similarly, the incidence of rotavirus has been well documented for humans from all continents of the world which indicates the ubiquitous nature of the agent.
Table 0.3  Detection of rotavirus in animals

<table>
<thead>
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<th>Species of Animal/Country</th>
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<td>Australia</td>
<td>Turner et al., (1973)</td>
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<td>Papua New Guinea</td>
<td>Vankammen and Kila (1979)</td>
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<td>Switzerland</td>
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<td>West Germany</td>
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**Chicken**

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</table>
Table 0.3 (Continued)

Turkey
Northern Ireland

Wild Animals
Fawns (Pronghorn antelope)
U.S.A.

Impala (Aepyceros malampus )
Thomson's Gazelle ) (Gazella thomsonii )
Addax (Addax nosomaculatus )

Deer
Australia

Monkeys
South Africa
U.S.A.

Mice
U.S.A.

McNulty et al., (1978a)
Reed et al., (1976)
U.S.A. Eugster et al., (1978b)
Tzipori and Caple (1976)
Malherbe and Strickland-Cholomley (1967)
Stuker et al., (1979a)
Cheever and Mueller, (1947)
Much and Zajac (1972).
0.3.3 EPIDEMIOLOGY

(A) ANIMALS

As noted earlier rotaviral gastroenteritis occurs only in young animals within first few weeks of birth and is most serious within the first 14 days in farms. It has been described for calves (Afshar and Tadayon, 1979; Burgess and Simpson, 1976; England et al., 1976; Haralambiev et al., 1978; Mebus et al., 1971b; Morin et al., 1974; Van Opdenbosch et al., 1979; Woode and Bridger, 1975), pigs (Bachmann et al., 1979; Lecce et al., 1976; McNulty et al., 1976c; Woode et al., 1976a), mice (Cheever and Mueller, 1947), foals (Flewett et al., 1975a), lambs (Snodgrass et al., 1976a) and in wild animals (Eugster et al., 1978b). Reinfection may occur a few weeks later but usually symptoms are then mild in nature (Van Opdenbosch et al., 1979). There is no direct evidence of the carrier state of adult animals but antibody has been often detected in them (Bachmann et al., 1979; Lecce and King, 1978; Petric et al., 1978) and if disease occurs it is usually less severe than in young (Bridger and Woode, 1975; Lecce et al., 1976; Snodgrass et al., 1976b). Rotavirus has also been detected by electronmicroscopy (EM) in calves scouring between 6 - 18 months (Pastoret et al., 1978; Vankammen and Kila, 1979) and recurrent outbreaks of rotaviral diarrhoea in calves have been recorded (Durham et al., 1979; Van Opdenbosch et al., 1979).

(B) MAN

In man, although there is well documented evidence of rotavirus association in adult diarrhoea (Haug et al., 1978; Oishi et al., 1979; Rodriguez et al., 1979), rotavirus associated gastroenteritis has mainly been recorded in infants and the long and short term hospital studies showed that about half of the hospitalised cases of
childhood diarrhoea was due to rotavirus infection. The rotavirus involvement in summer diarrhoea may be 0 - 20% while in winter may reach as high as 70 - 80% (Banatvala et al., 1978; Bishop et al., 1974; Bryden et al., 1975 Sack et al., 1980; Seigneurin et al., 1979) and in epidemics in Japan more than 90% of the cases were shown to be due to the rotavirus (Chiba et al., 1979a; Konno et al., 1977). Epidemiological studies from developing world are scanty. However, the detection of rotavirus showed a similar pattern to that noted above with a winter peak. For instance, at Vellore in South India Maiya et al., (1977) detected 26% rotavirus positive cases by EM on average but could not detect a single case in summer. In a comparative study of hospitalised school children below eight years of age, Heiber et al., (1978) reported that the excretion of rotavirus in gastroenteritis patients from a temperate region like Dallas (U.S.A.) or a tropical area like San Jose (Costa Rica) was about same (50 - 60%) in winter (December - February). However, in summer there was no virus excretion in Dallas while in San Jose the rate of excretion maintained at 30 - 40% throughout the year. It is probably relevant that the temperature of San Jose was about 20°C throughout the year whereas at Dallas it ranged from 10°C in winter to 27°C in summer.

The cross-sectional epidemiological study of the population in Boston (Blacklow et al., 1976) and Melbourne (Gust et al., 1977) showed an initial high prevalence of antibody in infants which fell away by six months of age but rose again by the age of 2 - 3 years and remained elevated until late adulthood. Similar serological evidence has also been recorded in Toronto (Tallett et al., 1977). It is postulated that the initial titers represent maternal antibody, the childhood rise is due to primary infection and the adult plateau results from repeated exposure or asymptomatic infections.
In a serological survey for the prevalence of rotavirus antibodies in black and white population of South Africa (Schoub et al., 1978) showed that it was 93% in the black adults but only 77% in the white adults. However, Schnagl et al., (1979) could not find any difference of excretion rates of rotavirus in aboriginal and non-aboriginal infants, children or adults although more aboriginal infants excreted rotavirus.

Seroepidemiological study of 3 - 4 years old children in different parts of the world revealed various levels of antibodies to rotavirus. In Bangladesh it was 93% (Sack et al., 1980), 87% in India (Jesudoss et al., 1978), 100% in Kenya (Metselaar et al., 1978) and 80% in U.S.A. (Kapikian et al., 1975).

Nosocomial infections with rotavirus have been widely reported. Ryder et al., (1977) noted the development of diarrhoea in 17% of infants who were admitted without diarrhoea to two pediatrics hospital ward in Atlanta in winter. Similarly one third of hospitalised children developed rotaviral diarrhoea in Malmo (Tufvesson and Johnsson, 1976). Hospital acquired rotaviral diarrhoea has also been reported from New Zealand (Scrimgeour et al., 1979). Transmission of infection from baby to baby has been observed (Bishop et al., 1979; Van Renterghem et al., 1980) and from adult visitors to babies (Bishop et al., 1979). Furthermore, outbreaks involved hospital staff and medical students are known to have occurred (Kim et al., 1977; Von Bonsdorff et al., 1978) also between geriatric hospital inmates and staff (Cubitt and Hozel, 1980).

In man rotavirus infection usually does not occur before six months of age and rare after 2 years of age (Gomez-Barreto et al., 1976; Seigneurin et al., 1979; Tallett et al., 1977). Epidemic rotavirus infection have been reported from nurseries in London (Banatvala et al.,
1978; Totterdell et al., 1976) and Melbourne (Bishop et al., 1976 and 1979). However, the diarrhoea was self limiting without necessity for any treatment. Such outbreaks occurred in winter months and the proportion of asymptomatic excretors may reach as high as 52% (Totterdell et al., 1976). A long-term survey from nursery patients has shown endemic rotavirus infections in hospitals at Grignon (Seigneurin et al., 1979), London (Banatvala et al., 1978; Chrystie et al., 1978), Melbourne (Bishop et al., 1979; Davidson et al., 1975) and Sydney (Crewe and Murphy, 1980; Murphy et al., 1975). The pattern of the shedding of rotavirus by the infants varied from 30 - 50% in the affected nurseries and only a few of the infants (8 - 30%) had diarrhoeal symptoms, in contrast Cameron et al., (1978) reported that 77% infants were symptomatic virus shedders. The excretion of rotavirus was usually for a very short period rarely exceeding 5 days and most of these infections did not appear to confer any lasting immunity (Crewe and Murphy, 1980; Davidson et al., 1975). Breast feeding apparently reduces infection in nurseries because breast fed babies excrete less rotavirus than bottle fed babies (Banatvala et al., 1978; Chrystie et al., 1975 and 1978). However, some other workers could not find any difference in excretion levels of rotavirus in breast fed or bottlefed babies (Brookfield et al., 1979; Crewe and Murphy, 1980). There are few outbreaks of rotaviral diarrhoea affecting as high as 90% of inmates of an infant home (Chiba et al., 1979a) in children 0 - 4 years of age (Konno et al., 1977) and in primary school children (Oishi et al., 1979).

The outbreak of rotavirus infection in adults usually seems to be associated with infection of their children. Infection within the families involving children and their symptomatic or asymptomatic parents have been recorded in U.S.A. (Rodriguez et al., 1979), U.K. (Wyn-Jones et al., 1978), in Kenya (Mutanda et al., 1979), in Japan (Oishi et al., 1979) and in Norway (Haug et al., 1978). Haug et al., (1978) stated that all proven adult contacts showed symptoms,
however, other studies reported symptoms in only 13% (Kim et al., 1977) or 25% (Tufvesson et al., 1977) of the adults. There are also reports of highly symptomatic cases in adults in Helsinki (Van Bonsdorff et al., 1978) and in Sweden (Lycke et al., 1978). In an outbreak of adult diarrhoea in hospital staff only 40% were positive for rotavirus either by EM or complement fixation (CF) test and a rise in antibody titer was recorded by complement fixation (CF) test (Van Bonsdorff et al., 1978). There is a report of rotaviral diarrhoea amongst army trainees in Finland (Sarkkinen et al., 1979) and serological evidence of rotavirus infection from symptomatic (Wenman et al., 1979) and asymptomatic (Tallett et al., 1977) adults have been recorded in Canada. Rotavirus was incriminated in a small epidemic outbreak of gastro-enteritis in a small Swedish town affecting 30% of population belonging to all age groups in a short span of 2 weeks (Lycke et al., 1978). Rotavirus has also been identified from 24% symptomatic and 15% asymptomatic students newly arrived in Mexico City from the U.S.A. (Vollet et al., 1979). Rotavirus may therefore have some role in "travellers' diarrhoea".

0.3.4 CLINICAL FEATURES

(A) ANIMALS

The clinical features of naturally occurring and experimental infections in animals have been well described (Durham et al., 1979; Mebus et al., 1969; Pearson and McNulty, 1977; Snodgrass et al., 1976b; Woode et al., 1976a). The prominent symptoms in animals are voluminous diarrhoea, pasty to watery consistency, sometimes mucoid or blood flaked and generally with little smell. The colour of the faeces ranged from white to yellow, although at times green or grey. The infected animals showed depression, anorexia, dehydration, either febrile or afebrile condition. In young wild animals (Eugster et al., 1978b) and in foals (Tzipori and Walker, 1978) the above symptoms
may be accompanied by respiratory symptoms as evidenced by clear mucoid nasal discharge and vomiting has been reported for pigs (Lecce et al., 1976; McNulty et al., 1976b; Schwartz and Glock, 1978; Wood et al., 1976a). The course of the disease is usually 4 - 8 days and death may occur in severe infection (Durham et al., 1979). The incubation period in experimentally infected piglets (Pearson and McNulty, 1977) is shorter (16 - 24 hours) than in calves (20 - 36 hours) (Mebus, 1976).

(B)-INFANT AND CHILDREN

The incubation period is 48 - 72 hours after infection (Shepherd et al., 1975; Oishi et al., 1979; Ørstavik et al., 1974; Zissi et al., 1976) and the illness is characterised by abrupt eruption of vomiting and diarrhoea. The children may also show respiratory symptoms in 20 - 40% cases (Campbell and Lang, 1979; Dominick and Maass, 1979; Goldwater et al., 1979; Jonas et al., 1979; Seigneurin et al., 1979; Tallett et al., 1977), and may be associated in up to 66% cases with upper respiratory symptoms (Lewis et al., 1979). However, rotavirus has neither been isolated in cell culture nor identified by EM from nasopharyngeal secretions (Goldwater et al., 1979; Lewis et al., 1979; Ørstavick et al., 1974). Mild to severe vomiting is recorded in almost all the cases studied, which usually precedes diarrhoea and mild fever around 38°C was found in the majority of cases (Delage et al., 1978; Tufvesson and Johnsson, 1976; Von Bonsdorff et al., 1978). However, higher temperature (39.5 - 40°C) has also been recorded (Gomez-Barreto et al., 1976). Along with vomiting, diarrhoea, and fever, abdominal pain (Von Bonsdorff et al., 1978), muscular pain and headache (Lycke et al., 1978) have been associated with symptomatic rotaviral infection in adults. Mild to moderate dehydration has been recorded in patients with diarrhoea (Carlson et al., 1978) and in some cases may be as high as 47% dehydration leading to hospitalisation.
In severe cases of dehydration, electrolyte imbalances leading to cardiac arrest was believed to be major factor causing death (Carlson et al., 1978). Symptoms of irritability or lethargy a few hours before death have been recorded in severe cases (Carlson et al., 1978).

Fecal leucocytes were found in rotaviral diarrhoea stool (Heiber et al., 1978; Rodriguez et al., 1977). However, studies from Bangladesh (Ryder et al., 1976), Boston (Echeverria et al., 1975) did not reveal the presence of faecal leucocytes and this discrepancy may be due to presence of undetected bacterial pathogen.

The course of rotaviral gastroenteritis in hospitalised patients especially children is usually short. The fever and vomiting lasts for up to 2 days while diarrhoea may persist up to 8 days (Delage et al., 1978; Flewett et al., 1975b; Ryder et al., 1976) and most hospitalised children recover within a week of admission (Rodriguez et al., 1977; Shepherd et al., 1975; Tallett et al., 1977). The excretion of rotavirus occurs usually from 3rd to 8th day of illness (Davidson et al., 1975). However, excretion as long as 23 days has been recorded (Flewett et al., 1975b).

Rotavirus infection has also been implicated in Rey's syndrome (Salmi et al., 1978) in encephalitis (Salmi et al., 1978) and in intussusception (Konno et al., 1978a). In one report (Lewis et al., 1979) it was suggested that symptoms of red throat or bulging tympanic membranes was seen more often in rotavirus infected patients than others in hospitals and these authors emphasized that the association of rotavirus in upper respiratory tract illness was more important than diarrhoea.

Recurrent rotavirus infection in infants due to a different serotype has been recorded (Brandt et al., 1979; Konno et al., 1978b; Rodriguez et al., 1978).
0.3.5 **PATHOLOGY**

Most of the information about the pathological changes caused by rotavirus infections has been studied in experimental animals (Bachmann *et al.*, 1979; Dubourguier *et al.*, 1978; Hall *et al.*, 1976; Lecce *et al.*, 1976; Mebus *et al.*, 1971b; Pearson and McNulty, 1977; Snodgrass *et al.*, 1977a; Theil *et al.*, 1978; Woode *et al.*, 1974). These experiments have shown that the villous epithelial cells of the small intestine are the principal site of virus replication, although virus antigen was also detected by immunofluorescence in the epithelial cells of the colon and caecum of infected lambs (Snodgrass *et al.*, 1977a) and histopathological changes in the colon and caecum of gnotobiotic calves has also been recorded (Dubourguier *et al.*, 1978). Besides intestinal lesions, histopathological changes have been found in the stomach and lungs of pigs (Hall *et al.*, 1976; Pearson and McNulty, 1977), desquamation of abomasal ridges of infected calves (Dubourguier *et al.*, 1978) in the lungs of infected lambs (Snodgrass *et al.*, 1977b) and wild animals (Eugster *et al.*, 1978b). However, these changes have not been confirmed by specific rotaviral immunofluorescence. Histopathological changes in liver, thymus, mesenteric lymphnodes and spleen, but not brain, have been seen in 21 fatal cases of rotaviral diarrhoea in infants (Carlson *et al.*, 1978).

The main changes in the small intestine consisted of a shortening or stunting of the villi, replacement of columnar epithelial cells by cuboidal or squamous cells and an increase in the number of reticulum like cells in villous lamina propria. The histopathological changes in children (Bishop *et al.*, 1973) are more or less similar to those of calves (Morin *et al.*, 1974; Pearson *et al.*, 1978). In absence of visible rotavirus particles, the histological architecture reversed back to normal 4 - 8 weeks after the onset of diarrhoea (Bishop *et al.*, 1973). The infected cells are lost from the tip of the villi and are replaced by immature cells from the crypt (Mebus *et al.*, 1971b) and these cells are resistant to reinfection (Mebus *et al.*, 1971b) possibly due to the
lack of receptors for the virus (Holmes et al., 1976).

0.3.6 TRANSMISSION STUDIES

The experimental transmission of human rotavirus to gnotobiotic animals resulted in varying degrees of success. Human rotavirus has been readily transmitted to gnotobiotic piglets with the development of clinical symptoms and virus excretion in the faeces (Middleton et al., 1975; Torres-Medina et al., 1976; Tzipori and Mankin, 1978) and subclinical infection resulting in virus excretion has also been recorded (Bridger et al., 1975). Human rotavirus has also been transmitted to the puppy (Tzipori and Mankin, 1978), to colostrum deprived monkeys but not juvenile monkeys (Holmes et al., 1974; Wyatt et al., 1976b); to gnotobiotic lambs (Snodgrass et al., 1977a) but not to thymus deficient suckling mice (Bridger et al., 1975). Mebus et al., (1976) were able to transmit human rotavirus (U.S.A. strain) to gnotobiotic calves with the development of symptoms only after serial passaging in calves. Direct inoculation of bacteria free filtrate of faeces containing human rotavirus to the duodenum of an adult human volunteer resulted in the development of mild clinical symptoms, the excretion of virus in faeces and a rise in antibody titer to the rotavirus (Middleton et al., 1974).

The pig rotavirus readily infect gnotobiotic piglets (Barrow et al., 1979; Woode et al., 1976a); hysterectomy piglets (Pearson and McNulty, 1977), newborn piglets (Bachmann et al., 1979; Chasey and Lucas, 1977; Lecce et al., 1976) and to weanling pigs (Lecce and King, 1978) with the development of characteristic clinical symptoms. However, the pigrotavirus does not infect guineapigs, hamsters, mice and rat (Lecce et al., 1976) but gnotobiotic piglets appear to be a good laboratory model for study of rotavirus infection because they were readily infected by human, calf, pig, deer and mouse rotavirus (Australian strain) with the development of varying degrees of clinical symptoms and excretion of virus in faeces (Tzipori and Walker, 1978).
Snodgrass et al., (1976b, 1977a) successfully transmitted the lamb rotavirus to gnotobiotic lamb with development of profuse watery diarrhoea. They also found out that one-day-old lambs were more susceptible than 12 day old animals (Snodgrass et al., 1976b). However, McNulty et al., (1976b) detected viral replication in villous epithelial cells of mucosa by immunofluorescence test without development of clinical symptoms in colostrum deprived lambs. Similarly, the calf rotavirus has been successfully transmitted to gnotobiotic calves (Mebus et al., 1971b; Woode, et al., 1974) to colostrum deprived calves (DeLeeuw et al., 1977; Morin et al., 1974) and to gnotobiotic piglets (Tzipori and Walker, 1978).

Foal rotavirus produced clinical symptoms with virus excretion, when a bacteria free filtrate was inoculated orally to the foal (Kanitz, 1976) but tested this way in gnotobiotic piglets caused rise in neutralising antibody which suggested that infection occurred (Tzipori and Walker, 1978).

Rabbit rotavirus has been successfully transmitted to one month old non-immune rabbits with the development of clinical symptoms and with the virus shedding lasting for 2 - 5 days. Specific viral antigen was detected in the gut epithelium by indirect immunofluorescence test (Petric et al., 1978) but the rabbit was insensitive to human, SA11 rotaviruses. Snodgrass et al., (1979b) successfully transmitted kitten rotavirus to kitten with development of symptoms and excretion of virus in faeces.

The mode of transmission within the herd or community has not yet been fully established. However, speculative suggestions have been put forward by various workers. The most likely mode of transmission is the faecal-oral route (Bishop et al., 1979; Rodriguez et al., 1979) and probably respiratory route (Lewis et al., 1979; Oishi et al., 1979; Vollet et al., 1979) and a close family contact is important with either transmission from child to parents or vice versa or within siblings (Haug et al., 1978; Mutanda et al., 1979; Oishi et al., 1979; Tallet et al., 1977; Wyn-Jones et al., 1978; Zissi et al., 1976). Transmission also occurs readily between babies in hospital (Van Renterghem et al., 1980).
from non family adult visitors to babies in hospital (Bishop et al., 1979). Food, water and air may play a role in the transmission of rotavirus in a community (Vollet et al., 1979).

In the Spring of 1964 an interesting epidemiological study was related to outbreaks of rotavirus diarrhoea in an isolated Pacific Island. 3,439 cases were reported. The retrospective study of the outbreak showed a high attack rate (62%), rapid rate of spread within the families and between population groups with limited direct personal contact. Consequently the possibility of a respiratory route of transmission was suggested (Foster et al., 1980). Similarly, in a typical outbreak of acute gastroenteritis in a small Swedish town, rapid spread of the disease was recorded with 2,172 cases in 2 weeks and there was a high attack rate with 30% of the population affected. The mode of transmission was traced to contamination of a fresh water supply with sewage effluent (Lycke et al., 1978).

In the adult, rotavirus infection is often mild or asymptomatic and probably provides a reservoir in temperate zone populations (Meuerman and Laine, 1977; Van Bonsdorff et al., 1976) because the excretion of massive quantities of rotavirus in stools of symptomless adults is known (Wyn-Jones et al., 1978) and it is possible that asymptomatic carriers exist (Tallett et al., 1977) in which the quantity of virus in stools is small and escapes detection by EM. The virus may also persist in a non human reservoir (Heiber et al., 1978). In the lamb the continued excretion of rotavirus after clinical recovery, suggests that a clinically normal lamb may serve as a source of infection for other lambs (Snodgrass et al., 1976b). Cross-infection study, already noted, indicates that many rotaviruses do not exhibit a strict species specificity and in the course of such experiment an increased virulance of the heterologous rotavirus isolate has been observed (Mebus et al., 1976) and it is suggested that the virus is possibly a zoonotic agent (Mayr, 1975).
The main approaches for diagnosis of rotaviruses are either the demonstration of virion in the faeces or serological evidence of infection and an account of the different methods for diagnosis will be judged on the basis of their reliability, reproducibility and ease of performance.

The electronmicroscopic identification of the virion in faeces is reliable because a massive amount of it is usually excreted which may be as great as \(10^9 - 10^{10}\) particles/gm of faeces in symptomatic cases (Chrystie et al., 1975). However, since at least \(10^6\) virus particles/gm of faeces (Chrystie et al., 1979; Madeley, 1979a) are required for visualisation of virus by EM there is a lower limit to its value. Although electronmicroscopy is a simple and reliable technique, it is time consuming and also requires expensive specialised equipment and trained personnel. Immunoelectron microscopy (IEM) (Flewett et al., 1974a; Konno et al., 1977; McNulty et al., 1976d) is also used for identification of rotavirus. The sensitivity of this technique can be enhanced by absorbing clarified virus suspension to agar containing specific rotavirus antisera (Lamontage et al., 1980). However, this method has it's limitations as it is time consuming and only a few samples can be processed in a day. However, EM has the unique advantage that besides rotavirus, other stool viruses can be recognised simultaneously (Birch et al., 1979; Seigneurin et al., 1979).

Routine use of cell culture techniques for identification of rotavirus is very limited because few grow readily. However, some animal rotavirus antigen can be detected in infected cells by indirect immunofluorescence test (Brade and Schmidt, 1979; Ellens et al., 1978; McNulty et al., 1976d) but identification of human rotavirus is difficult since the virus does not easily replicate (Wyatt et al., 1976a). Furthermore, a large number of samples cannot be processed quickly. Low speed centrifugation of partially clarified rotavirus on to cells (Banatvala et al., 1975)
or treatment with trypsin (Almeida et al., 1978b) has been used to increase uptake of the virion by cells and the partially synthesized antigen which then occurs may be revealed by indirect immunofluorescence test.

The haemagglutination (HA) and haemagglutination inhibition (HI) tests have been developed for detection of some rotaviruses (Fauvel et al., 1978; Spence et al., 1976) but not all rotaviruses possess haemagglutinin (Spence et al., 1978) so these techniques cannot be used routinely. Advances have been made in the development of the immune adherence haemagglutination (IAHA) test which makes it 16 - 32 times more sensitive than complement fixation (CF) test (Matsuno et al., 1977a) and approaches the sensitivity of EM (Matsuno and Nagayoshi, 1978).

The counter immunoelectroosmophoresis (CIEOP) first described by Middleton et al., (1976) for detection of rotavirus antigen in faeces has been found to be a useful technique. However, considerable discrepancies exist as regards its sensitivity over EM. One group (Grauballe et al., 1977) found the technique was more sensitive than EM for identification of rotavirus from stools which was in contrast to the findings of other groups (Birch et al., 1977; Ellens et al., 1978; Middleton et al., 1976). For CIEOP test SA11 antisera was found to be more sensitive than the Nebraska calf diarrhoea virus (NCDV) antisera for the detection of human stool antigen (Jesudoss et al., 1979). In some studies the immunodiffusion test was found to be more sensitive than EM for detection of stool antigen (Van Opdenbosch et al., 1978).

Kapikian et al., (1975) developed a CF test for seroepidemiological survey of the incidence of rotavirus amongst human population using NCDV as complement fixing antigen. The CF test was found to be equally efficient for the detection of rotavirus antigen in stool as EM (Konno et al., 1978b). The use of the 'O' agent as CF antigen was found to be more efficient than that derived from SA11, NCDV, EDIM. It is
easy to prepare and does not need concentration (Kapikian et al., 1976a). Although the CF test is a sensitive method for the detection of antibody (Chiba et al., 1979a) it is more tedious to perform than immunodiffusion and is of limited use because some faecal samples contain anticomplementary substances to rotavirus (Ellens et al., 1978; Mohammed et al., 1978).

The two most sensitive test systems for detection of rotavirus in stools are solid phase radioimmunoassay (RIA) and enzyme linked immunoabsorbent assay (ELISA). The RIA for detection of rotavirus antigen in stool has been described by Middleton et al., (1977a) who found it to be 10 times more sensitive than EM (Middleton et al., 1977a), 50 - 100 times more sensitive than CF (Sarkkinen et al., 1979) and 1000 times more sensitive than CIEOP for detection of rotavirus antibody (Mohammed et al., 1978). The ability of RIA to measure different classes of antibody (IgM, IgA and IgG) makes it possible to investigate the immunity and epidemiology of rotavirus infection and also the diagnosis of recent infection (Sarkkinen et al., 1979). However, the disadvantage of this test is use of hazardous radioactive material and the need for expensive gammacounters and trained staff.

Yolken et al., (1977) were first to adapt ELISA test for detection of rotavirus in stools and was found to be more sensitive than EM (Birch et al., 1979; Ellens et al., 1978; Seigneurin et al., 1979; Stuker et al., 1979b), as it can detect $10^4$ particle/ml (Chrystie et al., 1979) or 1 ng of viral protein/ml (Ellens and De Leeuw, 1977) or as small as 3 μls of antisera (Yolken et al., 1978a). This means that the ELISA test is more sensitive than CF, the fluorescent antibody (FA) test, serum neutralisation and CIEOP methods (Ellens et al., 1978; Yolken et al., 1978a) and it is also capable of determining IgG and IgM subclasses of human anti-rotavirus antibody using a single dilution of serum (Yolken et al., 1978a). The specific activity of some faecal samples can be overcome by pretreatment of stool with 20%
N-acetyl cystine (reducing agent) without reducing the sensitivity of the test system (Yolken and Stopa, 1979). The sensitivity of the test can also be increased by coating the solid matrix with antibody to one species of virus and use of another species rotavirus antibody for preparation of enzyme conjugate, i.e. use of two hyperimmune sera than one (Bishai et al., 1979; Zissi and Lambert, 1980). The test system once standardised is easy to perform and the results can be read by the naked eye.

0.3.8 IMMUNOLOGY

The role of passively acquired serum antibody is uncertain as both field and experimental observations indicate that such antibody in calves do not protect against rotavirus infection (Mebus et al., 1973b; Woode et al., 1975). Similarly passively acquired serum antibodies do not protect neonates against rotavirus infection (Brandt et al., 1979; Totterdell et al., 1980). Protection against rotavirus infection appears to depend on a local cellular resistance, initially this resistance may be due to an interference phenomenon and later due to local antibody production (Mebus, 1976). The continued presence of antibody to rotavirus in the gut of a neonatal ruminant has been shown to be important in protecting against clinical disease associated with infections (Wells and Snodgrass, 1978). It is well known that the lack of immunological defences at birth necessitates the immediate ingestion of colostrum which provides a passive supply of gammaglobulin, and the feeding of colostrum to ruminants containing high titer rotavirus IgG appears to protect against subsequent challenge (Lecce et al., 1978; Well and Snodgrass, 1978; Woode et al., 1975). McNulty et al., (1976e) suggested that calves fed sufficient colostrum to produce serum IgG levels in excess of 30 mg/ml were protected against the development of clinical symptoms. A similar effect has also been achieved by feeding immune serum at the rate of 2.5 ml/kg body weight twice daily and also intraperitoneal injection of immune serum presumably due to
transfer of antibody to gut (Snodgrass and Wells, 1978).

In a model system human rotavirus antibody containing IgG was fed orally to gnotobiotic lambs 24 - 72 hours after birth. The animals when infected with a lamb passaged human rotavirus at 30 hours after feeding, did not develop diarrhoea. Furthermore, virus excretion was delayed and the excretion period was shortened which was taken to imply that anti-rotavirus IgG may be useful to protect children in an outbreak (Snodgrass et al., 1977b).

The presence of antirotavirus IgA has been detected in human colostral samples and milk by RIA or ELISA from women in Boston (Cuker et al., 1978), Guatemala (Simhon and Mata, 1978) also in similar samples from Costa Rica, Guatemala and U.S.A. (Yolken et al., 1978b). However, the exact role of this secretory IgA in the prevention of rotavirus infection in neonates is uncertain.

An oral live attenuated vaccine (Scourvax-Reo) has been developed and was found to be protective in field trials in reducing the incidence of rotaviruses in calves and to subsequent challenge with virulent strain (Mebus et al., 1973b). However, in a challenge study Woode et al., (1978) reported the vaccine induced protection in 5 gnotobiotic calves when challenged 7 - 21 days with pathogenic rotavirus (U.K. strain) but did not protect when 3 calves challenged after 3 - 5 days of vaccination. The calf vaccine also did not protect any of the neonatal colostrum deprived piglets when challenged with a virulent strain of pig rotavirus, since the vaccine strain did not replicate to a significant level in the gut (Lecce and King, 1979).

Since there are at least 4 serotypes of human rotavirus (Flewett et al., 1978) and multiple types in other hosts probably also occur (Spence et al., 1978) there could be a need for a polyvalent vaccine. It is certainly known that immunity induced by two human serotypes is not cross
protective (Yolken et al., 1978c) and this partly explains the repeated attacks of human rotavirus gastroenteritis of children.

0.4 CORONAVIRUSES

The transmissible gastroenteritis (TGE) of pigs, a coronavirus (Tajima, 1970) may cause 100% mortality in piglets under one week old. The clinical severity of the disease overshadows all other gastroenteritis infections of pigs (Woode, 1979) and apart from TGE, serologically different coronavirus infection in pigs and calves are more virulent than the other viruses associated with gastroenteritis (Bohl, 1975; Gouet et al., 1978; Mebus et al., 1973a). However, the pathogenicity under different conditions are different. For instance, the U.K. and Danish isolates of bovine coronaviruses appear to cause relatively mild infection compared with the French and U.S.A. isolates (Bridger et al., 1978a; Gouet et al., 1978). In U.S.A., the neonatal calf diarrhoea coronavirus (NCDC) is highly pathogenic to calves and the annual average economic loss due to coronavirus infection is around 27.6%, much higher than that due to rotavirus (6.15%) (House, 1978).

Besides pigs and calves, the coronavirus has been incriminated in enteritis of various other animals and man (Table 0.4). The coronavirus enteritis usually occurs in calves of 3 months old or older (Durham et al., 1979; Stair et al., 1972). However, in some instances infection has been recorded in 1 - 3 month old calves (Langpap et al., 1979). Coronavirus infections are also associated with older children, adult and in tropical sprue in children in South India (Mathan et al., 1975). At Bristol, the proportion showing excretion in the faeces was 15 (4.2%) of 355 adults with diarrhoea and 5.2% (5 of 96) of adults without diarrhoeic symptoms (Clarke et al., 1979). In an extensive survey of the incidence of coronaviruses amongst aboriginal and non-aboriginal infants, children and adults in Australia,
Table 0.4  Coronaviruses associated with gastroenteritis of man and animals

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>HOST</th>
<th>PROVED CAUSE OF GASTROENTERITIS</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transmissible gastroenteritis (TGE) virus</td>
<td>Pig</td>
<td>Yes</td>
<td>Tajima (1970)</td>
</tr>
<tr>
<td>Coronavirus of pig</td>
<td>Pig</td>
<td>Yes</td>
<td>Chasey and Cartwright (1978)</td>
</tr>
<tr>
<td>Neonatal calf diarrhoea coronavirus (NCDC)</td>
<td>Calves</td>
<td>Yes</td>
<td>Stair et al., (1972)</td>
</tr>
<tr>
<td>Bovine enteric Coronavirus (BEC)</td>
<td>Calves</td>
<td>Yes</td>
<td>Wood and Bridger (1978a); Gouet et al., (1978)</td>
</tr>
<tr>
<td>Dog enteritis coronavirus (DEC)</td>
<td>Dog</td>
<td>Yes</td>
<td>Keenan et al., (1976)</td>
</tr>
<tr>
<td>Transmissible enteritis (blue comb) virus</td>
<td>Turkey</td>
<td>Yes</td>
<td>Panigrahy et al., (1973)</td>
</tr>
<tr>
<td>Mouse hepatitis virus (MHV)</td>
<td>Newborn mouse</td>
<td>Yes</td>
<td>Broderson et al., (1976)</td>
</tr>
<tr>
<td>Foal enteritis virus (FE V)</td>
<td>Foal</td>
<td>?</td>
<td>Bass and Sharpee (1975)</td>
</tr>
<tr>
<td>Human enteric coronavirus (HEC)</td>
<td>Children and adult</td>
<td>?</td>
<td>Mathan et al., (1975); Caul and Clarke (1975)</td>
</tr>
<tr>
<td>Enteric coronavirus of sheep and deer</td>
<td>Sheep and Deer</td>
<td>?</td>
<td>Durham et al., (1979)</td>
</tr>
<tr>
<td>Simian coronavirus</td>
<td>Monkey</td>
<td>?</td>
<td>Caul and Egglestone (1979)</td>
</tr>
</tbody>
</table>
Schnagl et al., (1979) found that the rate of excretion of coronavirus in clinically normal aboriginal infants (45.5%), children (84.8%) and adults (67.2%) was higher than diarrhoic infants (30%), children (66.5%) and adults (53.5%). However, they also found that non-aboriginal symptomatic as well as asymptomatic infant children and adults were much less excretors of coronavirus than aboriginal, but the rate of excretion in non-aboriginal individuals was more or less the same. Calves with diarrhoea excreted more virus than asymptomatic animals (Haralambiev et al., 1979).

Experimental or naturally occurring coronaviral diarrhoea in calves usually starts with sudden onset of profuse watery diarrhoea with occasional blood flakes, the calves were frequently, febrile, dehydrated and in extreme cases death occurs in as high as 30% (Durham et al., 1979; Gouet et al., 1978). The coronavirus infects the columnar epithelial cells of small intestine resulting in degeneration and desquamation of epithelial cells and also infect absorptive and crypt cells of the colon (Gouet et al., 1978; Mebus et al., 1973a, Morin et al., 1974). In ultrathin sections, the virus particles were seen in cytoplasm of the damaged cells and in cysternae of smooth endoplasmic reticulum of damaged crypt cells in pigs (Chasey and Cartwright, 1978) in man (Clarke et al., 1979). The calf and pig coronaviruses have been successfully transmitted to calves (Gouet et al., 1978; Mebus et al., 1973a) and pigs (Chasey and Cartwright, 1978) with development of typical disease.

Varying degrees of success have been achieved in growing coronaviruses in cell and organ cultures. The bovine coronavirus has been cultured in foetal bovine kidney cells (Mebus et al., 1973a), in primary embryonic calf kidney cells (Laporte et al., 1979), in Vero, MDBK and porcine kidney (PK15) cells (Dea et al., 1980) and to a very high efficiency in HRT 18 (human rectum adenocarcinoma cell line) cells but not BHK-21, CHO or MDCK cells (Laporte et al., 1979).
The bovine coronavirus has also been cultured in intestinal organ culture (Bridger et al., 1978b) and in intestinal loop of newborn calves (Haralambiev et al., 1979). However, only limited successes have been achieved in growing human enteric coronavirus in human embryonic intestine organ culture (Caul and Egglestone, 1977) and in human embryo kidney monolayer (Clarke et al., 1979). Laporte et al. (1979) reported to have succeeded in growing human enteric coronavirus in HRT 18 cells.

The antigenic differentiation of different enteric coronaviruses has not been undertaken, however, by HI test the bovine enteric coronavirus isolated in France is antigenically distinct from NCDC of U.S.A. (Gouet et al., 1978).

0.5 PARVOVIRUS-LIKE AGENT

A 27 nm virus-like particle was identified by IEM in the stools of volunteers ill with diarrhoea following oral administration of Norwalk agent (Kapikian et al., 1972). This agent was derived from the rectal swabs collected from elementary school children and their adult contacts in an outbreak of gastroenteritis at Norwalk, Ohio, U.S.A. in 1968 (Adler and Zickl, 1969). The Norwalk strain, the prototype of this group has been associated most frequently with epidemics and family outbreaks of gastroenteritis in older children and adults (Greenberg et al., 1979a). The Norwalk agent is stable to treatment with ether, acid and heat, so on the basis of its stability and size the agent closely resembled parvoviruses (Dolin et al., 1972). The buoyant density of the particle in cesium chloride was 1.38 - 1.40 gms/cm³ a property also consistent with that of parvovirus (Kapikian et al., 1973).

In the beginning the agent was thought to be confined to U.S.A., however the ubiquitous distribution is now recognised (Table 0.5). In a comparative serological study, antibody to Norwalk agent was identified in Bangladesh, Belgium,
<table>
<thead>
<tr>
<th>Country/Agent</th>
<th>Size (nm)</th>
<th>Buoyant density (gms/cm³)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Australia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norwalk-like (Oyster) agent</td>
<td>27 - 30</td>
<td>1.38</td>
<td>Murphy et al., (1979)</td>
</tr>
<tr>
<td>Parramatta agent</td>
<td>23 - 26</td>
<td>-</td>
<td>Christopher et al., (1978)</td>
</tr>
<tr>
<td><strong>Japan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>United Kingdom</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ditchling agent</td>
<td>25 - 26</td>
<td>1.38 - 1.40</td>
<td>Appleton et al., (1977)</td>
</tr>
<tr>
<td>Small round agent</td>
<td>25 - 26</td>
<td>1.40</td>
<td>Appleton and Pereira (1977)</td>
</tr>
<tr>
<td>Cockles agent</td>
<td>25 - 26</td>
<td>1.40</td>
<td>Appleton and Pereira (1977)</td>
</tr>
<tr>
<td>Norwalk-like particles</td>
<td>32 - 34</td>
<td>-</td>
<td>Caul et al., (1979)</td>
</tr>
<tr>
<td>Norwalk-like particles</td>
<td>32</td>
<td>-</td>
<td>Egglestone (1980)</td>
</tr>
<tr>
<td>Norwalk-like particles</td>
<td>-</td>
<td>-</td>
<td>Curry and Roberts (1980)</td>
</tr>
<tr>
<td><strong>United States of America</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norwalk agent</td>
<td>27</td>
<td>1.38 - 1.40</td>
<td>Kapikian et al., (1972)</td>
</tr>
<tr>
<td>Hawaii agent</td>
<td>26</td>
<td>1.38 - 1.39</td>
<td>Thornhill et al., (1977)</td>
</tr>
<tr>
<td>Montgomery County (MC) agent</td>
<td>26 - 27</td>
<td>1.37 - 1.41</td>
<td>Morens et al., (1979)</td>
</tr>
<tr>
<td>Colorado agent</td>
<td>27</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Ecuador, Nepal, Switzerland, U.S.A. and Yugoslavia and antibody acquisition approached 70% in all countries studied, and the antibody acquisition occurred in very early life in developing countries (Greenberg et al., 1979b).

In a recent serological survey the Norwalk agent appeared to be the cause of more than 25% of nonbacterial gastroenteritis outbreaks in families and communities (Greenberg et al., 1979a). In a survey of 308 residents at Massachusetts, Blacklow et al., (1979) showed that serum antibody was rarely present in childhood but 50% of the adults studied had antibody. They also found 20% serum antibody in 0 - 3 month old babies which was presumably of maternal origin and rapidly declined to 5% for rest of the childhood.

The Norwalk-like agents Hawaii and Montgomery County (MC) agents have been associated with two separate family outbreaks of non-bacterial gastroenteritis and the serum antibody response was recorded by IEM in patients and volunteer studies (Thornhill et al., 1977). Outbreak of "winter vomiting disease" affecting children and staff of a primary school at Ditchling, England (Appleton et al., 1977) at Paramatta, Australia (Christopher et al., 1978) and in children (8 months to 4 years of age with 67.9% attack rate) in Japan (Kogashaka et al., 1980) have all been shown to be associated with the Norwalk-like agent. The Norwalk-like agents have also been identified from hospital outbreaks affecting patients and staff (Caul et al., 1979) and morphologically indistinguishable particles in outbreaks affecting all age groups were recorded (Curry and Roberts, 1980; Egglestone, 1980).

The parvovirus-like agents have also been incriminated in food poisoning after consumption of cockles (Appleton and Pereira, 1977) and in an Australian outbreak involving at least 2,000 people who had consumed oysters (Murphy et al., 1979). The transmission of virus seems to be faecal-oral
route, however, transmission of oyster agent from sewage to oyster to human has been suggested (Murphy et al., 1979).

The oyster agent is antigenically similar to Norwalk agent (Murphy et al., 1979) and the MC agent is also serologically similar to Norwalk agent but Hawaii agent is different (Thornhill et al., 1977). By IEM, the Ditchling agent appeared to differ from Norwalk and Hawaii agents (Appleton et al., 1977). Similarly, the small round virus (SRV) is antigenically different from Norwalk agent, 'W' agent and caliciviruses (Kogasaka et al., 1980).

None of the above agents have been cultivated in cell cultures (Appleton et al., 1977; Kogasaka et al., 1980; Thornhill et al., 1977; Wyatt et al., 1978b). However, their host range appears to be human and chimpanzees (Wyatt et al., 1978b). The Norwalk agent has been successfully transmitted in serial passages through multiple human volunteers without significant alteration in rate of illness or specific symptoms (Dolin et al., 1971). The symptoms of experimentally induced illness are indistinguishable from that of naturally occurring disease. The incubation period is 18 - 48 hours and symptoms last for 24 - 48 hours (Dolin et al., 1971). The predominant symptoms in experimentally or naturally occurring disease are sudden onset of nausea, rapidly followed by abdominal cramps either vomiting or diarrhoea or both and additionally less frequent symptoms are headache, sore throat, cough or fever (Christopher et al., 1978; Dolin et al., 1971; Kogasaka et al., 1980).

The immunoelectron microscopy (IEM) (Kapikian et al., 1972), solidphase microtiter radioimmunoassay (RIA) and immune adherence haemagglutination assay (IAHA) for Norwalk agent antigen and antibody detection have been developed (Greenberg et al., 1978; Kapikian et al., 1978).
The parvoviruses which are associated with enteritis of animals include bovine parvovirus, canine parvovirus, feline panleukopenia virus (FPV) and mink enteritis virus (MEV); (Apple et al., 1978; Rose, 1974). MEV appears to be closely related to, or identical with FPV based on physical properties, serological tests and cross challenge studies of immunity (Johnson, 1967; Wills, 1952). The parvovirus isolated from dogs with vomiting and diarrhoea is serologically indistinguishable from FPV and is also associated with leukopenia in infected dogs (Apple et al., 1978). The FPV vaccine protected dogs against challenge with virulent canine parvovirus, so a close antigenicity exists between parvoviruses of cat and dog (Apple et al., 1979).

The bovine parvovirus was first isolated from diarrhoic calves in 1959 in Maryland, U.S.A. (Abinanti and Warfield, 1961). Since then bovine parvoviruses have been detected in faeces of symptomatic and asymptomatic calves and also serological evidence of infection has been recorded in Algeria, Austria, England, Japan and U.S.A. (Storz and Leary, 1979). The parvoviruses have also been identified from faeces of human patients with gastroenteritis and clinically normal people. The antigen agglutinated with convalescent sera, however, the role of the virus in typical acute gastroenteritis is uncertain (Paver et al., 1975, Flewett et al., 1974b, Kjeldberg, 1977; Schnagl et al., 1979). On the other hand, in an outbreak in dogs morbidity and mortality rates may reach 50 - 100% and 10 - 50% respectively and may be even higher in pups (Eugster et al., 1978a). Similarly, the fatality in the cat may reach as high as 50 - 90% in an outbreak (Carpenter, 1971). However, clinically normal carriers of parvovirus exists in cattle, pig and cats (Siegl, 1976).

The clinical symptoms in dogs is usually fever, vomiting, greyish or yellowish diarrhoea which is often
haemorrhagic, mucoid or watery with a foetid odour, and dehydration results (Burtonboy et al., 1979; Eugster et al., 1978a; Gognon and Povey, 1979; McNulty et al., 1980). Upper respiratory symptoms have also been observed (Eugster et al., 1978a). A significant mortality occurs within the first 48 hours of the onset of symptoms (Gognon and Povey, 1979).

The virus has been successfully transmitted to specific pathogen free beagle pups (Apple et al., 1979; Gognon and Povey, 1979) and to germ free as well as conventional dogs (Burtonboy et al., 1979) with the development of clinical symptoms in the first 3 days. Two to eight months old calves become diarrhoic 4–7 days after oral or intranasal inoculation with bovine parvovirus Type 1 (Spahn et al., 1966).

The bovine parvovirus isolates have been cultivated in rapidly replicating bovine foetal kidney cells or calf testicle cells (Storz and Leary, 1979). Various cell lines are also susceptible to canine parvovirus infection and the virus replication was identified by indirect immunofluorescence test (Apple et al., 1979).

0.7 ASTROVIRUS

Astroviruses were first described by Madeley and Cosgrove in 1975 as 28 nm particle with a five or six pointed star-shaped surface structure seen in negatively stained preparations by EM. The astrovirus have an unbroken outline with a smooth circular outer edge, occasionally some preparations showed rodlike structures (5 nm in diameter) between virus particles (Madeley, 1979b). Similar morphologically distinctive viruses have been observed in the faeces of diarrhoic children (Ashley et al., 1978; Kurtz et al., 1977; Maass et al., 1978; Madeley et al., 1977; Schnagl et al., 1978) in lamb (Snodgrass and Gray, 1977) and calves (Woode and Bridger, 1978b). The virus has now been identified as a RNA virus (Gray et al., 1980).
The astroviruses have been observed in babies below 2 years of age as well as some normal babies (Madeley and Cosgrove, 1975). In a recent survey Madeley (1979a) found an association of astrovirus in 80% of children with diarrhoea and also 20% of asymptomatic babies. Similarly, Kurtz et al., (1977) detected astrovirus in 63% of children with diarrhoea but not in 10 asymptomatic controls. They also found virus in the faeces of 25.5% asymptomatic staff members. Maass et al., (1978) detected astrovirus in faeces of 78 newborn babies in an epidemic of acute nonbacterial gastroenteritis. In a serological survey 77% of a group of 70 young adults had antibodies to astrovirus (Kurtz and Lee, 1978).

The astrovirus has been associated with outbreaks of diarrhoea in lamb and 47% of lamb faeces was positive for rotavirus in a survey (Snodgrass and Gray, 1977).

Serial transmission of astrovirus to adult volunteers was successful with the development of diarrhoea in one of the 17 volunteers. In addition three asymptomatic volunteers were rotavirus excretors and 81% volunteers developed seroresponse to astrovirus detected by immunofluorescence (Kurtz et al., 1979). They concluded that astrovirus possess a low pathogenicity for adults since 77% adult possess antibody to astrovirus. However, lamb astrovirus was highly pathogenic to gnotobiotic lamb with development of diarrhoea in second passage, with virus excretion in the faeces (Snodgrass and Gray, 1977). However, calf astrovirus was only mildly pathogenic to gnotobiotic calves without development of symptoms and replication of the antigen was detected in the small intestinal sections by indirect immunofluorescence test (Wood and Bridger, 1978b). The lamb astrovirus appeared to replicate in villous epithelial cells of small intestine (Gray et al., 1980; Snodgrass et al., 1979a).

Astrovirus has not yet been isolated in cell culture, however, the replication of astrovirus was detected by
indirect immunofluorescence after infection of human embryonic kidney cells with human astrovirus (Kurtz et al., 1979).

0.8 CALICIVIRUS

Calicivirus particles are isometric about 30-35 nm in diameter with a capsid showing clear evidence of cubic symmetry with 32 hollows which have dark centres in negatively stained preparation (Madeley and Cosgrove, 1976). The association of caliciviruses in gastroenteritis was first reported by Madeley and Cosgrove (1976) during examination of stools of infants from symptomatic as well as asymptomatic babies.

Madeley (1979a) reported identification of 53% calicivirus in the stools of infants associated with gastroenteritis and similar numbers (47%) in asymptomatic cases. Recently, the association of calicivirus in outbreaks of winter vomiting disease in London school children was clearly implicated by detection of virus in stools and rising antibody to calicivirus was demonstrated in several children by immuno-electron microscopy (Cubitt et al., 1979). Similarly, in an outbreak of acute infectious diarrhoea in an infant home at Sapporo, Japan which experienced 77% attack rate, the calicivirus virus was detected only in 28% of faeces but seroconversion of 95% symptomatic and 75% asymptomatic infants was demonstrated by IEM showing the subclinical nature of infection.

Calicivirus has also been associated with acute gastroenteritis of calves (Wood and Bridger, 1978b) and pigs (Saif et al., 1980). The animal caliciviruses have been successfully transmitted to their respective host and virus transmission was shown by the development of symptoms and excretion of virus in faeces (Saif et al., 1980; Wood and Bridger, 1978b).

Attempts to grow enteric caliciviruses in cell culture were unsuccessful (Chiba et al., 1979b; Saif et al., 1980; Wood and Bridger, 1978b).
0.9  **ADENOVIRUS**

Uncultivable enteric adenoviruses have been detected in 5 - 8% stools of infants with or without diarrhoea (Davidson *et al*., 1975; Scott *et al*., 1979; White and Stancliff, 1975) and have also occasionally been associated with hospital outbreaks of diarrhoea (Flewett *et al*., 1975b; Madeley, 1979a; Middleton *et al*., 1977b). Two fatal cases of adenoviral gastroenteritis have been reported by Whitlaw *et al*., (1977). In one isolated study 15% of hospitalised viral associated gastroenteritis cases were attributed to adenovirus (Retter, *et al*., 1979). Recently, Johansson *et al*., (1980) developed an ELISA test for adenovirus and they reported that enteric adenoviruses are antigenically different from the 35 established adenovirus serotypes. They suggested that the enteric adenovirus represent a new serotype which appears to be associated with gastroenteritis without clear-cut respiratory symptoms.

Limited success in cultivation of enteric adenovirus in HAE-70 cells (human amnion cell line) when clarified faecal filtrate was centrifuged onto cells and replication of antigen was detected by indirect immunofluorescence staining (Retter *et al*., 1979).

0.10  **MISCELLANEOUS SMALL ENTERIC VIRUS-LIKE AGENT ASSOCIATED WITH ACUTE GASTROENTERITIS OF HUMANS**

The 'minireovirus' a 30 nm virus like particles was first described by Middleton *et al*., (1977b) and later recognised as 'minirotavirus' (Spratt *et al*., 1978) was associated with acute infantile gastroenteritis. The intact particles possess a double capsid with a complete outer capsid or with a rim like structure having 2 - 3 nm external projections. Out of 669 cases of acute gastroenteritis studied minireovirus was found in only 104 cases and 50% cases considered to be nosocomial origin (Middleton *et al*., 1977b). Spratt *et al*., (1978) also described association of either
minirotavirus or calicivirus in 20 cases of infantile gastroenteritis.

In an institutional outbreak of acute gastroenteritis of mentally retarded adults (22 - 51 years of age), 32 (34.8%) of 92 inmates and 2 (6.3%) of 32 teachers experienced acute gastroenteritis with symptoms of nausea, vomiting and diarrhoea with recovery in 1 - 3 days. An uncultivable 'Otefuke agent' of 35 - 40 nm in diameter was identified from 5 of 7 stool samples examined by IEM and rise in antibody titer was also demonstrated against the agent. The agent does not share common antigenicity with either Norwalk agent, 'W' agent or caliciviruses by IEM (Taniguchi et al., 1979).
CHAPTER 1

DEVELOPMENT AND CHARACTERISATION OF A CALF KIDNEY

DIPLOID CELL LINE
# CONTENTS OF CHAPTER 1

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Introduction</strong></td>
<td>48</td>
</tr>
<tr>
<td><strong>Materials and methods</strong></td>
<td>51</td>
</tr>
<tr>
<td>1.1 Media</td>
<td>51</td>
</tr>
<tr>
<td>(a) Growth medium</td>
<td>51</td>
</tr>
<tr>
<td>(b) Maintenance medium</td>
<td>51</td>
</tr>
<tr>
<td>(c) Phosphate buffer saline</td>
<td>51</td>
</tr>
<tr>
<td>(d) Trypsin and versene solution</td>
<td>52</td>
</tr>
<tr>
<td>1.2 Cell culture</td>
<td>52</td>
</tr>
<tr>
<td>(a) Initiation culture</td>
<td>52</td>
</tr>
<tr>
<td>(b) Subculture procedure</td>
<td>52</td>
</tr>
<tr>
<td>(c) Preservation of cells</td>
<td>53</td>
</tr>
<tr>
<td>(d) Recovery of cells</td>
<td>53</td>
</tr>
<tr>
<td>(e) Test for contaminating agents</td>
<td>53</td>
</tr>
<tr>
<td>(f) Chromosomal analysis of cells</td>
<td>54</td>
</tr>
<tr>
<td>1.3 Viruses</td>
<td>55</td>
</tr>
<tr>
<td>1.4 Viral cytopathology</td>
<td>55</td>
</tr>
<tr>
<td>1.5 Assay of viral infectivity</td>
<td>55</td>
</tr>
<tr>
<td>(a) Assay by cytopathology</td>
<td>55</td>
</tr>
<tr>
<td>(b) Assay by immunofluorescence of rotavirus</td>
<td>57</td>
</tr>
<tr>
<td>1.6 Characterisation of virus by electron-microscopy</td>
<td>58</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td></td>
</tr>
<tr>
<td>1.7 Development and characterisation of calf kidney diploid cell line.</td>
<td>59</td>
</tr>
<tr>
<td>1.8 Cell morphology and karyotype.</td>
<td>65</td>
</tr>
</tbody>
</table>
1.9 Susceptibility of cell culture to viruses.  
(a) Bovine rotavirus (U.K. strain).  
(b) Infectious bovine rhinotracheitis virus  
(c) Bovine enterovirus  
(d) Poliovirus type 1.  

1.10 Replication of viruses in cell culture  

Discussion  
1.11 Cultural characteristics  
1.12 Susceptibility to viruses
DEVELOPMENT AND CHARACTERISATION OF A CALF KIDNEY DIPLOID CELL LINE

INTRODUCTION

Only limited successes have been obtained in developing diploid cell strains and the definitive work of Hayflick and Moorhead (1961) with human diploid cell strains paved the way for the successful development of other human and animal diploid cell strains such as WI* -38 (Hayflick, 1965), MRC* -5 (Jacobs et al., 1970), FCDC* -2 (Mirchamy et al., 1977), BEL* -2 and SMBE* -3 by Kalinina et al., (1979).

The era of tissue culture began towards the end of the last century as a by-product of techniques used in experimental biology. The first successful in vitro transplant was achieved by Roux (1885), who kept alive a chick medullary plate in a physiological salt solution. About the same time Arnold (1887) succeeded in studying the survival and migratory activity of frog leucocytes maintained in fragments of alder piths soaked in a physiological salt solution. Ten years later Ljungren (1898) demonstrated by reimplantation that human skin could survive in vitro for many days if stored in ascitic fluid.

The first successful tissue culture, in the modern sense, was realised by Harrison (1907), who aseptically removed small fragments from the wall of the frog neural crest and cultivated them in coagulated frog lymph in a hanging drop preparation. After a few weeks naked nerve fibres sprouted from the piece of cord and grew out into lymph. He not only described the technique but also suggested its several biological implications.

*WI (Wistar Institute), MRC (Medical Research Council), FCDC (Foetal calf diploid cell), BEL (Bovine embryonic kidney), SMBE (Skin muscle of bovine embryo).
But the work was not followed up until his pupil, Burrows (1910) explanted fragments of embryonic chick tissue in blood plasma and obtained growth of cells. Burrows was also the first to describe mitosis in vitro and to pinpoint that cells were able to grow as far as the limits of coagulated plasma. In collaboration with Carrel in the following year (Carrel and Burrows, 1911), he discovered that embryo extract had strong growth promoting action for certain cells. Carrel further advanced the tissue culture techniques in 1912 and demonstrated that cells could be made to proliferate indefinitely in culture by adding a saline extract of embryonic tissue to the plasma and transplanting cells to fresh medium at suitable intervals. Thereafter, in collaboration with several workers he studied the composition of media by identifying different substances favouring cell growth. A significant contribution by Carrel was the introduction of surgical methodology in the handling of such tissues, particularly asceptic and antiseptic techniques. Carrel later (1923) developed the method of growing cells in flask and this procedure proved to be the basis for modern tissue culture.

The introduction of tissue trypsinization by Moscona (1952) was an important development in tissue culture technology. He digested fragments of chicken embryo in a 3% solution of trypsin and observed that the cellular clumps obtained were still able to grow in vitro. However, earlier work of Vogelaar and Elischman (1939) had already shown that trypsin could detach cells from the walls of the roller tubes in which they had been grown. Trypsinization of tissue fragments to form cell suspensions was demonstrated by Dulbecco (1952) and the work of Youngner (1954) now forms the basis of the preparation of primary tissue cultures.

Lewis and Lewis (1911) attempted to develop liquid media with some specified components such as different salt and peptones. They also tested sea water, serum and aqueous embryo extracts in different concentrations but although
the cells grew as a thin layer, they survived only for a short period. Vogelaar and Erlichman (1933) introduced the use of a laboratory formulated synthetic medium, composed of peptone, haemin, cystine, insulin, thyroxin and glucose. Several workers have since searched vigorously for a better synthetic medium. Medium 199 of Morgan et al., (1950) formed the basis for much subsequent development of the different media in use today. The long patient work of Parker et al., (1954), Eagle (1955 and 1959), Waymouth (1959), is a testimony to the wide use of tissue culture.
MATERIALS AND METHODS

1.1 MEDIA

(a) **Growth medium (GM).** This was Hank's balance salt solution (10 X concentrated, Wellcome Reagents Ltd.) supplemented with 0.5% (W/V) Lactalbumin hydrolysate (Sigma, London Chemical Co. Ltd. pfs enzymatically hydrolysed; stock solution 5% (W/V) in deionized water), 0.1% (W/V) D(+) Galactose (anhydrous B.D.H. Chemical Ltd., stock solution 1% W/V in deionized water); 0.066% sodium bicarbonate (Wellcome Reagents Ltd., 4.4% W/V) and 5% (V/V) foetal calf serum (Flow Laboratories Ltd.). The antibiotics, Benzyl penicillin (sodium BP Glaxo, 100 IU/ml) and streptomycin sulphate BP (Glaxo, 100\u03bcg/ml) were used at 1% (V/V) with 0.5% (V/V) mycostatin (E.R. Squibb and Sons; 25 IU/ml).

Stock solution of lactalbumin hydrolysate and D (+) Galactose were sterilised by filtration and stored at 4°C. Foetal calf serum and stock solutions of penicillin, streptomycin and mycostatin were prepared in sterile deionised water and stored at -25°C.

(b) **Maintenance medium (MM).** This differed from GM in that Earle's balance salt solution (Flow Laboratories Ltd.) was used instead of Hank's balance salt solution. Furthermore, the foetal calf serum was lowered to 2% (V/V) and the sodium bicarbonate increased to 0.088% (W/V). The other constituents remained the same as in the growth medium.

(c) **Phosphate buffer saline (PBS) pH 7.3.** Throughout the study PBS without calcium and magnesium was used and was prepared by dissolving the following salts in deionised water to obtain a volume of one litre: Sodium chloride, AR (B.D.H. Chemical Ltd.), 8.0 gms, Potassium chloride, AR (Hopkins and Williams Ltd.), 0.2 gms, di-sodium hydrogen
orthophosphate anhydrous AR (B.D.H. Chemical Ltd.), 1.15 gms, Potassium dihydrogen orthophosphate, AR (B.D.H. Chemical Ltd.), 0.2 gms. The solution was sterilised by autoclaving (10 lbs/20 minutes) and was stored at room temperature.

(d) Trypsin and Versene solution. Trypsin (Wellcome Reagents Ltd., 1:300) was reconstituted with 10 ml of sterile deionised water to provide a 5% (W/V) solution. After distribution of 1 ml volumes to bijoux bottles it was stored at -25°C. Versene (Wellcome Reagents Ltd., 1:5000) was stored at 4°C. When necessary 1 ml of trypsin solution was thawed and added to 100 ml of Versene, this mixture was stored at 4°C for up to 2 weeks after which it was discarded.

1.2 CELL CULTURE

(a) Initiation of culture. One ml volumes of packed primary calf kidney cells suspended in 20 ml of GM were received by post (Central Veterinary Laboratory, Weybridge). The cells were deposited by centrifugation (800 Xg for 8 minutes at room temperature) and the supernatant fluid was discarded. The packed cells were resuspended in 250 ml of growth medium at 37°C and the suspension was distributed in 7.5 ml volumes to 2 oz medical flat bottles. The cultures were incubated at 37°C for 48 hours. The medium was then discarded and the cell sheet was washed twice with PBS. The culture was refed 7.5 ml of maintenance medium and incubated at 37°C until confluent by 5 days.

(b) Subculture procedure. Once the primary monolayer was confluent a subculture schedule of a 1:2 split was adopted. When the cells were confluent, the maintenance medium was discarded and the cell sheet was washed twice with PBS. Four ml of the trypsin versene solution was added to the culture which was then intermittently rocked for one minute before the excess medium was discarded, leaving about 0.5 ml in the bottles. The culture was incubated for 15 minutes at 37°C and the stripped cells were resuspended in
15 ml of prewarmed (37°C) growth medium. Two fresh sterile 2 oz medical flat bottles were each seeded with 7.5 ml of the cell suspension and the cultures were incubated at 37°C.

(c) Preservation of cells. Cultures of actively growing cells of near confluency were selected for preservation. They were trypsinized as described earlier and the cells were resuspended in growth medium containing twice the serum concentration plus 7.5% (V/V) dimethyl sulfoxide (SLR, Fison Laboratory Reagents). The concentration of cells was adjusted to about 3 x 10⁶/ml and 0.6 ml volumes of cell suspension were put into sterile glass ampoules which were sealed. The cell suspensions were equilibrated at 4°C for one hour before slow freezing in the vapour phase of liquid nitrogen. The rate of freezing was slowed by placing the ampoules in a polystyrene box, but the following day the ampoules were removed from the box and stored approximately at -136°C.

(d) Recovery of frozen cells. Ampoules were removed from vapour phase of liquid nitrogen and placed directly in a water bath at 37°C. Once the cell suspension was thawed, the ampoule was opened and the cell suspension was pipetted into 7 ml of prewarmed (37°C) growth medium in a 2 oz medical flat bottle. The cells were thoroughly mixed with the medium and the culture was incubated at 37°C overnight after which the growth medium was discarded and the culture was refed with new growth medium. If the medium became alkaline quickly, it was discarded and replaced by fresh medium. The culture was incubated until it was confluent.

(e) Test for contaminating agents. Cell culture fluid from the 32nd, 42nd and 52nd passages was subjected to testing for contaminating bacteria, fungi and mycoplasmas as described in Appendix I.
(f) Chromosomal analysis of cells. Actively growing cells at about 48 hours of age were selected for chromosomal studies by modification of the method of Whitaker (1972). Colcemid (Grand Island Biological Co.), was added to give final concentration of \(0.04 \mu g/ml\) of medium and the culture was incubated at 37°C for 5 hours after which the medium was discarded and the cell sheet was washed twice with PBS. The cells were stripped with 5 ml of prewarmed (37°C) trypsin versene solution by incubation at 37°C for 10 minutes. The cells were harvested in a tapered glass centrifuge tube at room temperature by centrifugation (800 X g for 5 minutes). The supernate was decanted and the pelleted cells were resuspended in 3.5 ml of hypotonic solution, (11% (V/V) foetal calf serum in distilled water) and incubated for 30 minutes at room temperature. The cells were sedimented by centrifugation as above, the supernate was discarded and 0.5 ml of freshly prepared fixative (1 part of glacial acetic acid and 3 parts of absolute methyl alcohol) was added. Incubation for a further period of 30 minutes at room temperature was allowed after which the cells were carefully resuspended by a pasteur pipette and resedimented by centrifugation as before. The supernate fixative was decanted and the cells were carefully resuspended in 0.5 ml of fresh fixative. The cells were again deposited by centrifugation and after decanting, sufficient fixative was added to make a slightly opalescent cell suspension. A drop of cell suspension was allowed to slowly run down on a clean wet slide. The excess fluid was blotted and the slide was thoroughly dried at 37°C.

A 2% orcein solution was prepared (described in Appendix I) and flooded over the slides for 45 minutes at room temperature. At the end of staining, the slides were washed thoroughly in running tap water, air dried and examined under the oil immersion objectives of a microscope. Suitable spreads were photographed and analysed.
1.3 **VIRUSES**

Viruses used in this study were as in Table 1.1.

1.4 **VIRAL CYTOPATHOLOGY**

Cells were grown on coverslip cultures in Leighton's tubes. The growth media from confluent monolayer was decanted and the cell sheet was washed twice with PBS. A virus suspension of 0.1 ml was inoculated to each of 12 coverslip cultures which were incubated at 37°C for 1 hour. After this 1.9 ml of maintenance medium was added and the cultures incubated at 37°C. Then, depending upon the development of cytopathic effects (CPE) tubes were removed from the incubator at selected intervals. One tube from each infected culture was frozen at -25°C and from another the medium was decanted and the cells were fixed for staining with Haematoxylin and Eosin (H and E) (Appendix II).

1.5 **ASSAY OF VIRAL INFECTIVITY**

Virus was recovered from coverslip cultures in Leighton's tubes by subjecting the culture to three successive periods of freezing (-25°C) and thawing at room temperature. The cellular debris was removed from the suspensions by centrifugation (3000 Xg for 10 minutes). The supernatant fluid was distributed in 1 ml volumes in bijoux bottles and stored at -25°C.

(a) **Assay by cytopathology.** Assay of infectivity of viruses were carried out by the standard microtitration method, Kuchler (1977) in calf kidney diploid cell line. A cell suspension containing $1 \times 10^6$ cells/ml was made in growth medium and 0.1 ml of suspension was added to each well of the microtitre plate (Linbro). The plates were sealed by plate sealer (Titertek) and incubated for 3 days at 37°C to form a confluent monolayer. A serial 10 fold dilution of each virus suspension was made in maintenance
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Strain</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Calf rotavirus</td>
<td>Prof. G.N. Wood, ARC Research Institute on Animal Diseases, Compton.</td>
</tr>
<tr>
<td>2</td>
<td>Calf rotavirus</td>
<td>Dr. M.S. McNulty, The Veterinary Research Institute, Belfast.</td>
</tr>
<tr>
<td>3</td>
<td>Infectious bovine rhinotracheitis Oxford strain</td>
<td>Miss M.H. Lucas, Central Veterinary Laboratory, Weybridge.</td>
</tr>
<tr>
<td>4</td>
<td>Bovine enterovirus</td>
<td>Miss M.H. Lucas, Central Veterinary Laboratory, Weybridge.</td>
</tr>
<tr>
<td>5</td>
<td>Poliovirus Type 1 Vaccine strain</td>
<td>Dr. John S. Slade, Thames Water Authority, London.</td>
</tr>
<tr>
<td>6</td>
<td>Coxsackie A9</td>
<td>Dr. John S. Slade, Thames Water Authority, London.</td>
</tr>
</tbody>
</table>
medium prepared without serum. The growth medium in the microtitre plates was decanted, the cell sheets were washed with Earle's balance salt solution then, 0.04 ml of each dilution of virus was added with 8 replicate wells for each dilution. Finally, 0.1 ml of maintenance medium (prepared without serum) was added to each well, the plates were sealed and were incubated at 37°C. The development of CPE was recorded and the infectious titre was calculated by the Kärber equation to provide the tissue culture infection dose 50/ml (TCID 50/ml).

(b) **Assay by immunofluorescence of rotavirus.** An indirect immunofluorescence test based on Gardner and McQuillin (1974) was used to identify replication of rotavirus in the calf kidney diploid cell line. Coverslip cultures in Leighton tubes were inoculated with rotavirus (described in section 1.4). At 24 hours intervals after infection, coverslip cultures were removed, washed twice in PBS and fixed with cold acetone (B.D.H. Chemicals Ltd.) for 15 minutes. The coverslips were then air dried in a 37°C incubator for 30 minutes and stored at 4°C until required for immunofluorescence staining. For this the acetone fixed preparations were soaked in PBS for 10 minutes. Excess PBS was removed and a 1:20 dilution of calf rotavirus antiserum (199), diluted in PBS was flooded over the cells which were then incubated in a humidified chamber at 37°C for 1 hour. The cells were washed three times in PBS, flooded with fluorescein isothiocyanate (FITC) antiovine IgG conjugate (Nordic Immunological Laboratories) diluted 1:40 in PBS and incubated as above. Excess conjugate was drained off and then cells were washed in three changes of PBS. The coverslips were mounted on a glass slide in PBS glycerine (9 parts glycerine and 1 part PBS pH 7.3) and were examined in a Leitz orthomat microscope fitted with an ultraviolet lamp source and a camera. The excitation and barrier filters were BG12 and K530 respectively. The fluorescing cells were photographed using a Kodak Ektachrome Transparency, 400 ASA. The transparencies were processed by a commercial company. (Appendix III).
1.6 CHARACTERISATION OF VIRUSES BY ELECTRONMICROSCOPY

Clarified suspensions of virus were centrifuged (145,000 Xg for 45 minutes at 4°C). The supernatant fluid was discarded and tubes were drained for 2 hours at 4°C. The pellets of virus was resuspended in 1 to 2 drops of distilled water. A drop of the suspension was placed on a grid (Appendix IV), left for 2 to 3 minutes and then excess fluid was removed. A drop of 3% tungsto phosphoric acid (PTA) pH 6.6 was placed on the grid for 30 to 45 seconds and excess stain was removed. The grids were examined in a JEOL 100B electronmicroscope operating at 80 or 100 KV, and suitable preparations were photographed, processed and printed. (Appendix III).
RESULTS

1.7 DEVELOPMENT AND CHARACTERISATION OF CALF KIDNEY DIPLOID CELL LINE

The primary cell culture became confluent in 5 days after seeding. The following 4 subcultures also produced confluent cultures in 3 to 4 days but thereafter, the rate of growth was slower and it was only practicable to subculture once a week. At this stage, the growth medium was changed at least every 48 hours. By the tenth subculture, islands of rapidly growing cells amongst cellular debris were noticed (Fig. 1.1) and in subsequent passages, two types of cells, epithelial-like and fibroblast-like grew together in patches (Fig. 1.2). An attempt to ensure better growth was made by varying the medium and in particular the type of serum (Table 1.2).

The cells grew well only in the Hank's lactalbumin galactose medium containing either foetal calf or lamb serum and they grew best in the presence of lamb serum. By the thirteenth passage the fibroblast-like cells became dominant (Fig. 1.3) and ultimately the epithelial-like cells disappeared (Fig. 1.4). Under these conditions the growth was again rapid and a subculture schedule of 1:2 split twice a week was readopted. From sixteenth and later passage levels samples of cells were preserved in liquid nitrogen. The growth rate of cells slowed down markedly after the 55th passage and it was necessary to subculture once a week until the 65th passage. However, beyond this passage level, it became very difficult to maintain the cells, a weekly schedule of subculture of the whole cell population to a fresh bottle was introduced. Various efforts to induce growth after the 65th passage by changing medium or serum were of no avail and the culture was abandoned after the 81st passage.
### TABLE 1.2

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hank's balance salt solution 10X</td>
<td>10 ml. 0 ml.</td>
</tr>
<tr>
<td></td>
<td>Lact albumin hydrolysate</td>
<td>0.5%</td>
</tr>
<tr>
<td></td>
<td>D (þ) Galactose</td>
<td>0.1%</td>
</tr>
<tr>
<td></td>
<td>Sodium bicarbonate</td>
<td>0.066%</td>
</tr>
<tr>
<td></td>
<td>Penicillin and streptomycin</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Foetal calf serum</td>
<td>5%</td>
</tr>
<tr>
<td>2</td>
<td>All the constituents of the growth media were the same as in (1) except foetal calf serum was substituted by Lamb serum (Flow Laboratory Ltd.,)</td>
<td>5%</td>
</tr>
<tr>
<td>3</td>
<td>All the constituents of the growth media were the same as in (1) except foetal calf serum was substituted by horse serum (Wellcome Reagents Ltd.,)</td>
<td>5%</td>
</tr>
<tr>
<td>4</td>
<td>Medium 199 (Wellcome Reagents Ltd., 10X)</td>
<td>10 ml. 5 ml.</td>
</tr>
<tr>
<td></td>
<td>Foetal calf serum</td>
<td>5%</td>
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<td></td>
<td>Sodium bicarbonate</td>
<td>0.11%</td>
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<td></td>
<td>Penicillin and streptomycin</td>
<td>1%</td>
</tr>
<tr>
<td>5</td>
<td>MEM (Glaxo modification with Earle's salt)</td>
<td>10 ml. 1 ml.</td>
</tr>
<tr>
<td></td>
<td>(Flow, 10X)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non essential amino acid (Flow, 100X)</td>
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</tr>
<tr>
<td></td>
<td>L - glutamine (Flow 200 mM)</td>
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</tr>
<tr>
<td></td>
<td>Foetal calf serum</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>Penicillin and streptomycin</td>
<td>1%</td>
</tr>
</tbody>
</table>

All the above media was made up to 100 ml in sterile deionised water for working strength of growth media.
Fig. 1.1 Areas of rapidly growing cells (a) amongst cellular debris (b). Live 69 X

Fig. 1.2 Growth of epithelial-like and fibroblastic cells together. Live 69 X
Fig. 1.3 Fibroblast-like cells
Live 69 X

Fig. 1.4 Epithelial cells
Live 69 X
Once the monolayer was confluent evidence of cell replication stopped and at no time were multilayers of cells developed. With periodic change of the maintenance medium containing 2% lamb serum, the monolayer could be maintained for up to three months but if medium was not changed, the cells survived for about one month without showing degenerative changes.

During the active state of cell replication there was considerable acid production evidenced by a drop in pH within 24 hours of seeding but in culture after 65th passage mitotic activity as well as acid production was greatly reduced. The cells lost contrast and became filamentous and star shaped with markedly increased granularity of cytoplasm.

It is possible to represent graphically the activity of the culture throughout its history (Figure 1.5). Arbitrarily, the whole process of cell growth can be divided into three stages. Stage 1 lasted until the tenth passage with peak of cell growth occurring at fifth passage. After a "Crisis" in cell cultivation between tenth and thirteenth passage, there was a long period of stability with optimum cell growth at the 35th passage. The final stage of senescence lasted for about twenty passages.

Cultures regenerated from the frozen stocks behaved in the same way as the parent culture.

The senescence of the culture often passaged 65 was not related to detectable contamination with bacteria, fungi and mycoplasma (Fig. 1.6) because repeated attempts to isolate such organisms in different media, both aerobically and anaerobically and at various temperatures, were unsuccessful.
Fig. 1.5  DIAGRAMATIC REPRESENTATION OF BEHAVIOUR OF THE CALF KIDNEY DIPLOID CELL LINE IN CULTURE

RELATIVE NUMBER OF CELLS

NUMBER OF SUBCULTIVATIONS
1.8 CELL MORPHOLOGY AND KARYOTYPE

The microscopy of stained cells showed that the single nucleus contained 1 to 6 nucleolus which were either oval or branching bodies (Figure 1.7). The individual cells were transparent with characteristic cytoplasmic granularity and only rarely were multinucleated cells seen. The cultured cells adhered firmly to glass surface within 2 hours of subculture and by 24 hours the fibroblast-like cells were oriented in random directions. Between 24 and 48 hours highly refractile cells, representing various stages of mitosis were noticed.

No standardised method for the classification of bovine chromosome was available, so an analysis based on an arrangement of the chromosomes in order of descending length was made. The chromosomal numbers were predominantly 60 (2n = 60) which was characteristic for the bovine. There were 58 acrocentric autosomes and two submetacentric 'X' chromosomes (Fig. 1.8 and 1.9). However at phase III of growth cycle (See Fig. 1.5) one isochromosome (Fig. 1.10) was apparent in the cells. Presence of two 'X' chromosome indicated that, cells were derived from a female calf.

1.9 SUSCEPTIBILITY OF CELL CULTURE TO VIRUSES

(a) Bovine rotavirus (U.K. strain). Within 24 hours of inoculation scattered sickle shaped cells were observed floating in the medium without other signs of overt cytopathic effects (CPE). However, by 48 hours, distinctly thin areas had developed presumably due to localised shedding of cells and many more floating cells, plus cellular debris were observed (Fig. 1.11). There were areas where the cells appeared as if stretched and intracytoplasmic inclusion bodies were sometimes found in such cells (Fig. 1.12). The CPE was progressive and by 72 hours many more areas of CPE developed associated with piknotic nuclei and rounded
Fig. 1.6 Absence of mycoplasma at the interjunction (arrow) of the cells.
Orcein stain 1333 X

Fig. 1.7 Monolayer of calf kidney diploid cells.
Arrow shows nucleus with 6 oval nucleolus.
Phase contrast, stained H & E, 219 X
Fig. 1.8 Chromosomal spread of calf kidney diploid cell line: 58 autosomes and two 'X' chromosomes. Orcein stain. 1333 X

Fig. 1.10 Arrow indicates presence of isochromosome. Orcein stain. 1333 X
Fig. 1.9 (Facing page) Arbitrary arrangement of chromosomes of calf kidney diploid cells in descending order. Twenty nine pairs of autosomes and two 'X' chromosomes. Orcein stain. 3063 X
Fig. 1.11 At 48 hours of bovine rotavirus infection areas of coalesced cells (arrows) were seen. Stained H & E. 137X.

Fig. 1.12 At higher magnification at 48 hours, some cells were swollen, stretched and presence of eosinophilic intracytoplasmic inclusion body (arrow) was seen. Stained H & E. 549X.
cells free floating in the medium. By 96 hours (Fig. 1.13) about 50% cells had detached and ultimately (120 hours) virtually all the cells had disappeared leaving a clean background. The susceptibility of cell line at different passage levels was the same.

The immunofluorescent studies provided confirmation of the presence of rotavirus. Bright green intracytoplasmic fluorescence was observed in a few cells 24 hours after infection and the number of such cells rapidly increased (Fig. 1.14) until by 96 hours all the surviving cells were seen to fluoresce.

(b) **Infectious bovine rhinotracheitis virus (Oxford strain)** At about 18 hours after inoculation 50% of the cells showed cpe's (Fig. 1.15). Some cells were rounded with piknotic nuclei, others were elongated and intranuclear basophilic inclusions were commonly observed. By 48 hours only a few cells survived. The detached cells were rounded and some cells tended to clump together.

(c) **Bovine enterovirus (MC strain).** Eighteen hours after inoculation only about 5% of the cells appeared infected but after 42 hours infected cells were shrunken in size with piknotic nuclei. Highly refractile degenerative elongated cells rounded up and floated off into the medium (Fig. 1.16). Twenty four hours later 90% of the cells had detached from the glass surface.

(d) **Poliovirus type 1 (vaccine strain and Coxsackie A9)** The development of a cytopathic effect was slow and it was not before about 70 hours after inoculation that areas of rounded, refractile, degenerative cells were seen (Fig. 1.17). This process continued slowly but relentlessly so that by 128 hours all the cells were detached from the glass surface. Piknotic nuclei and intracytoplasmic inclusion bodies were observed.
Fig. 1.13 Healthy living monolayers of calf kidney cells. Phase contrast 219 X

Fig. 1.13 Infected. At 96 hours highly elongated cells were seen. Some cells have piknotic nuclei (A) and some rounded floating cells (B) were also seen. Living, phase contrast. 219 X.
Fig. 1.14  At 24 hours after infection with rotavirus infected cells showing bright granulated intracytoplasmic fluorescence.
Fig. 1.15 Cells infected with infectious bovine rhinotracheitis virus. At 18 hours after infection some cells were elongated, some showed basophilic intranuclear inclusion bodies (B) and some had piknotic nuclei (A) Phase contrast stained H & E. 219 X

Fig. 1.16 Cells infected with bovine enterovirus. At 24 hours after infection rounded cells (D) floating in the medium. Some cells had piknotic nuclei (C). Stained H & E. Phase contrast 219 X
Fig. 1.17 Cells infected with Poliovirus Type 1 strain. At 70 hours areas of degenerative cells among healthy monolayer were seen. Intracytoplasmic inclusion body (E) and rounded cells (F) were noticed. Stained H & E. Phase Contrast 219 X
No cytopathic effect was developed even three weeks after inoculation of Coxsackie A9 virus.

1.10 REPLICATION OF VIRUSES IN THE CELL CULTURE

Infectious bovine rhinotracheitis virus (IBR) produced the highest infectivity titre (Table 1.3) and characteristic viral particles were demonstrated by electronmicroscopy (Fig. 1.18). The rotavirus (U.K. strain) replicated well and the complete particles, about 70 nm diameter with distinct hexagonal capsomers, were observed (Fig. 1.19). Some preparations showed complete particles with penetration of the strain to the inner capsomers (Fig. 1.20). The bovine enterovirus and poliovirus grew to about the same infectivity titre and characteristic small round particles were observed (Fig. 1.21).
TABLE 1.5

Infectivity titre of viruses grown and titrated in calf kidney diploid cell line.

<table>
<thead>
<tr>
<th>Virus</th>
<th>$10^{\text{g}<em>{10}} \text{ TCID}</em>{50}/\text{ml} $</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine rotavirus (U.K. strain)</td>
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<tr>
<td>Infectious bovine rhinotracheitis (Oxford strain)</td>
<td>8.4</td>
</tr>
<tr>
<td>Bovine enterovirus (MC strain)</td>
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<tr>
<td>Polio virus type 1 (Vaccine strain)</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Fig. 1.18 Electronmicrograph of Infectious bovine rhinotracheitis virus. 196,000 X

Fig. 1.21 Electronmicrograph of bovine enterovirus. 248,108 X
Fig. 1.19  Electronmicrograph of complete particle of bovine rotavirus with internal hexagonal subunit structure visible. 398,565 X

Fig. 1.20  Electronmicrograph of complete particles of bovine rotavirus with penetration of stain to internal structure. 324,000 X
DISCUSSION

1.11 CULTURAL CHARACTERISTICS

The cell growth pattern observed during development of the calf kidney diploid cell line was not quite like that of human diploid lung fibroblast as reported by Hayflick and Moorhead (1961), Hayflick (1965), and Jacobs et al. (1970) and foetal bovine lung diploid fibroblast by Mirchamy et al. (1977), Kalinina et al. (1979). These workers reported three distinct phases of growth. Phase I was simply the establishment of the cells with first subculture and was followed by a rapid luxuriant growth in Phase II. Finally, there was a protracted decline in growth rate evidenced by decreased mitotic rate until senescence of cell line. In the case of the calf kidney diploid cell line described in this chapter, the first phase included a decline phase between passages 5 and 10. The peculiarity of this stage was the development of actively growing cells amongst degenerative cells and such a phenomenon is sometimes assumed to be a sign of development of a transformed cell with heteroploid chromosomal number. Spontaneous malignant change of cells in culture, had been first observed by Gey (1956) and later by Hayflick (1961) but in the case of calf kidney diploid cell line reported here, although the cell transformation phenomenon was observed, the cells continued to retain their apparent diploid chromosomal character. It was only in the final degenerative phase that chromosomal abnormality was observed. The other two developmental stages were without exceptional features. The highest mitotic rate was at the 35th subculture but the final degeneration, so typical of diploid cell lines, was not obviously attributable to any deficiency of the medium, since the use of different media and sera failed to reverse the senescence. Similar studies and conclusions were made by Swim and Parker (1957), Hayflick and Moorhead (1961) in their study of human diploid cell. It was interesting to
note that on one occasion when calf kidney diploid cell culture was grown in another laboratory it also went into a phase of senescence at about passage 50 (Mr. T. Hall, Wellcome Research Laboratory, personal communication).

Aging or senescence in diploid fibroblast is probably a normal phenomenon of the cell, but there is no obvious correlation between mean maximum life span of donor and normal in vitro population doubling of the cells (Hayflick, 1976). Medvedev (1967) and Orgel (1972) proposed a hypothesis that, over a period of time, information in the information-process-system, represented by the transcription and translation of the genetic message in DNA to RNA and into enzyme and other protein molecules, might be increasingly subjected to error. Such error would give rise to faulty enzyme molecules and lead to a decline in the functional abilities of the cells. A second hypothesis proposed by Medvedev (1972) was that the repeated sequences are normally repressed but that, if an active gene is extensively damaged, it is replaced by one of the identical reserve genes. The redundancy of the DNA might therefore provide insurance against the system's inherent vulnerability to random molecular accidents, lengthening the time before a sufficient number of errors could accumulate to confound the genetic message. Ultimately, however, all the repeated genes would be used up, error would accumulate and physical deficiencies leading to age changes would arise. The third genetic hypothesis of aging proposes that age changes are simply a continuation of the normal genetic signals regulating the development of an animal from the moment of its conception until its sexual maturation. There may even be an 'aging gene' that slows or shuts down biochemical pathways in a sequesterial manner leading to the predictable expression of what are recognised as age changes. The genetically programmed events may differ in the time of their expression in different cell types. The root of aging might therefore result from deficiencies in a few key cell types whose rate of aging is the fastest and has
the greatest effects (Hayflick, 1980).

All these hypothesis of aging are applied equally to the demise of normal animal cells in tissue culture and aging of intact animals.

The method of karyology followed did not reveal details of autosomal abnormalities. However, it gave a good account of chromosomal number which was sufficient to identify the diploid chromosomal configuration. The number of chromosomes demonstrated in the calf kidney diploid cell line conformed with the findings of Yamanka (1977) and Logue (1978) who showed that there were 58 acrocentric autosomes and 2 submetacentric 'X' chromosomes. These observations are also in agreement with those of Melander (1959) and Monnier-Cambon (1964) that cattle autosomes are acrocentric and Jorge's (1974) observations that, cattle 'X' chromosomes are always submetacentric.

Although cattle chromosomes have been studied by G-banding (Schnedl and Czaker, 1974; Yamanka, 1977 and Logue, 1978) and Q-banding by Schnedl (1972), the order of arrangement has not yet been agreed upon.

1.12 SUSCEPTIBILITY TO VIRUSES

The development of cytopathic effects caused by rotavirus have been studied in various cell lines. Fernelius et al., (1972) were able to grow both strains of the Nebraska virus in a variety of cell culture and reported development of CPE in cultures of PK*-15, embryonic bovine trachea, lamb kidney and L-cells. However, Welch and Twiehaus (1973) using Nebraska isolate, reported that the virus grew only in bovine embryonic kidney. The U.K. strain of bovine rotavirus grew well in primary calf kidney cells

* PK = Porcine Kidney
(Woode et al., 1974) so it was not surprising that it also grew well in the calf kidney diploid cell line. Furthermore, it was interesting that its characteristic cytopathic effects were similar to those noted by other workers using the Nebraska strains (Welch and Twiehaus, 1973, Kurogi et al., 1976). The Northern Ireland strain of bovine rotavirus (McNulty et al., 1976a) produced a similar CPE as the U.K. strain and had been reported to grow in secondary bovine kidney monolayer and MDBK cells. However, it did not replicate in BHK-21, Vero and PK-15 beyond first passage (McNulty et al., 1978b). The development of intracytoplasmic eosinophilic inclusion bodies in culture infected with the U.K. strain were similar to those reported by McNulty et al., (1977) in secondary calf kidney cells and by Estes et al., (1979a) who studied SA11 virus in MA 104 cells.

The development of immunofluorescence reported in this chapter corresponds closely with that reported by McNulty et al., (1977) in their isolate and by Estes et al., (1979a) with SA11 virus.

The growth and CPE caused by infectious bovine rhinotracheitis virus were predictable and are in agreement with similar observations made by Stevens and Groman (1964) in MDBK cells. Similarly, the development of CPE in calf kidney diploid cell line by bovine enterovirus was expected and was in agreement with the observations of McFerran (1962), Pleva and Mesároš (1967) who infected primary bovine kidney cell cultures with the virus. The only slight difference was the relatively slower rate of growth of enterovirus in the calf kidney diploid cells and this may have been due to strain differences.

The results of studies on Poliovirus was quite interesting because generally this virus does not grow in non primate cells. It is not clear what was the mechanism
by which it occurred. The host range for piliovirus is apparently determined by a specific interaction between viral capsid proteins and host cell receptors (Holland and Hoyer, 1962). However, Holland et al., (1959) demonstrated that, poliovirus would replicate in non-primate cells when infectious viral RNA is introduced into them. Similarly, Ozaki (1978) reported growth of poliovirus in a non-primate (PS cell) cell after artificial adsorption.
CHAPTER 2

THE ENHANCEMENT OF VIRAL REPLICATION BY SALTS AND CHEMICALS

VIRUS ISOLATION
## CONTENTS OF CHAPTER 2

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>87</td>
</tr>
<tr>
<td>Material and methods</td>
<td>90</td>
</tr>
<tr>
<td>2.1 Cells</td>
<td>90</td>
</tr>
<tr>
<td>(i) Calf kidney diploid cell line</td>
<td>90</td>
</tr>
<tr>
<td>(ii) Human embryonic lung (HEL) cell line</td>
<td>90</td>
</tr>
<tr>
<td>(iii) Vero cell line</td>
<td>91</td>
</tr>
<tr>
<td>2.2 Stock virus.</td>
<td>91</td>
</tr>
<tr>
<td>2.3 Inoculation of Leighton's tube</td>
<td>92</td>
</tr>
<tr>
<td>2.4 Isolation of rotavirus from faeces</td>
<td>92</td>
</tr>
<tr>
<td>(a) Calf rotavirus</td>
<td>92</td>
</tr>
<tr>
<td>(b) Human rotavirus</td>
<td>94</td>
</tr>
<tr>
<td>Results</td>
<td>97</td>
</tr>
<tr>
<td>2.5 Enhancement of virus replication</td>
<td>97</td>
</tr>
<tr>
<td>(a) Effect of added salt during virus adsorption to the cells</td>
<td>97</td>
</tr>
<tr>
<td>(b) The effect of temperature on growth of rotavirus</td>
<td>100</td>
</tr>
<tr>
<td>(c) The effect of 5-iodo - 2-deoxyuridine (IUDR).</td>
<td>103</td>
</tr>
<tr>
<td>(d) The influence of hypotonic treatment of cells</td>
<td>103</td>
</tr>
<tr>
<td>(e) The effect of different growth medium on cells for virus growth.</td>
<td>105</td>
</tr>
<tr>
<td>2.6 Development of persistently infected cells</td>
<td>110</td>
</tr>
<tr>
<td>2.7 Isolation of rotavirus</td>
<td>113</td>
</tr>
<tr>
<td>(a) Calf rotavirus</td>
<td>113</td>
</tr>
<tr>
<td>(b) Human rotavirus</td>
<td>113</td>
</tr>
<tr>
<td>Discussion</td>
<td>119</td>
</tr>
</tbody>
</table>
INTRODUCTION

Malherbe and Strickland-Cholomley (1967) were the first to report the successful isolation of a rotavirus SA11 in primary monkey kidney cell culture from rectal washings of an apparently healthy Vervet monkey and a similar virus 'O' (Offal) agent from abattoir effluent of sheep. However, it was not until much later (Woode et al., 1976b. McNulty, 1978) that these two viruses were defined as rotaviruses. This was followed by the classical work of Mebus et al., (1969) who isolated two strains of virus, reported as Nebraska calf diarrhoea virus (NCDV). The viruses were first passaged in colostrum deprived calves and later successfully adapted to foetal bovine kidney culture (Mebus et al., 1971a).

The epizootic diarrhoea of infant mice (EDIM) which was first observed in various laboratories of U.S.A. in 1944 (Flewett and Woode, 1978) was thought to be of bacteriological origin. However, Adams and Kraft (1967) revealed the details of its viral pathogenicity in laboratory mice but it was not until 1971 that successful cultivation of virus in organ cultures of mouse ileum and caecum was achieved but the virus failed to grow in mouse fibroblast monolayer (Rubenstein et al., 1971).

Cell culture adaptation of other calf rotaviruses at that time was largely unsuccessful despite the presence of large numbers of virus particles in selected faecal specimen. However, serial cultivation of some of these viruses have been achieved in primary and secondary bovine kidney cell culture (Bridger and Woode, 1975, L'Haridon and Scherrer, 1976, McNulty et al., 1976a and Sato et al., 1978). The incorporation of trypsin in the cell culture maintenance medium or the treatment of the faecal suspension with trypsin before adsorption to the cells resulted in additional successes (Babiuk et al., 1977, Theil et al., 1977, Almeida et al., 1978b) with rotavirus isolation from faeces.
of both calves and pigs. Since then incorporation of trypsin in maintenance medium has resulted in successful isolation of other calf rotaviruses by workers in several parts of the world (Frey et al., 1979, Köves, 1979, Theodoridis et al., 1979). McNulty et al., (1979) also reported successful isolation of rotaviruses from Turkey and Chicken in chicken embryo liver cell culture by incorporation of trypsin, but they were unsuccessful in their attempt to isolate lamb (McNulty et al., 1976a) and pig (McNulty et al., 1978b) rotaviruses without incorporation of trypsin in the medium.

Since the discovery of human rotavirus in ultrathin sections of duodenal mucosa at Melbourne by Bishop et al., (1973) and simultaneous identification in faeces of babies at Birmingham by Flewett et al., (1974c) and in Toronto by Middleton et al., (1974) many attempts to isolate human rotaviruses in cell culture have been vigorously pursued. However, all efforts to isolate virus either in human embryonic gut organ culture (Wyatt et al., 1974) in monolayers of human embryonic gut (Purodham et al., 1975), in foetal bovine intestinal monolayer (Albrey and Murphy, 1976) or in human embryonic kidney cell culture (Wyatt et al., 1976a) have resulted at best only in incomplete replication, detected by immunofluorescence. Such infectivity was sometimes detected for a few passages using the infected virus suspensions to fresh monolayer. An improvement in the detection level was achieved by application of low speed centrifugation of faecal filtrate on to the cells (Banatvala et al., 1975) and the modification of this method to cell culture in microtitre system (Bryden et al., 1977) has become a diagnostic tool. However, Drozdov et al., (1979) finally succeeded in cultivating human rotavirus in primary green monkey cell culture by combining the use of trypsin and low speed centrifugation of faecal filtrate onto cells. But Wyatt et al., (1980) had to passage 11 times in gnotobiotic pigs before adaptation of human rotavirus type 2 to grow in
primary monkey kidney cells with incorporation of trypsin in maintenance medium.

Recently, Stuker et al., (1980) reported isolation of two new strains of simian rotavirus (S: U.S.A.: 78:1) and (S: U.S.A.: 79:2) in primary cynomolgus monkey kidney (CMK) cell culture from infant Rhesus monkeys with diarrhoea.

It is surprising that, primary isolation of rotavirus from faeces of monkey did not require any special treatment of either virus or cells for replication of virus. However, with much difficulty only a few strains have been isolated from animals and man with the use of trypsin.

The enhancement of virus replication requires addition of different chemical agents or physical treatment of cells either during adsorption of virus to the cells or their presence in the maintenance medium (Lonberg-Holm and Philipson, 1974) so, in the present work some of the chemical agents were added or cells were subjected to physical treatment to enhance rotavirus replication in the calf kidney cells.
MATERIAL AND METHODS

2.1 CELLS

The following cells were used in the study of growth conditions for replication of the virus.

(i) Calf Kidney diploid cell line

The stock cells were grown in 4 oz medical flat bottles in Hank's balance salt solution as base medium as described earlier (Methods in Chapter 1). The maintenance medium was in Earle's balance salt solution as base instead of Hank's salt solution. However, in studies of influence of basal medium on cell growth on virus replication, the cells were grown in Hank's as well as Earle's balance salt solution throughout that particular experiment.

The coverslip cultures in Leighton's tube were prepared by adding 2 ml of $2 \times 10^5$ cells/ml to each tube and the confluent cell sheet was formed in three days at $37^\circ\text{C}$.

(ii) Human embryonic lung (HEL) cell line

The HEL cell was received from Pfizer Inc. They were grown in 2 oz plastic bottles (Nuncoln) and the growth media consists of 10% (V/V) Eagle medium (Basal, Wellcome Reagents Ltd., 10 x), 10% (V/V) Foetal calf serum (Flow Laboratories Ltd.), 0.11% sodium bicarbonate (Wellcome Reagents Ltd.), 1% (V/V) Penicillin and Streptomycin (final concentration of 100 IU and 100\mu g/ml of medium). Sterile deionized water was added to make up to 100 ml of media. The maintenance medium was the same as the growth media except that serum was omitted from the media. The subculture of cells was carried out by a 1 : 2 split twice weekly as described previously (Methods in Chapter 1).
(iii) **Vero Cells** (Vervet monkey kidney cells)

Vero cells were grown in the medium consisting of 10% (V/V) Medium 199 (Wellcome Reagents Ltd. 10 X), 5% (V/V) Newborn calf serum (Flow Laboratories Ltd.), 0.11% (W/V) Sodium bicarbonate (Wellcome Reagents Ltd.), 1% (V/V) Penicillin and Streptomycin (Glaxo Laboratories Ltd.), at the final concentration of 100 IU and 100 µg/ml of medium and sterile deionised water was added to make up to 100 ml of medium. After three days of incubation of the culture at 37°C the growth medium was replaced by maintenance medium which consisted of all the components of the growth medium except the sodium bicarbonate concentration was raised to 0.22% (W/V) and the serum concentration was reduced to 2% (V/V). The cells were passaged by a 1 : 4 split once a week as described earlier (Method in Chapter 1).

The coverslip cultures were prepared in Leighton's tubes by adding 2 ml of 1 x 10^6 cell/ml to each tube and incubated at 37°C until confluency.

### 2.2 **STOCK ROTAVIRUS**

The growth medium of confluent culture of calf kidney diploid cells was changed to maintenance medium approximately 24 hours prior to virus inoculation. The medium was discarded and the cell sheet was washed twice with PBS. A 0.3 ml volume of virus suspension was adsorbed to the cells at 37°C for one hour with intermittent rocking of the bottle. The bottles were refed with maintenance medium and incubated at 37°C for 96 hours. The stock virus suspension was prepared by three cycles of freezing (-25°C) and thawing at room temperature (20°C) followed by low speed centrifugation (800 X g for 20 minutes) at 4°C. The virus suspension was passaged five times in fresh culture. The final virus suspension was distributed in 1 ml quantities in bijoux bottles and stores at vapour phase (-136°C) of liquid nitrogen. The stock virus was titrated as described
in Appendix VI. The titre of stock virus was \(4.7 \times 10^{10} \text{TCID}_{50}/\text{ml}\).

2.3 **INOCULATION OF LEIGHTON'S TUBE**

The confluent cover slip cultures in Leighton's tubes were selected, the cell sheet was washed twice in PBS and a 0.1 ml of virus suspension was adsorbed to the cell at 37°C for one hour with intermittent rocking of the tube. Then, 1.9 ml of maintenance medium was added to each tube and incubated at 37°C and, at 24 hour intervals, the infected tubes were removed and were stored at -25°C until termination of the experiment. The virus was harvested as described earlier and infectivity titration was carried out as described in Appendix VI.

2.4 **ISOLATION OF ROTAVIRUS FROM FAECES**

(a) **Calf Rota Virus**

Twelve specimens known to contain calf rotavirus by electronmicroscopy (Table 2.1) were received from Miss M.H. Lucas of the Central Veterinary Laboratory, Weybridge. They were processed as described below before being inoculated to calf kidney cells for virus isolation. One gram or 0.5 ml of faeces were stirred well in 4.5 ml of PBS pH 7.3 with a glass rod. The coarse particles were removed by low speed centrifugation (800 X g for 15 minutes) at 4°C. A 0.5 ml mixture of penicillin and streptomycin (final concentration of 1000 IU and 1000 μg/ml) was added to each supernate and incubated at 37°C water bath for 30 minutes, then centrifuged at (7,500 X g for 20 minutes) at 4°C to remove bacteria. The supernate fluid was distributed in 0.6 ml quantities in bijoux bottles and stored at -25°C until use.
### Table 2.1

**Calf Faecal Specimens**

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Register Number of sample/year:</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>551/77</td>
<td>Large intestinal content</td>
</tr>
<tr>
<td>2</td>
<td>1477/77</td>
<td>Faeces</td>
</tr>
<tr>
<td>3</td>
<td>1575/77</td>
<td>Large intestinal content</td>
</tr>
<tr>
<td>4</td>
<td>1753/77</td>
<td>Large intestinal content</td>
</tr>
<tr>
<td>5</td>
<td>1795/77</td>
<td>Faeces</td>
</tr>
<tr>
<td>6</td>
<td>1851/77</td>
<td>Large intestinal content</td>
</tr>
<tr>
<td>7</td>
<td>1952/77</td>
<td>Faeces</td>
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<tr>
<td>8</td>
<td>1955/77</td>
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</tr>
<tr>
<td>12</td>
<td>13128/77</td>
<td>Faeces</td>
</tr>
</tbody>
</table>
Inoculation and passaging of specimen

Twenty four confluent coverslip cultures were washed twice with PBS, a 0.2 ml of specimen was inoculated in duplicate and the suspension was adsorbed to the cell for 90 minutes at 37°C with intermittent rocking of the tube. The cell sheet was washed twice with PBS and a 2 ml of maintenance medium was added to each tube. They were then incubated at 37°C for three weeks or until destruction of the cell sheet, whichever was earlier.

The inoculated first passage material often developed cytopathic effects (CPE) at which time they were frozen at -25°C. If no CPE developed then at the end of three weeks of uneventful incubation the cultures were frozen at -25°C. The harvesting of the virus suspension and passaging was carried out as described earlier. The passaging was continued at intervals of three weeks for the first three passages and then at one week intervals for subsequent passages.

Immunofluorescence detection of viral isolate

The infected coverslip cultures from different samples and passages were fixed in cold acetone and immunofluorescence staining and identification was carried out as described in Chapter 1.

(b) Human Rotavirus

A faecal sample of yellowish watery consistency was kindly received from Prof. J.E. Banatvala, St. Thomas's Medical School, London and had been seen to contain rotavirus by electronmicroscopy. The sample was divided into four parts and was diluted as follows: (i) $\frac{1}{10}$ in borate KCl buffer (Appendix V) containing 0.01 M MgSO$_4$ $7H_2O$ (AR. B.D.H.) pH 8.4 and 10% lamb serum, (ii) as before
without lamb serum, (iii) $1/10$ in borate Kcl buffer containing $0.01 \text{ M MgSO}_4 7\text{H}_2\text{O}$ pH 7.9 with 10% foetal calf serum; (iv) as (iii) but without serum. All four samples were sonicated at 13 amplitude for 60 seconds (at about $-2^\circ\text{C}$). The samples were clarified by centrifugation ($7,500 \times \text{g}$ for 20 minutes) at $4^\circ\text{C}$ and the supernate was filter sterilised ($0.45/\mu\text{m}$ membrane filter, milipore Inc).

A 0.5 ml sample of sterile supernatant fluid (i) and (ii) was inoculated to confluent bottle cultures of calf kidney cells and a similar amount of sample (iii) and (iv) was inoculated to the HEL cells. The virus adsorption was carried out at $37^\circ\text{C}$ for 90 minutes with intermittent rocking of bottles. Appropriate maintenance medium was added and incubated at $37^\circ\text{C}$ along with uninfected controls.

Blind passaging of the infected material

The infected calf kidney culture was incubated for three days and HEL for five days after which the possible virus was recovered by three successive cycles of freezing ($-25^\circ\text{C}$) and thawing (room temperature) followed by sonication for 30 seconds and clarified by centrifugation ($1200 \times \text{g}$ for 15 minutes) at $4^\circ\text{C}$. A $1/10$th proportion of $0.1 \text{ M MgSO}_4 7\text{H}_2\text{O}$ in borate Kcl buffer pH 8.4 and 7.9 was added to the virus suspension. Controls were also treated similarly as above. The materials were inoculated into fresh cultures of the same cell type, an adsorption period was allowed for 90 minutes at $37^\circ\text{C}$ and after adding maintenance medium, the cultures were incubated at $37^\circ\text{C}$ for the period as described earlier. After the 5th passage, further passaging in the HEL cells was discontinued, whereas in the calf kidney cells it was continued up to the 17th passage.

Identification of virus by immunofluorescence

The 6th passage infected calf kidney cells were scrapped from the bottle and after a low speed centrifugation,
a portion of pellet was smeared on a glass slide, air dried and fixed with cold acetone for 15 minutes. After air drying thoroughly, the slides were stained for immunofluorescence identification of virus and examined for fluorescing cells as described earlier (Method in Chapter 1). Besides staining the calf antirotavirus sera (G 199), some slides were also stained with human antirotavirus sera (Pig III antihuman rotavirus sera raised in gnotobiotic pig at ARC Research Institute on Animal Diseases, Compton) and the fluorescin isothiocyanate (FITC) conjugated antihuman IgG (Nordic Immunological Laboratories) raised in pigs.

Infectivity of isolate to vero cells

Coverslip cultures of vero cells were prepared and inoculated with clarified cell culture fluid from infected calf kidney cultures as described earlier. After 48 hours the infected cultures were removed, fixed, stained and photographed as described in Appendix II and III.

Electronmicroscopy of Isolate

Concentration of infected cell culture fluid, preparation of specimen, examination of the virus was carried out as described (Method in Chapter 1). The photographic recording was made according to method described in Appendix III.
RESULTS

2.5 ENHANCEMENT OF VIRUS REPLICATION

The enhancing effect of virus replication by addition of salt, drug or physical shock was carried out only in calf kidney cells. The infectivity titre was determined as described in Appendix VI.

(a) Effects of added salt during adsorption of virus to cell

The results of this test are summarised in Table 2.2 and Fig. 2.1. The divalent salts solutions of MgSO\textsubscript{4} \(7\text{H}_2\text{O}\) (AR. B.D.H.), MgCl\textsubscript{2} \(6\text{H}_2\text{O}\) (AR. B.D.H.) and BaCl\textsubscript{2} \(2\text{H}_2\text{O}\) (AR. B.D.H.) were prepared in borate kcl buffer pH 8.4 (Appendix V) and were added to virus suspension (9 parts of virus suspension and 1 part of salt solution) so as to give 0.01M and 0.001M final concentration of added salt in the virus suspension during adsorption. A 0.11 ml of virus suspension was inoculated to each confluent monolayers of coverslip cultures of calf cells, adsorbed and incubated as described earlier.

The higher concentration of barium salt was toxic to the cells and the virus only replicated to a low titre never reaching more than 3.5 \(\log_{10}\) TCID\textsubscript{50}/ml. Much better replication occurred at a low concentration of barium salt with the titre of virus reaching 4.7 \(\log_{10}\) TCID\textsubscript{50}/ml within 24 hours of inoculation. After this time it inclined to a lower plateau. However, with both MgSO\textsubscript{4} and MgCl\textsubscript{2} the replication of virus was slow at first but reached the highest level by 96 hours. The development of cytopathic effects was most pronounced in the presence of BaCl\textsubscript{2} especially at higher concentration of the salt. By 24 hours 60% of cell sheet was destroyed at the higher concentration of BaCl\textsubscript{2} and virtually all cells were destroyed by 72 hours. The development of cytopathic effects were
TABLE 2.2. Effect of different salts on virus adsorption.

<table>
<thead>
<tr>
<th>SALT</th>
<th>Concentration of extra salt in adsorbing virus suspension</th>
<th>Time of incubation in hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>BaCl₂·2H₂O</td>
<td>0.01M</td>
<td>2.2 *</td>
</tr>
<tr>
<td></td>
<td>0.001M</td>
<td>4.7</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.01M</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>0.001M</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.01M</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>0.001M</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Log₁₀ TCID₅₀/ml
Fig. 2.1 THE EFFECT OF SALTS ON VIRUS REPLICATION

- **MgSO₄**
- **MgCl₂**
- **MnSO₄**
- **MnCl₂**
- **BaCl₂**

**Time of Incubation in Hours**

**Titer (Logₐ TCID₅₀/mL)**

- 0.01M
- 0.1M

---

*Note: Graph shows the replication of virus under different concentrations of salts.*
much slower when the cells were treated with either MgSO$_4$ or MgCl$_2$ and by 48 hours only 30% of the cells were affected.

(b) **The effect of temperature on growth of rotavirus**

The calf kidney cultures were grown in sterile tube cultures as described for preparation of coverslip cultures. The tubes were incubated at 37°C for three days in a 30° angle stationary culture. The tubes were inoculated as described for coverslip cultures and incubated in a water bath running at 33, 37, 38.5 and 40°C in an inclined position along with uninfected cells as controls.

The cells incubated at 33°C did not develop any characteristic cytopathic effects after 120 hours of inoculation except for a few rounded and sickle shaped cells floating in the medium. However, in the cultures incubated at 37, 38.5 and 40°C, the cytopathic effects were evident by 48 hours and almost all the cells became detached from the glass by 120 hours. The cytopathic effect was more severe in the cells incubated at 40°C than 37 or 38.5°C.

The infectivity titre of virus at the different incubation temperatures parallel the development of cytopathic effect (Table 2.3 and Fig. 2.2) except at 40°C. Although virus was probably released by 24 to 48 hours at 33°C, the titre was below the level of detection by the method used here for virus titration. The maximum virus titre was recorded by 72 hours at 33 and 40°C but not until 96 hours at 37 and 38.5°C. The replication of virus was more rapid at 38.5°C and at 24 hours by a factor of about 1.6 log$_{10}$ TCID$_{50}$/ml more than 37 or 40°C. After the maximum titre was reached at 37 and 38.5°C by 96 hours there was a subsequent drop in titre, whereas a plateau of replication was noted with cells incubated at 33 and 40°C after 72 hours.
TABLE 2.3  Effects of temperature on virus replication

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>33°C</td>
<td>ND</td>
</tr>
<tr>
<td>37°C</td>
<td>1.6</td>
</tr>
<tr>
<td>38.5°C</td>
<td>3.2</td>
</tr>
<tr>
<td>40°C</td>
<td>1.6</td>
</tr>
</tbody>
</table>

ND = Not detectable

* = $\log_{10} \text{TCLD}_{50}/\text{ml}$
Fig 2.2  THE EFFECT OF INCUBATION TEMPERATURE ON VIRUS REPLICATION.
(c) The effects of 5-iodo-2'-Deoxyuridine (IUdr)

The pretreatment of confluent cultures with drugs was done by adding 20, 50, 100 or 200μg/ml of IUdr* (Sigma, London Ltd.) in maintenance medium for either 24 or 72 hours at 37°C. Subsequently IUdr was removed by bathing the cells in PBS pH 7.3 for four hours, then virus was adsorbed to cells as described earlier.

Pretreatment with IUdr for 72 hours resulted in the destruction of the cell sheet by virus by 24 hours leaving only 5% of the cells attached to the glass surface. However, the virus only replicated to the low titre of 3.2 log₁₀ TCID₅₀/ml (Table 2.4). In those cells pre-incubated with IUdr for 24 hours, the concentration of IUdr and treatment of cells similar results were obtained but the development of cytopathic effect was slower so that it was not until 48 hours that 95% of the cells were destroyed, nevertheless, the replication of virus was ten times greater.

* A solution of IUdr was prepared by dissolving 1 mg of IUdr/ml of deionised water and filtered through 0.22μm membrane filter, Milipore Inc. and stored in an amber coloured bottle at 4°C.

(d) The influence of hypotonic treatment of cells

The confluent coverslip cultures were washed twice with PBS, one set of cultures were subjected to hypotonic (1 : 8 lamb serum in sterile deionised water) treatment for 5, 10, 30, 60 and 90 minutes and another set of cultures to different hypotonic solution (1 : 16 lamb serum in sterile deionised water) for 30, 60 and 90 minutes. At the end of each time, the hypotonic solution was discarded and the cell sheet was washed quickly in PBS (Ca²⁺ and Mg²⁺ free ions). The virus inoculation, harvesting and titration of virus was then carried out as described previously.
TABLE 2.4  Effects of IUdR on virus replication.

<table>
<thead>
<tr>
<th>Hours of pre-incubation with IUdR</th>
<th>Concentration of IUdR in μg/ml of media</th>
<th>Time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>24</td>
<td>20</td>
<td>4.2*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.4</td>
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<tr>
<td></td>
<td>100</td>
<td>4.2</td>
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<tr>
<td></td>
<td>200</td>
<td>4.2</td>
</tr>
<tr>
<td>72</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not Done

* = $\log_{10} \text{TCID}_{50}$/ml.
When the cells were exposed to the 1:8 hypotonic solution for 5 or 10 minutes before adsorption of the virus, the infectivity titre reached $4.7 \log_{10} \text{TCID}_{50}/\text{ml}$ within 24 hours and remained more or less at that level until the experiment was terminated at 96 hours. There was not much difference in virus replication when the cells were exposed for 30 minutes with hypotonic solution. However, as the pretreatment time of hypotonic solution increased to 60 or 90 minutes the infectivity titre of the virus in the cells exposed to the higher dilution (1:16) of hypotonic solution was much lower than the lower dilution (1:8) (Table 2.5).

(e) The effect of different growth medium on cells for virus growth

The cells were grown in media prepared with two different balance salt solutions: (i) Hank's balance salt solution, and (ii) Earle's balance salt solution as base media throughout the period of study. The other components of the media were the same as described in Chapter 1. The virus adsorption to confluent coverslip culture and harvesting was carried out as described earlier. The 24 hour infected coverslip cultures from the cells grown in Earle's base, 24 and 120 hours cultures from cells grown in Hank's base were fixed, stained with H & E (Appendix II) and photographed (Appendix III).

The development of CPE in the cells grown in medium with Hank's balance salt solution as a base medium was very slow (Fig. 2.3), thus after 24 hours there was no detectable effect (Fig. 2.4). However, by 96 hours, the cytopathic effect was so extensive that only a few cells remained (Fig. 2.5). The release of virus was also slow reaching its highest level at 96 hours after which there was a slight fall in virus yield (Fig. 2.3).
<table>
<thead>
<tr>
<th>Dilution serum in deionised water</th>
<th>Time (hours)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:8</td>
<td>Pre-treatment of cells in hypotonic solution in minutes</td>
<td>5</td>
<td>4.7*</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>4.7</td>
<td>4.7</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>4.7</td>
<td>3.7</td>
<td>3.7</td>
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<tr>
<td></td>
<td></td>
<td>90</td>
<td>2.7</td>
<td>3.5</td>
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<td></td>
<td>30</td>
<td>3.2</td>
<td>3.7</td>
<td>4.5</td>
</tr>
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<td></td>
<td></td>
<td>60</td>
<td>3.2</td>
<td>3.7</td>
<td>4.5</td>
</tr>
<tr>
<td>1:16</td>
<td></td>
<td>90</td>
<td>2.7</td>
<td>ND</td>
<td>2.5</td>
</tr>
</tbody>
</table>

ND = Not detectable

* = Log10 TCID50/ml.
Fig 2.3 THE EFFECT OF TWO DIFFERENT GROWTH MEDIA ON VIRUS REPLICATION.
Fig. 2.4 Uninfected calf kidney diploid cells.
Stained H & E. 291 X.

Fig. 2.4 Cells infected with bovine rotavirus after 24 hours. Stained H & E 291 X.
Fig. 2.5 Extensive cytopathic effect after 96 hours of infection with bovine rotavirus.
Stained H & E. 137 X
An entire different situation was observed, when the cells were grown in the medium containing Earle's base continuously for 5 or 6 passages, the development of cytopathic effect after virus inoculation was very quick and release of virus was detected as early as 4 hours and the maximum titre of virus was reached by about 20 hours (Fig. 2.3). The cytopathic effect was detected as early as 8 hours and only a few cells remained on the glass surface at 24 hours (Fig. 2.6).

### 2.6 DEVELOPMENT OF PERSISTENTLY INFECTED CELLS

The infection of confluent monolayers in 2 oz medical flats and harvesting of infected fluid was carried out as described earlier (Method in this chapter). From each passage level the infected cell culture fluid was collected and preserved at -25°C and whole cells were subcultured to new bottles as described in Chapter 1. However, from the 4th passage onwards both cell free and cell associated and plus cell free virus was collected and preserved at -25°C. The infectivity titre (Table 2.6) of cell free, or cell associated plus cell free virus at different passage levels was titrated (Method in Appendix VI).

For the first three passages the cytopathic effect was developed by 48 hours and at each subculture the cells became confluent by 48 hours. However, as the passage number increased the cells showed less and less cytopathic effect even on the 8th day of subculture but although there was no development of characteristic cytopathic effect, virus replication continued with the release of infectious virus to medium. The infectivity titre of cell free or cell associated plus cell free virus remained more or less the same throughout subculture of the cells. At the 10th passage, the cells died within 12 hours of subculture for unexplained reasons so further passaging was impossible.
<table>
<thead>
<tr>
<th>Passage Number</th>
<th>Cell Free Virus</th>
<th>Cell associated Cell + free virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.2*</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>3.2</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>3.7</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>3.2</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>3.2</td>
<td>4.2</td>
</tr>
<tr>
<td>6</td>
<td>3.2</td>
<td>4.2</td>
</tr>
<tr>
<td>7</td>
<td>3.2</td>
<td>4.5</td>
</tr>
<tr>
<td>8</td>
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<td>4.2</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>4.2</td>
</tr>
<tr>
<td>10</td>
<td>TOXIC</td>
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</tr>
</tbody>
</table>

ND = Not Done

* + Log₁₀ TCID₅₀/ml.
Fig. 2.6 Uninfected healthy calf kidney diploid cells. Stained H & E, Phase Contrast 219 X.

Fig. 2.6 Cells infected with bovine rotavirus, at 24 hours few remained cells were either rounded or elongated and swollen. Stained H & E, Phase contrast 219 X.
2.7 ISOLATION OF ROTAVIRUS

(a) Calf rotavirus

The results of the isolation of calf rotaviruses in calf kidney cells is summarised in Table 2.7. Samples 1 and 4 were toxic to the cells within 12 hours, so this material was discarded. Indeed, whenever culture samples developed signs of cytotoxicity within 24 hours, subsequent passaging was discontinued. Some samples showed mild cytopathic effects but not so specific as those seen in the earlier studies reported on the Compton strain of calf rotavirus infection in calf kidney cells (Chapter 1). However, seven samples were positive by immunofluorescence staining in the first passage, although after the third passage only three samples remained positive by immunofluorescence and this positive effect continued until the 7th passage after which further replication could not be detected by immunofluorescence. Subsequent passaging of these cultures was discontinued. In each passage only a few cells showed intracytoplasmic fluorescence and without spread of infectivity to other cells.

(b) Human Rotavirus

The HEL cells did not apparently support the replication of virus for the first five passages. But from the fourth passage onwards an agent was isolated which grew to a titre of 6.3 $\log_{10}$ TCID$_{50}$/ml in calf kidney cells. By electron-microscopy the structure was indistinguishable from that of a rotavirus (Fig. 2.7). However, no immunofluorescence in the cell was detected by use of either human or bovine rotavirus antisera. (Neutralisation of the virus against reoviruses was not carried out).

A cytopathic effect developed within 42 hours of infection of the calf kidney cells. The infected cells were elongated and some cells had piknotic nuclei, while
### TABLE 2.7 Serial passaging of the rotavirus in the calf kidney cells.

<table>
<thead>
<tr>
<th>Passage 1</th>
<th>Passage 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours of post inoculation</td>
<td>Hours of post inoculation</td>
</tr>
<tr>
<td>12 39 60 115</td>
<td>18 61 112 160</td>
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<tr>
<td>179 203 229 253</td>
<td>184 206 233 282</td>
</tr>
<tr>
<td>301 325 349</td>
<td>330 368 392</td>
</tr>
<tr>
<td>373 391 444 468</td>
<td>419 439 463 507</td>
</tr>
</tbody>
</table>

**Toxic Passage discontinued**

- + + + + + + + + + + + + + + + + + + + + + + Frozen
- + + + + + + + + + + + + + + + + + + + + + + Frozen
- + + + + + + + + + + + + + + + + + + + + + + Frozen
- + + + + + + + + + + + + + + + + + + + + + + Frozen
- + + + + + + + + + + + + + + + + + + + + + + Frozen
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**IF-VE**

- + + + + + + + + + + + + + + + + + + + + + + Frozen
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<table>
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<th>Passage 3</th>
<th>Passage 4</th>
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<td>Hours of post inoculation</td>
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<td>185 212 233 257 302 350 401 473 502</td>
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<td>- + + + + + + + + + + + + + + + + + + + + + + Frozen</td>
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<td>- + + + + + + + + + + + + + + + + + + + + + + Frozen</td>
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<td></td>
</tr>
</tbody>
</table>

**IF-VE**

- + + + + + + + + + + + + + + + + + + + + + + Frozen
- + + + + + + + + + + + + + + + + + + + + + + Frozen
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**FROZEN**

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**IF+VE**

- + + + + + + + + + + + + + + + + + + + + + + Frozen
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- + + + + + + + + + + + + + + + + + + + + + + Frozen

**Passage discontinued**

- + + + + + + + + + + + + + + + + + + + + + + Frozen
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- + + + + + + + + + + + + + + + + + + + + + + Frozen

+ = Immunofluorescence +VE for rotavirus
- = Immunofluorescence -VE
++ = Cytopathic effect seen in 50% cells.
+++ = Cytopathic effect seen in 75% cells.
Fig. 2.7 Electronmicrograph of human reovirus-like agent.
151,000 X.
others were rounded and seen floating in the medium (Fig. 2.8). When the virus was inoculated to vero cells areas of infected cells were observed. Some cells were rounded and others were seen floating in the medium (Fig. 2.9).
Fig. 2.8 Live, uninfected calf kidney diploid cells. 176 X

Fig. 2.8 Cells infected with human reovirus-like agent. At 48 hours, highly elongated cells (G) and some rounded cells (H) floating in the medium. Live. 176 X
Fig. 2.9 Uninfected live Vero cells
176 X

Fig. 2.9 Vero cells infected with human reovirus-like agent. At 48 hours areas of infected rounded cells (arrow) free floating in the medium.
Live, 176 X.
2.8 DISCUSSION

Some explanation is required of the behaviour of rotavirus in cell culture, especially for instance, the great difficulty experienced in their replication. It may be instructive to consider the general evidence for factors influencing viral replication and the interaction of viruses and cells, before considering the problems of rotavirus.

The inhibition of host cell protein synthesis in cells infected with viruses is a phenomenon that has aroused interest for sometime (Martin and Kerr, 1968). Carrasco and Smith (1976) and Carrasco (1977) suggested that after virus infection, viral proteins were able to alter the gradient of monovalent ions maintained by the cell membrane by increasing the concentration of sodium ions in the cytoplasm and causing inhibition of initiation of cellular protein synthesis. This new environment would facilitate translation of viral messenger RNA (m-RNA) because there would not be any competition from host m-RNA and thus the binding of viral RNA would be favoured by an increase in monovalent ion concentration. However, Cusack and Morser (1979) as a result of their study of alphavirus in mouse myeloma cells thought it is unlikely that the cut off of host cell protein synthesis was caused by an influx of monovalent cations, but other groups (Durham and Butler, 1975; Durham, 1977; Durham et al., 1977; Durham, 1978) emphasized the role of Ca$^{2+}$ in disassembly of plant viruses and it was generally suggested that, there are calcium binding sites present in most, perhaps all, plant viruses and the evolutionary purpose of these sites is to bind calcium.

The role of Ca$^{2+}$ in cell activation has been reviewed extensively (Gomperts, 1976; Whitfield et al., 1976; Rebhun, 1977). The ionic changes cause transformed phenotypes in the cells particularly an elevation of Ca$^{2+}$ concentration in
the cytoplasm (Dulbecco and Klkington, 1975; Whitfield et al., 1976 and Gupta et al., 1979) and this phenomenon can be explained due to increase RNA synthesis. Calcium apparently acts in a special way, since this effect cannot be achieved with Sr\(^{2+}\), Ba\(^{2+}\), Mg\(^{2+}\) or Mn\(^{2+}\) (Dulbecco and Klkington, 1975). Calcium is known to be necessary for the stability of the cell membrane (Carafoli, 1975) and is instrumental in the association of hydrophilic (extrinsic) protein with the membrane (Carafoli and Crompton, 1978).

With regard to the attempts at the isolation of rotavirus from faeces, it is known that the infected faeces contain very large numbers of rotavirus particles, yet this adaptation to grow in serial passages in tissue culture has proved to be extremely difficult (McNulty, 1978). However, there have been several reports of evidence for the synthesis of viral antigens for a few passages only as detected by immunofluorescence (Wyatt et al., 1974; Purdham et al., 1975; McNulty et al., 1976b, McNulty et al., 1976c and Wyatt et al., 1976a). Electronmicroscopic studies of such cells revealed the appearance of large numbers of coreless particles which implied that incomplete replication had occurred (McNulty et al., 1976c; Esparza et al., 1980). If such coreless particles are indeed defective particles the difficulty in adapting rotaviruses to serial passages in cell culture may be due to not only lack of appropriate receptors for entry of the virus but also to nonpermissiveness of the cells to subsequent events in virus replication (McNulty, 1978). Fully infectious rotavirus particles require free Ca\(^{2+}\) ion for the activation of RNA polymerase and this effect cannot be achieved with Mg\(^{2+}\) or other di or monovalent cations (Cohen et al., 1979) and it was interesting in the present study, that there were enhanced cytopathic effects with an increase in virus yield when the calf rotavirus (U.K. strain) was grown in the presence of high rather than in low Ca\(^{2+}\) concentration in the medium, that is in Earle's rather than Hank's balance salt solution as base medium. Similarly, when the virus was grown in cells
supported by Hank's salt base virus replication was slow and the cell sheet was not destroyed until after five days, whereas when the cells were grown in Earle's base medium throughout the experiment, the virus replicated to a high titre and 90% of the cell sheet was destroyed within 24 hours, and it is suggested that the difference in the concentration of CaCl₂ in Hank's and Earle's base medium may have accounted for improved cytopathic effect and growth of the virus.

In addition to the role of the Ca²⁺ ion in the structural integrity of the cells, it is uniquely important in the regulation of cell function, since it acts as the messenger that co-ordinates a multiplicity of intracellular reactions. Indeed the number of processes known to be affected by changes in the ambient concentration of ionised Ca²⁺ grows continuously (Carafoli and Crompton, 1978). The uptake of Ca²⁺ by the tissue is prompted by a decrease in Na⁺ concentration of the medium or by an increase content in the tissue (Cooke and Robinson, 1971; Stahl and Swanson, 1972). For instance in barnacle muscle fibres, the influx of Ca²⁺ is stimulated by an increase in the Na⁺ in/Na⁺ out ratio and is accompanied by Na⁺ efflux (DiPolo, 1973). Thus in the present work, when the virus was adsorbed to the cells after treatment of hypotonic solution for 5 or 10 minutes, the influx of water could well have resulted in efflux of some Na⁺ to the medium to maintain the balance of the osmotic pressure and during virus adsorption the Ca²⁺ from virus suspension medium might have entered the cytoplasm due to an increased permeability of the membrane to Ca²⁺ in exchange for sodium. However, when the cells were exposed for 30, 60 and 90 minutes to hypotonic solution the influx of Ca²⁺ from virus suspension was less because more Na⁺ leaked out of the cell during prolonged exposure to hypotonic solution so Na⁺ was not available for exchange with Ca²⁺, and this was correlated with slower replication of the virus. It should be noted here that serum, which was also present,
is known to stimulate DNA synthesis in resting cells, but the increased replication of virus observed here cannot be attributed to the effect of serum on the cells because such stimulation of DNA synthesis described for chicken embryo fibroblasts required interaction of serum with the cells over a period of several hours (Temin, 1971).

The observations made in the present work lead, therefore, to the following explanations. When the cells were grown in Hank's salt base, a high Na\(^+\) and low Ca\(^{2+}\) concentration medium, the uptake of Ca\(^{2+}\) by cell organelles was low leading to low RNA synthesis and low free ionised Ca\(^{2+}\) content at the cytoplasm. But, if 24 hours prior to infection, the medium was changed to Earle's base (High Ca\(^{2+}\) and low Na\(^+\) concentration) the high concentration of intracellular Na\(^+\) facilitated the influx of Ca\(^{2+}\) from extracellular medium to the cytoplasm in exchange for Na\(^+\). This Ca\(^{2+}\) appears to move across the inner membrane in response to its electrochemical gradients (Baker et al., 1971) and it is estimated that, when extracellular fluid has a Ca\(^{2+}\) concentration of 1mM, the intracytoplasmic free ionic Ca\(^{2+}\) concentration is about 1μM (Baker et al., 1971; Bygrave, 1977). A level of ionic Ca\(^{2+}\) much above this value is deleterious to the normal metabolic operations of the cells (Coleman and Terepka, 1972; Kimmich and Rasmussen, 1969). This places an important constraint on the transcellular transport of bulk quantities of Ca\(^{2+}\). Any excess Ca\(^{2+}\) ion in cytoplasm must be sequestered by cytoplasmic organelles like mitochondria, endoplasmic reticulum, microsomes and such sequestered concentration of Ca\(^{2+}\) might appear as discrete localisation (Warner and Coleman, 1975). The rough endoplasmic reticulum which is studded with ribosomes is distributed throughout the cytoplasm and these ribosomal sites are the site of protein synthesis (Madge, 1975) so a hypothesis for rotavirus replication can be that the attachment of rotavirus is enhanced by divalent cations at higher pH during adsorption. Penetration of the cell is then by pinocytosis and the virus is uncoated by passing
through electrochemical gradients across the membrane but the actual mechanism of this is not known. After uncoating, viral RNA polymerase is activated by free ionic \( Ca^{2+} \) of cytoplasm and viral synthesis takes place at the rough endoplasmic reticulum any other place in the cytoplasm wherever ribosomes are present. These hypothetical situations can be correlated with electronmicroscopic observations of the presence of rotavirus in the rough endoplasmic reticulum in various cell lines (Stair et al., 1973; Hall et al., 1976; Chasey, 1977; Theil et al., 1977; Altenburg et al., 1980; Esparza et al., 1980). It is also worth noting that it has been reported that \( Ca^{2+} \) is required for assembly, maturation and liberation of several bacterial (Snipes et al., 1974; Takai, 1966) and mammalian viruses Matsumura and Yamashita, 1978). So, in this study rotavirus replication in low \( Ca^{2+} \) medium was slow, as less ribosomes were available for rotavirus protein synthesis and less free ionic \( Ca^{2+} \) for activation of RNA polymerase was available than in high \( Ca^{2+} \) containing medium. What is not yet clear is the role of \( Ca^{2+} \) ion is the maturation and liberation of rotaviruses from the cell.

In relation to these ideas about calcium and rotavirus replication are those observations on the role of trypsin. Enhancement of infectivity of rotaviruses have been seen by incorporation of trypsin to the medium (Babiuk et al., 1977; Almeida et al., 1978b; Clark et al., 1979). A similar enhancing effect of the proteolytic enzyme on reoviruses replication was recorded earlier (Spendlove and Schaffer, 1965). The proteolytic enzymes may remove or alter the viral capsid structure which may lead to enhancement of viral infectivity (Spendlove et al., 1970). However, incorporation of trypsin is not necessary for the plaque assay of U.K. strain of calf rotavirus (Wyatt et al., 1978a; Naik and Butler, 1979). Furthermore, the U.S.A. strain of calf rotavirus formed plaques in MA 104 cells as efficiently in presence of trypsin as in its absence provided the concentration of diethylaminoethyl dextran (DEAE-dextran)
was increased to $300\mu g/ml$ (Matsuno and Mukoyama, 1979) and trypsin had little or no effect on the infectivity of tissue culture adapted Nebraska calf diarrhoea virus (NCDV) or SA11 (Schoub and Bertran, 1978; Schoub et al., 1979). The presence of trypsin ($10^{-6} M$) in the medium makes the cell membrane permeable to extracellular divalent cations, particularly $\text{Ca}^{2+}$ ions (Loewenstein, 1967; Loewenstein et al., 1967) so it is conceivable that trypsin has two complimentary functions, one on assisting viral uncoating, the other on facilitating $\text{Ca}^{2+}$ uptake by cells which facilitate viral replication.

It has been reported that when clarified human faeces containing rotavirus was centrifugated onto cells, limited replication of viral antigen can be detected by immunofluorescence without release of virus to medium (Banatvala et al., 1975; Thouless et al., 1977). It has been suggested that the centrifugation alters the properties of the cell surface permitting viral uptake perhaps by a mechanism similar to trypsin (Almeida et al., 1978b). However, trypsin does not apparently bring about any structural changes to cells (Matsuno and Mukoyama, 1979) so it is suggested that the centrifugal force brings about membrane permeability to the divalent cations, particularly to $\text{Ca}^{2+}$ ions, thereby increasing the level of free $\text{Ca}^{2+}$ ion in the cytoplasm which activates the viral genome so that limited replication of rotavirus can take place. But enough ribosomes are not available at the endoplasmic reticulum for replication and maturation of rotavirus, so there is no release of virus to medium. It has also been observed that, if cells are infected sometime after centrifugation the cells remain uninfected. The explanation for this phenomenon is that $\text{Ca}^{2+}$ pulses are generally spontaneous and reversible (Rose and Lowenstein, 1975), so after centrifugation $\text{Ca}^{2+}$ will be pumped out of the cells to maintain the original equilibrium. During centrifugation, although the presence of ionised $\text{Ca}^{2+}$ activated the viral genome, the sequestered $\text{Ca}^{2+}$ to endoplasmic reticulum
reversed back to the extracellular fluid, so Ca\(^{2+}\) was not available for maturation and release of rotavirus.

An analogous situation exists at the intestinal wall of babies and young animals. Ca\(^{2+}\) is absorbed at the intestinal mucosa against an electrochemical gradient by active transport is energy dependent. Vitamin D synthesizes a specific calcium binding protein (CaBP) at the intestinal goblet cells and at the absorptive surface of epithelial cells, which acts as an microvillar Ca\(^{2+}\) concentrator by sequestering Ca\(^{2+}\) from the lumen (Taylor and Wasserman, 1970 and 1974). These suggestions have been correlated by the presence of a high concentration Ca\(^{2+}\) in the goblet cells by electron probe analysis of intestinal mucosa (Warner and Coleman, 1975). The border microvilli facing the lumen is lined with acid mucopolysaccharide material which carries a net negative charge at the pH of the intestine, therefore Ca\(^{2+}\) should collect on this coat by exchange and adsorption reaction prior to absorption (Wasserman, 1968; Forstner et al., 1974).

The most rapid rate of Ca\(^{2+}\) transport occurs in the dudenum, followed by the jejunum and ileum (Schachter and Rosen, 1959; Harrison and Harrison, 1969) but Vitamin D also improves intestinal Ca\(^{2+}\) absorption even in the colon (Harrison and Harrison, 1969). Because of the time during which Ca\(^{2+}\) is subjected to absorption, it seems evident that the distal portion of small intestine is primarily responsible for bulk of the intestinal Ca\(^{2+}\) absorption. Estimation have shown that in rats and dogs most Ca\(^{2+}\) is absorbed from the ileum, followed, in order, by the jejunum (Hurwitz and Bar, 1966). Calcium absorption across the intestine in the young chick and human is much greater than in the adult (Wasserman and Taylor, 1968; Ireland and Fordtran, 1973).

In studies of rotavirus infection by examination of intestinal sections of animals, killed sequentially after
infection, fluorescence of the cells can be seen at the tip of the duodenum, followed by a severe infection of jejunum and ileum and finally less severe damage of the colon (Mebus et al., 1973b; Stair et al., 1973; Hall et al., 1976, Chasey, 1977). These observations clearly correlate with the absorptive pattern of Ca\(^{2+}\) at the intestinal mucosa. This pattern is that Ca\(^{2+}\) is absorbed and transported to the circulation more rapidly from the duodenum. This means its concentration of Ca\(^{2+}\) in the cytoplasm of these epithelial cells is much lower than that in the cells of the jejunum and ileum. In these two regions of intestine the bulk of the Ca\(^{2+}\) is absorbed leading to a higher intracellular concentration. Thus, the higher the concentration of cytoplasmic Ca\(^{2+}\) the more severe is the infection of the cells.

Replacement of enterocytes or absorptive cells takes place every 1.4 to 2.8 days, depending upon the species of animals (Wilson, 1962). The infected cells lost to the lumen are replaced by cuboidal and squamous type of cells with irregular microvilli (Stair et al., 1973; Hall et al., 1976). When infected calves were reinoculated with a virulent strain of calf rotavirus, 24 hours after diarrhoea had ceased, the enterocytes remained normal (Mebus et al., 1971b) so, it is thought that after diarrhoea, there was removal of the mucopolysaccharide which was lining microvilli, followed by a reduction in retention time of food in the lumen and a lack of synthesis of CaBP as vitamin D was excreted. All these factors contributed towards a lack of Ca\(^{2+}\) absorption at the absorptive surface consequently, there would be a low Ca\(^{2+}\) concentration in the cytoplasm of newly generated enterocytes. It has also been suggested that IgA may play an inhibitory role but it is unlikely that, within 24 hours of infection there is synthesis of secretory IgA, so, one can draw the inference that, due to the low concentration of ionised Ca\(^{2+}\) in the cytoplasm newly inoculated rotavirus is unable to replicate.
In a study Graham and Estes (1980) tried to enumerate the mechanism(s) of proteolytic enhancement of rotavirus (SA11) infectivity in MA-104 cells and reported that trypsin acts extracellular environment by converting non-infectious particles to infectious particles. In their study, they found that trypsin acts early in replicative cycle (between 0 - 3 hours) after adsorption of virus onto the cells but not after 4 hours and again at 10 hours after infection. They also could not enhance virus replication after treating the cells with trypsin for 5 minutes and washing the cell sheet with diluent before infecting with virus. The explanations for these phenomenons is that, as reported elsewhere (in this Chapter) when trypsin added to the maintenance medium (between 0 - 3 hours) after virus adsorption, the cell membrane was permeable to Ca$^{2+}$ from the medium to the cytoplasm and the higher concentration of intracytoplasmic free ionised Ca$^{2+}$ activated the RNA-dependent RNA polymerase so virus replicated to produce more infectious particles. However, addition of trypsin after 4 hours of infection did not help activation of viral enzymes as by that time uncoating and activation process might have been completed. On the other hand, when trypsin was added at 10 hours after virus adsorption, the polymerase enzyme of newly generated virus might have been activated by higher concentration of ionised Ca$^{2+}$ in the cytoplasm. As noted elsewhere (in this Chapter) the Ca$^{2+}$ pulses are transitory, once trypsin was removed and cell sheet was washed, the higher intracytoplasmic Ca$^{2+}$ might have reversed back to the medium in order to maintain equilibrium so when the cells were infected with virus after washing, higher intracytoplasmic concentration of Ca$^{2+}$ was not available for activation of viral enzymes.

It has been reported that proteases do not bring about structural changes of rotaviral polypeptides or cell surface (Matsuno and Mukoyama, 1979). However, it has been reported that when small concentration (3μg/ml) of trypsin are used on untransformed interphase cells, only a
few distinct surface protein changes are seen but quite strikingly a whole set of surface associated changes developed in transformed cells, such as density inhibited cultures of chick embryo fibroblast (Sefton and Rubin, 1970), mouse 3T3 cells in certain conditions (Burger, 1970) and serum depleted human fibroblast (Pohjanpelto, 1976) initiate another round of cell division. It has also been reported that confluent monolayers of mouse cell line Balb/C 3T3 (Dulbecco & Klkington, 1975) and Chinese hamster kidney (CHO) cell line (Gupta et al., 1979) can be induced to proliferate by addition of extra Ca$^{2+}$ in the medium. So it is conceivable that trypsin permeates Ca$^{2+}$ to the cytoplasm to initiate resting cell to proliferate.

Almeida et al., (1978b) reported that in the presence of the trypsin in the medium the replicative cycle of rotavirus was shorter (48 hours) and infectivity titer was higher than absence of it in primary calf kidney cells. Similar observations have been made in the preliminary study in the present work, when the cells were grown in Hank's balance salt solution as base medium and then infected with virus, in presence of 5μg/ml of trypsin in the maintenance medium, the virus grew to a maximum infectivity titer by 48 hours with development of extensive cytopathic effect. However, when the cells were grown only in Earle's balance salt solution as base medium, virus replicative cycle was shorter (20 hours) and virus yield was higher in absence of trypsin in the medium.

Another interesting observation has been made in the present work, when the cells were grown only in the medium with Hank's balance salt solution as base, the cell growth was much slower than with Earle's balance salt solution as base medium. So higher replication of the virus was not only due to presence of higher concentration of Ca$^{2+}$ in the cytoplasm but also due to faster rate of replication of the cell itself. In relation to this observation, it is known that polyomavirus replicated to a maximum titer when Balb-C-3T3 cells infected at or near the beginning of G1 stage of cell cycle (Thorne, 1973) and also freshly trypsinized cells
are more susceptible to rotavirus infection than resting cells (McNulty et al., 1976b; Woode et al., 1976b). So faster rate of replication of the cells to higher yield of virus can be correlated to the faster rate (2.4 – 2.8 days) of turnover of intestinal absorptive cells in vivo.

Success in the isolation of rotavirus is limited to the isolation from calf and pig faeces usually with incorporation of trypsin (Babiuk et al., 1977; Theil et al., 1977; Almeida et al., 1978b; Theodoridis et al., 1979). However, after the early unsuccessful attempts to isolate human rotavirus (Wyatt et al., 1974; Purdham et al., 1975; Wyatt et al., 1976a), Wyatt et al., (1980) finally succeeded in isolating human rotavirus after adaptation of the virus to pigs. Drozdov et al., (1979) were successful by a combination of centrifugation and trypsin treatment. However, an examination of the literature with regards to the contribution of the growth medium used for cell cultivation, it is concluded that, when isolation attempts were made in cells grown in Hank's base (low Ca$^{2+}$ concentration) medium there was rarely any virus isolation but when the cells were grown in Earle's base (High Ca$^{2+}$ concentration) a varying degree of success had been achieved provided there was also incorporation of trypsin (Babiuk et al., 1977; Almeida et al., 1978b, Theodoridis et al., 1979). So, in the present study when virus isolation was attempted in calf kidney diploid cell line grown in Hank's base medium without incorporation of trypsin, there was only limited replication of virus.

Another interesting and probably relevant observation is the influence of diet on susceptibility of babies to rotavirus infection. Cows milk which is commonly used contains four times more Ca$^{2+}$ and five times more Vitamin D than human milk (Darke, 1976), so intestinal Ca$^{2+}$ content
of babies fed on cow's milk will be greater than those of breast fed. So, it is interesting that, rotavirus infection in babies fed on cow's milk are more severe than in those which are breast fed and this is not apparently related to the presence of IgA in the human milk, as secretory IgA in milk does not afford protection against rotavirus infection (Totterdell et al., 1980). An incidental comment on this is that, it is not at all surprising that, adaptation of human rotavirus type 2 occurred in gnotobiotic pigs whose intestinal Ca\(^{2+}\) concentration was higher and certainly higher than in the growth medium used for growing cells to which the virus would not adapt. However, there is the probability that the virus might have mutated some way so as to grow in low Ca\(^{2+}\) concentration of Earle's base medium of primary African green monkey kidney (AGMK) cells with incorporation of trypsin.

It has been suggested that interferon or other cell product may be responsible for the inhibition of virus replication (Almeida et al., 1978b). In this connection it has been reported that production of interferon can be overcome by treating the cells with 5-iodo-2'-deoxyuridine (IUdR, Holmes et al., 1964). It has also been reported that, IUdR and 5-bromo-deoxyuridine (BUdR) are capable of activating the synthesis of both RNA and DNA containing viruses. (Lowy et al., 1971; Gerber, 1972; St. Jeor and Rapp, 1973). However, in the present study pretreatment of cells with IUdR resulted in reduction in virus titre probably due to nonavailability of enzymes necessary for virus replication.

The divalent cations used to increase attachment of virus to the cell resulted in a slight increase in attachment rate as the rate of attachment is dependent on optimum pH during adsorption (Tolmach and Puck, 1952; Tolmach, 1957). When the divalent cations in borate KCl buffer pH 8.4 was added to virus suspension the pH of suspension did not increase, so probably optimum pH was not achieved for
maximum attachment of virus to cell. There is no obvious explanation for the sudden increase in virus titre when 0.001M BaCl₂ was incorporated to virus suspension during adsorption.

The optimum temperature for replication of rotavirus in calf kidney cells was 38.5°C, however at the higher temperature of (40°C) incubation might have resulted in inhibition of cellular macromolecular synthesis so virus replicated to a low titre, although there was development of extensive cytopathic effect. Estes et al., (1979b) reported that SA11 infected MA104 cells grew to a similar titre irrespective of temperature of incubation whether at 34, 37 or 39°C. This might be due to adaptability of cells to grow at different temperatures.

Persistantly infected cells continued to release virus with decreased cytopathic effect on the cells as the subculture number increased. The cells were grown in Hank's base medium throughout this experiment and were subjected to trypsinization at each subculture. Since the cells were rounded after each trypsinization and there was no chance of Ca²⁺ entering the cells as versene and trypsin solution was free of any Ca²⁺ or Mg²⁺ ions, so the question of any effect of trypsin on virus probably does not arise. Since the concentration of intracytoplasmic Ca²⁺ was low, it might be expected that replication of the virus and development of cytopathic effect would also be slow.

Finally, it is reported that, primary and secondary cells in culture cannot maintain their DNA synthesis in Ca²⁺ deficient medium where as transformed cells lines are able to maintain (Swierenga and Whitfield, 1978) so it can be concluded that, human rotavirus isolation should be tried in primary cells which have a ten times more Ca²⁺ concentration than transformed cell lines (Swierenga and Whitfield,
(1978) and that any study should be carried out with a higher concentration of Ca$^{2+}$ in the medium than is usually present in the commercially available medium. The attachment of virus to the cell may be enhanced by adding higher concentration of Mg$^{2+}$ ion and the pH of virus suspension during adsorption should be around 8.0 for optimum activation of viral enzymes.
CHAPTER 3

DEVELOPMENT OF A PLAQUE ASSAY AND DIFFERENTIATION OF

ROTAVIRUSES
## CONTENTS OF CHAPTER 3

### 3.1 Introduction  
**Materials and methods**  

### 3.2 Cells  

### 3.3 Virus  
(i) Bovine rotavirus (U.K. strain)  
(ii) Bovine rotavirus (Northern Ireland strain)  

### 3.4 Rotavirus antisera  

### 3.5 (A) Development of plaque assay in the calf kidney cells  
(B) The use of LLCMk₂, BGM and Vero cell cultures for the plaque test  

### 3.6 The thermal stability test  

### 3.7 The neutralisation tests  

### Results  

### 3.8 Development and standardisation of the plaque assay  
(A) Factors influencing plaque formation  
(1) The cells  
(2) The growth medium of the cells  
(3) The cell seeding rate  
(4) Overlay medium  
(a) Agar  
(b) Serum and trypsin  
(c) DEAE-dextran  
(d) Neutral red  
(5) Effect of pH  
(6) Effect of temperature
(7) Incubation time 149
(8) Adsorption period and salt concentrations 149
(9) Inoculum size 154
(B) Applications of the plaque test 154
(1) Plaque formation by two calf rotavirus strains 154
(2) Comparison of plaque and microtiter assay 154
(3) Thermal stability of the U.K. strain 156
(4) Neutralisation kinetics. 156

Discussion
INTRODUCTION

3.1 Welch and Twiehaus (1973) were first to report on plaque formation by a bovine rotavirus (Neonatal calf diarrhoea virus, NCDV) in primary bovine embryonic kidney cells and found that sometimes the virus produced minute plaques (0.5 mm diameter) which were only visualised by light microscopy. They used an overlay media which contained either 0.9% Ionagar or 1.5% methyl cellulose along with DEAE - cellulose or DEAE-dextran and they also incorporated 5% foetal bovine serum to prevent degeneration of the cells.

Much better plaque production was reported four years later by Matsuno et al., (1977b) in Neonatal calf diarrhoea virus (NCDV) in monkey kidney cells (MA-104). They noted the necessity for special overlay conditions, for instance the need for incorporation of DEAE-dextran and trypsin at a particular concentration, along with 0.8% purified agar (Difco). A second similar overlay was required for the effective visualisation of the plaques, this contained neutral red (0.003%) and the incubation of the plates was carried out in CO₂ incubator at 37°C. Well defined plaques of 2-3 mm diameter were developed by the fourth day of incubation. They also reported that the plaques would develop without the incorporation of DEAE-dextran in the overlay medium. However, the presence of trypsin was essential for the development of the plaques but the plaque development was best when both compounds were incorporated in the overlay medium. They also reported the occasional development of plaques in some other cell culture systems, for instance MDBK, MDCK, FL, Vero, PS and LLCMK₂. However, later on Matsuno and Mukoyama (1979) reported that, NCDV would form plaque in absence of trypsin as efficiently as in its presence provided the concentration of DEAE-dextran in overlay medium was increased to 300μg/ml.
One year previously Wyatt et al., (1978a) had reported plaque formation by both the U.K. strain of bovine rotavirus and the simian rotavirus SA11 in primary African Green Monkey Kidney (AGMK) cells and without any need for the incorporation of enzymes in the overlay medium. They also reported plaque production in primary calf kidney, CV1 and MA-104 cells also without addition of any proteases in the overlay medium but the results were unreliable.

The plaque assay of SA11 has also been reported in various continuous cell lines by Smith et al., (1979) and by Ramia and Sattar (1979). The incorporation of the DEAE-dextran and trypsin in overlay medium was found essential and the virus only produced plaques in MA-104, CV1 and LLCMK₂ cell lines but not in Vero or BGM cells. The MA-104 cell was found to be the most sensitive cell line in respect to both the production of virus to a high titer and the efficiency of the plaque development. Recently, two new simian strains of the rotaviruses have been isolated and a plaque assay has been developed in BSC-1 cell culture with the incorporation of trypsin and DEAE-dextran in the overlay medium (Stuker et al., 1980).

A successful plaque assay for a human rotavirus in primary AGMK cells has been briefly reported (Wyatt et al., 1980) but without any description of the techniques in detail.

The main value of the plaque technique is for the assay of infectivity of virus and a particularly useful application is the determination of neutralisation of the infectivity by specific sera (see review Mandel, 1978 and 1979). A highly specialised modification of this test is neutralisation kinetics (Dulbecco et al., 1956; Lewentonn-Kriss and Mandel, 1972; McBridge, 1959), which may provide a method for separating closely related types of viruses Gould et al., 1980; Woods et al., 1962). Other applications of the plaque test have been to detect such
features as rate of thermal inactivation and the action of disinfectants (Estes et al., 1979b; Hajenian and Butler, 1980).

In the present work a plaque assay for bovine rotaviruses (the U.K. and the Northern Ireland, NI strains) has been developed mainly in order to study neutralisation kinetics of the viruses to identify intratypic variations.
MATERIALS AND METHODS

3.2 CELLS

The cell lines used in the attempt to develop the plaque assay have been summarised in Table 3.1. The table includes a detailed description of the source of the cell lines, the growth and maintenance medium used and the subculture procedure employed. All the stock cultures were grown in 4 oz medical flat bottles and incubated at 37°C.

3.3 VIRUS

Two strains of the bovine rotaviruses were used for this work.

(i) Bovine rotavirus (U.K. strain). The stock suspensions of bovine rotavirus was prepared as described previously (Chapter 2). The titer of the stock virus was $1.5 \times 10^5$ TCID$_{50}$/ml assayed as described in Appendix VI by the microtiter method. However, the same virus when assayed by plaque assay gave a titer of $4 \times 10^6$ PFU/ml.

(ii) Bovine rotavirus (Northern Ireland Strain). The virus was received from Dr. M.S. McNulty of The Veterinary Research Laboratory, Stormont, Belfast. The virus stock culture was grown in the calf kidney diploid cells. The growth and maintenance medium used for these cells was as described in Table 3.1 except that the use of serum was omitted from the maintenance medium. Three successive passages of virus were carried out and the stock virus was prepared as described previously (Chapter 2). The stock virus had an infectivity titer of $3.5 \times 10^5$ PFU/ml by plaque assay (Methods in this Chapter).

3.4 ROTAVIRUS ANTISERA

Three rotavirus antisera were provided and used in the study of neutralisation of the viruses.
<table>
<thead>
<tr>
<th>SERIAL NUMBER</th>
<th>CELL LINE</th>
<th>SOURCE</th>
<th>GROWTH MEDIA</th>
<th>MAINTENANCE MEDIA</th>
<th>SUB-CULTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Calf Kidney diploid cell line</td>
<td>Developed in this laboratory</td>
<td>10% (V/V) Earle's balanced salt solution (10 X Flow Laboratories Ltd); 0.5% (V/V) Lactalbumin hydrolysate (Sigma London Ltd.); 0.1% (V/V) D(+)-Galactose (BDH); 5% (V/V) lamb serum (Flow Laboratories Ltd.); 0.088% (V/V) Sodium bicarbonate (Wellcome Reagents Ltd.); 1% (V/V) Penicillin and streptomycin (100 IU and 100 μg/ml, Glaxo) and sterile deionised water added up to 100 ml.</td>
<td>Same as growth medium except concentration of lamb serum was reduced to 2%</td>
<td>1 : 2 twice a week</td>
</tr>
<tr>
<td>2</td>
<td>LLCMK2 (Kidney, Rhesus monkey, Macaca mulatta)</td>
<td>ARC Research Institute on Animal Diseases, Compton, Berkshire</td>
<td>10% (V/V) Eagle's MEM (10 X Wellcome Reagents Ltd.), 10% (V/V) Foetal bovine serum (Flow Laboratories Ltd.); 0.132 (V/V) Sodium bicarbonate (Wellcome Reagents Ltd.); 1% (V/V) Penicillin and Streptomycin (100 IU and 100 μg/ml final concentration, Glaxo); sterile deionised water added up to 100 ml.</td>
<td>All components as growth medium but foetal bovine serum concentration was reduced to 2%</td>
<td>1 : 4 once a week</td>
</tr>
<tr>
<td>3</td>
<td>BGM (Buffalo Green monkey)</td>
<td>Thames Water Authority, Rosebery Ave. London</td>
<td>10% (V/V) Eagle's MEM with glasgow modification (Flow Laboratories Ltd); 10% (V/V) Foetal bovine serum (Flow Laboratories Ltd.); 1% (V/V) L-Glutamine (200 mM, Flow Laboratories Ltd.); 0.11% (V/V) Sodium bicarbonate (Wellcome Reagents Ltd.); 1% (V/V) Penicillin and Streptomycin (100 IU and 100 μg/ml final concentration, Glaxo); sterile deionised water added up to 100 ml.</td>
<td>Same as growth medium except concentration of serum was reduced to 2%</td>
<td>1 : 4 once a week</td>
</tr>
<tr>
<td>4</td>
<td>Vero (Kidney African Green Monkey)</td>
<td>Central Veterinary Laboratory, Weybridge, Surrey.</td>
<td>10% (V/V) Medium 199 (Wellcome Reagents Ltd.); 5% (V/V) New born calf serum (Flow Laboratories Ltd.); 0.11% (V/V) Sodium bicarbonate (Wellcome Reagents Ltd.); 1% (V/V) Penicillin and Streptomycin (100 IU and 100 μg/ml final concentration, Glaxo) and sterile deionised water added up to 100 ml.</td>
<td>All components of media were same as growth media except concentration of newborn calf was reduced to 2% and concentration of sodium bicarbonate was raised to 0.22%</td>
<td>1 : 4 once a week</td>
</tr>
</tbody>
</table>
(1) **G 199.** Bovine rotavirus (U.K. strain) antisera raised in gnotobiotic calf by infecting 5 day old calf which was bled 6 weeks later.

**L-258.** Bovine rotavirus (U.K. strain) antisera raised in gnotobiotic calf by purified virus.

(Both antisera were given by Prof. G.N. Woode, ARC Research Institute on Animal Diseases, Compton).

(2) **Pig 111.** The human rotavirus antisera raised in gnotobiotic pigs by inoculating purified faecal extract containing human rotavirus. (The antisera was donated by Prof. J.E. Banatvala, St. Thomas Hospital, London).

(3) **A/sera/37.** Bovine rotavirus (Northern Ireland) antisera raised in hens, and was provided by Dr. M.S. McNulty, The Veterinary Research Laboratory, Belfast.

Some of these antisera were distributed in 0.2 ml volumes in sterile bijoux bottles and were stored at -25°C; others were distributed in 0.1 ml volumes in sterile V-bottom polyvinyl microtiter plates and were sealed with plate sealer. These plates were stored at vapour phase (-136°C) of the liquid nitrogen. Whenever necessary one well was cut out and the serum was thawed at the room temperature.

3.5

(A) **Development of the plaque assay in the calf kidney cells**

The stock cultures of the calf kidney cells were grown in 4 oz medical flat bottles. The cultures were maintained and sub-cultured as described in Table 3.1. Eight milliliter volumes of the cell suspension containing $2 \times 10^5$ cells/ml were seeded into 60 mm plastic petridishes (Falcon) and the cells were incubated in a CO$_2$ incubator at 38°C. The concentration of CO$_2$ was so adjusted as to keep the culture medium at pH 7.8 ± 0.2. The humidity of the chamber was
maintained by keeping a tray of water at the bottom of the incubator. The cells formed a confluent monolayer within three days. They were prepared for the plaque assay by being washed twice with Hank's balance salt solution after removal of the growth medium. Ten fold virus dilutions were made in borate kcl buffer pH 8.4 containing 0.01M MgSO4·7H2O (Appendix V). A 0.2 ml volume of each dilution was seeded in 3 replicate plates and the virus was allowed to adsorb at 38°C for 45 minutes with intermittent rocking of the plates. At the end of the incubation the cells were washed once with Hank's balance salt solution before addition of the overlay medium.

The purified agar (0.6 gm, Difco Laboratories Ltd.) was added to 49 mls of deionised water in 4 oz medical flat bottles. It was sterilised by autoclaving (15 lbs/15 minutes) and when cool was stored at 4°C until use. The agar was melted in boiling water for 15 minutes prior to use and was placed in the water bath at 43°C. When the agar was at 43°C, 1 ml of sterile DEAE-dextran (Sigma London Ltd.) was added (to give 100/~g/ml concentration of DEAE-dextran in final overlay medium). The other constituents of the overlay medium (50 ml) were prepared by adding twice the concentrations of the maintenance medium without serum and before mixing with agar, the temperature of the maintenance medium was raised to 43°C in the water bath.

Five ml volumes of the overlay medium were added to each plate and after it had solidified (at room temperature for 15 - 20 minutes), the plates were incubated in an inverted position in a CO2 incubator. A second similar overlay of 5 ml was added 72 hours later which consisted of all ingredients of first overlay with or without neutral red (0.003%, BDH Chemicals Ltd.). The plates were incubated for a total of 6 days, after which the overlay medium was removed, the cell sheet was fixed and stained with 0.05% crystal violet (Hopkin and William) in formal PBS (10% Industrial formal dehyde in PBS). The plaques were counted
by the naked eye or by use of the low power microscope if many small plaques were present. The plaque size was measured, when required, by use of an ordinary ruler. The mean value of the plaque count was obtained by counting a minimum of three replicate plates in any one test.

The stained plaques were photographed and the prints were made (Audio Visual Unit, The University of Surrey), for photographic records.

(B) The use of LLCMK₂BGM and Vero cell cultures for the plaque test

The growth and the maintenance medium as well as the subculture schedule for these cell cultures is recorded in the Table 3.1. Eight ml. volumes of a suspension of cells containing 1.5 - 2 X 10⁶ cells/ml in growth medium were seeded into 60 mm plastic petridishes. The plates were incubated at 38°C in a CO₂ incubator as described earlier and the confluent monolayers were formed in 3 - 4 days time. The inoculation of virus, the overlay conditions with their respective maintenance medium and incubation conditions were carried out as described for plaque assay in the calf kidney cells.

3.6 THE THERMAL STABILITY TESTS

The virus was diluted to 1000 PFU/0.2 ml in Earle's balance salt solution and distributed to sterile bijoux bottle in 1 ml quantities. The bottles were placed in water baths running at particular temperature and were withdrawn at intervals. When they were rapidly cooled down by placing them in the vapour phase of liquid nitrogen, but the bottles were removed before the liquid in them froze and they were then stored at 4°C. The residual infectious virus was titrated by diluting 10 fold in borate KC1 buffer pH 8.4 containing 0.01M MgSO₄.7H₂O and the plaque assay was carried out as described. The surviving virus was determined on a percentage of the original plaque count.
estimated at time 0 and the inactivation curve obtained by plotting the virus survival percentage against the time.

3.7 THE NEUTRALISATION TESTS

The antisera used in this study was first inactivated at 56°C for 30 minutes. The required dilutions of the serum were made in Earle's balance salt solution at pH 7.5. A dilution containing approximately 10,000 PFU/0.2 ml of virus was made in Earle's balance salt solution at pH 7.5 and an equal volume of virus and serum dilutions were incubated together for a specified period when small volumes (0.2 ml) of the serum-virus mixtures were withdrawn. These were diluted immediately by the addition of 0.8 ml of borate KCl buffer pH 8.4 containing 0.01M MgSO4.7H2O. The residual virus was titrated in triplicate by the plaque assay. The percentage of plaque survival was plotted by taking non neutralised virus as 100%
RESULTS

3.8 DEVELOPMENT AND STANDARDSATION OF THE PLAQUE ASSAY

(A) FACTORS INFLUENCING PLAQUE FORMATION

1. The cells. Under the conditions described for the development of the plaque assay, the rotavirus developed plaques only in the calf kidney cells but not in LLCMK₂, BGM or Vero cell monolayers.

2. The growth medium of the cells. When the cells were grown with Hank's balance salt solution medium as the base, plaques did not develop unless the growth medium was changed to maintenance medium (which contained Earle's balance salt solution as base) approximately 24 hours before plaque assay. However, when the cells were grown in growth medium with Earle's balance salt solution as the base, without the need to change the medium, there was development of the plaques.

3. The cell seeding rate. The optimum cell density for seeding good monolayers in 60 mm plastic petri dishes was found to be by use of 8 ml of cell suspension containing $2 \times 10^5$ cells/ml. A cell density in excess of this resulted in the clomping of the cells at the middle of the petridish with the development of only a thin layer of cells at periphery of the plate. The central cells matured earlier than those at the periphery, which resulted in their early degeneration. A volume of cells less than 8 ml was also found to produce a higher density of cells at the middle than at the periphery but gentle rocking of the cells prior to incubation could overcome this trouble.

4. Overlay medium

(a) Agar. Both 0.6% purified agar (Difco
Laboratories Ltd.) and 1% agarose (BDH Chemicals Ltd.) were tested in the overlay medium but no plaques developed under agarose at this concentration, subsequently, only agar was used.

(b) Serum and Trypsin. Incorporation of lamb serum (1.5% v/v) in the overlay medium resulted in a slight decrease in virus titer ($3.5 \times 10^6$ PFU/ml) but since the cells could be maintained without incorporation of serum in the overlay medium, it was omitted from the subsequent studies. The titer of the stock suspension of virus was $4 \times 10^6$ PFU/ml without incorporation of the serum in the overlay medium. Trypsin ($2 \mu$g or $5 \mu$g/ml) in the overlay medium neither increased the plaque size nor the count, so its incorporation was discontinued.

(c) DEAE-dextran. There was only an irregular development of a few plaques without the incorporation of DEAE-dextran but with its presence the plaque count increased to an optimum at $100 \mu$g/ml (Table 3.2) thereafter there was a slight fall in the plaque count and also a reduction in the plaque size. The incorporation polycations at $140 \mu$g/ml resulted in the destruction of the cell monolayers.

(d) Neutral red. The incorporation of neutral red (BDH Chemicals Ltd.) at 0.003% concentration in the second overlay medium resulted in an increase in plaque size (Fig. 3.1) but there was no effect on the number. In most subsequent studies neutral red was omitted.

5. Effect of pH

The pH of the incubator-indicator-medium during incubation was maintained at about $7.8 \pm 0.2$ by controlling the flow of $\text{CO}_2$ to incubator.
Fig. 3.1 (a) Plaque morphology of bovine rotavirus (UK strain) with incorporation of neutral red in second overlay medium.

Fig. 3.1 (b) Plaque morphology of bovine rotavirus (UK strain) without incorporation of neutral red in second overlay medium.
Fig. 3.1 (C) Plaque morphology of bovine rotavirus (Northern Ireland strain).
Plaques did not develop if the pH of the indicator medium fell below 7.4.

6. **Effect of temperature.** In preliminary study the incubation temperature for the cells was found to be important as few plaques developed at 35°C, however, the maximum number was achieved at 38°C.

7. **Incubation time.** Monolayers of cells infected with an inoculum of 0.2 ml were fixed and stained daily from 3 - 9 days. The plaque count increased steadily as the incubation period increased reaching the optimum number on the sixth day (Table 3.3, Fig. 3.2). However, three days later there was a surge in the count, probably due to the development of secondary plaques. Although plaque size was also increased with increased incubation period, the edges of the plaques became very diffuse after the sixth day, which was the chosen time for further work.

8. **Adsorption period and salt concentration.** The adsorption time for optimum plaque formation varied with respect to the use of concentrations of MgSO\(_4\). 7H\(_2\)O (Fig. 3.4). The most rapid virus adsorption occurred in the presence of 0.1M salt. However, the optimum adsorption of virus to cells was completed in 45 minutes, more or less, for all the tested concentration of the salt (Table 3.4, Fig. 3.3 and Fig. 3.4). The least adsorption occurred in the presence of 0.001M salt even after 90 minutes of adsorption (Fig. 3.4). Since the highest concentration of salt was deleterious to the cells, the intermediate concentration (0.01M) was selected. In a preliminary study similar results were obtained by use of 0.01M CaCl\(_2\) 2H\(_2\)O, BaCl\(_2\) 2H\(_2\)O and MgCl\(_2\) 6H\(_2\)O.
### Table 3.2  The effect of concentration of DEAE-dextran in the overlay medium on plaque count

<table>
<thead>
<tr>
<th>mg DEAE dextran/ml</th>
<th>Mean plaque count/0.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>194 ± 5.1</td>
</tr>
<tr>
<td>20</td>
<td>206 ± 5.6</td>
</tr>
<tr>
<td>40</td>
<td>276 ± 1.5</td>
</tr>
<tr>
<td>60</td>
<td>280 ± 1.4</td>
</tr>
<tr>
<td>80</td>
<td>288 ± 58</td>
</tr>
<tr>
<td>100</td>
<td>533 ± 65</td>
</tr>
<tr>
<td>120</td>
<td>530 ± 78.5</td>
</tr>
<tr>
<td>140 (cyto toxic)</td>
<td>500 approximately</td>
</tr>
</tbody>
</table>

### Table 3.3  The effect of the time of incubation on the plaque count

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Mean plaque count/0.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>52 ± 19.1</td>
</tr>
<tr>
<td>4</td>
<td>77 ± 21.9</td>
</tr>
<tr>
<td>5</td>
<td>81 ± 46.7</td>
</tr>
<tr>
<td>6</td>
<td>102 ± 12.0</td>
</tr>
<tr>
<td>7</td>
<td>100 ± 31.1</td>
</tr>
<tr>
<td>8</td>
<td>96 ± 36.8</td>
</tr>
<tr>
<td>9 (cell poor)</td>
<td>131 ± 68.6</td>
</tr>
</tbody>
</table>

### Table 3.4  The interrelationship between the time of adsorption and the concentration of magnesium ions on the plaque count

<table>
<thead>
<tr>
<th>Time of adsorption in minutes</th>
<th>Concentration of MgSO₄·7H₂O in borate KCl buffer pH 8.4 in inoculum diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.001M</td>
</tr>
<tr>
<td>0.5</td>
<td>6* ± 2.8</td>
</tr>
<tr>
<td>15</td>
<td>20 ± 8.5</td>
</tr>
<tr>
<td>30</td>
<td>43 ± 4.2</td>
</tr>
<tr>
<td>45</td>
<td>47 ± 32.81</td>
</tr>
<tr>
<td>60</td>
<td>52 ± 21.2</td>
</tr>
<tr>
<td>90</td>
<td>53 ± 25.45</td>
</tr>
<tr>
<td>120</td>
<td>43 ± 14.1</td>
</tr>
</tbody>
</table>

* Mean plaque count/0.2 ml
- Cell sheet destroyed
Fig. 3.2  THE EFFECT OF TIME OF INCUBATION ON PLAQUE COUNT.
Fig. 3.3 THE INFLUENCE OF MAGNESIUM IONS ON THE PLAQUE COUNT

![Graph showing the influence of magnesium ions on plaque count. The x-axis represents molar MgSO4 concentration, and the y-axis represents mean plaque count per 0.2 ml. The graph shows a linear relationship between magnesium ion concentration and plaque count.]
Fig. 3.4 THE INTERRELATIONSHIP BETWEEN THE TIME OF ADSORPTION AND CONCENTRATIONS OF MAGNESIUM IONS ON THE PLAQUE COUNT.
9. **Inoculum size**

The number of plaques increased corresponding to the volume of the inoculum up to 0.3 ml/plate (Table 3.5 and Fig. 3.5) but volume greater than this resulted in smaller number. The theoretical count on the basis of that obtained using 0.1 ml is also shown in the figure 3.5 and this indicates a deficiency in the method. The 0.2 ml volume was selected for future use on the basis that, it was closer to the maximum achieved but less far from the predicted value than that obtained with 0.3 ml.

(B) **APPLICATION OF THE PLAQUE TEST**

1. **Plaque formation by two calf rotavirus strains**

The plaques formed by the U.K. and the N.I. strain of virus differed strikingly in size and definition (Fig. 3.1). The plaques produced by N.I. strain were much larger, diffuse and were also more variable in size (2.0 - 5.0 mm diameter, Fig. 3.1C) than the U.K. strain (2.0 - 3.0 mm diameter, Fig. 3.1a). Neutral red in second overlay had no influence on the development of the large plaques by N.I. strain.

2. **Comparison of the plaque and microtitration assay**

The two rotaviruses (U.K. and N.I. strains) were grown in calf kidney cells (the cells were grown in Earle's base medium) and then titrated by both the plaque assay (as described earlier) and the microtiter assay (Method in Chapter 1). The U.K. virus had a titre that was 10 times higher titer by plaque test than microtiter assay (Table 3.6) but for the N.I. strain both assays gave about same titer.
Fig. 3.5 ACTUAL AND THEORETICAL PLAQUE COUNTS USING DIFFERENT VOLUMES OF INOCULA

- THEORETICAL PLAQUE COUNT
- ACTUAL PLAQUE COUNT

VOLUMES (ML) OF INOCULUM/PLATE

MEAN PLAQUE COUNT

0.1 0.2 0.3 0.4 0.5
3. Thermal stability of the U.K. strain

At 50°C there was a critical first order reaction followed by a slowing in rate of inactivation by 10 minutes to a plateau value (Table 3.7 and Fig. 3.6). Inactivation at 32.5°C followed a similar biphasic reaction although the degree of inactivation was much less.

4. Neutralisation kinetics

The serum neutralisation tests were carried out at room temperature (approximately 20°C) or at 4°C. All the neutralisation tests were carried out against 10,000 PFU/0.2 ml unless otherwise stated.

The rate of inactivation of the U.K. strain of rotavirus at 20°C with homologous serum dilutions (1 : 100, 1 : 200, 1 : 400) was biphasic with maximum inactivation occurring during the first 2½ minutes followed by a slow rate of neutralisation over the remaining period (Table 3.8 and Fig. 3.7). The rate of inactivation was apparently independent of the temperature of incubation, whether at 4°C or 20°C (Table 3.8, 3.9 and Fig. 3.8). The heterologous serum, human rotavirus antiserum had a relatively low activity against the calf rotavirus and the neutralisation curve was slightly different with a slower critical rate of reaction followed by a continuous slow fall in infectivity titer (Table 3.10 and Fig. 3.9).

The N.I. strain was neutralised completely by its homologous antisera at 1 : 100, 1 : 200 or 1 : 400 dilutions. However, when the U.K. and N.I. viruses (1,000 PFU/0.2 ml) were neutralised by a 1 : 400 dilutions of heterologous sera (A/Sera/37 and G199) respectively, the inactivations curves were similar.
Table 3.5  The effect of the size of the inoculum on the plaque count

<table>
<thead>
<tr>
<th>Volume inoculum (ml)*</th>
<th>Mean plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>168 ± 15.6</td>
</tr>
<tr>
<td>0.2</td>
<td>261 ± 18.4</td>
</tr>
<tr>
<td>0.3</td>
<td>333 ± 21.2</td>
</tr>
<tr>
<td>0.4</td>
<td>326 ± 21.2</td>
</tr>
<tr>
<td>0.5</td>
<td>306 ± 26.9</td>
</tr>
</tbody>
</table>

* per 60 mm plastic petridish

Table 3.6  Infectivity titer of rotaviruses by plaque and microtiter assay

<table>
<thead>
<tr>
<th>ROTAVIRUS</th>
<th>Plaque Assay PFU/ml</th>
<th>Microtiter Assay TCID 50/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK STRAIN</td>
<td>4 X 10^6</td>
<td>1.56 X 10^5</td>
</tr>
<tr>
<td>NI STRAIN</td>
<td>3.5 X 10^5</td>
<td>1.56 X 10^5</td>
</tr>
</tbody>
</table>

Table 3.7  Heat inactivation kinetics of UK strain of rotavirus

<table>
<thead>
<tr>
<th>Time in Minutes</th>
<th>Temperature (0°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>32.5</td>
</tr>
<tr>
<td>0</td>
<td>110 ± 14.7</td>
</tr>
<tr>
<td>5</td>
<td>101 ± 8.6 (92)</td>
</tr>
<tr>
<td>10</td>
<td>96 ± 22.6 (87)</td>
</tr>
<tr>
<td>20</td>
<td>96 ± 10.5 (87)</td>
</tr>
<tr>
<td>40</td>
<td>91 ± 17.2 (83)</td>
</tr>
</tbody>
</table>

* Mean plaque count/0.2 ml

Figures in brackets % survival.
Table 3.8  Inactivation of Rotavirus (UK strain) by homologous serum at 20°C

<table>
<thead>
<tr>
<th>Time in Minutes</th>
<th>SERUM DILUTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G199 1 : 100</td>
</tr>
<tr>
<td>2.5</td>
<td>107.0* ± 10.4</td>
</tr>
<tr>
<td>5</td>
<td>58.7 ± 10.7</td>
</tr>
<tr>
<td>10</td>
<td>31.7 ± 7.4</td>
</tr>
<tr>
<td>20</td>
<td>28.0 ± 3.5</td>
</tr>
<tr>
<td>30</td>
<td>21.0 ± 3.7</td>
</tr>
</tbody>
</table>

* Mean plaque count/0.2 ml.

Table 3.9  Inactivation of Rotavirus (UK Strain) by homologous serum at 4°C

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>SERUM DILUTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G199 1 : 200</td>
</tr>
<tr>
<td>2.5</td>
<td>181.3* ± 8.0</td>
</tr>
<tr>
<td>5</td>
<td>126.0 ± 11.1</td>
</tr>
<tr>
<td>10</td>
<td>79.0 ± 6.1</td>
</tr>
<tr>
<td>20</td>
<td>50.3 ± 1.5</td>
</tr>
<tr>
<td>30</td>
<td>45.3 ± 1.5</td>
</tr>
</tbody>
</table>

* Mean plaque count/0.2 ml
Fig. 3.6  HEAT INACTIVATION KINETICS OF U.K. STRAIN OF ROTAVIRUS.
Fig. 3.7 INACTIVATION OF ROTAVIRUS (U.K. STRAIN) BY TWO DIFFERENT HOMOLOGOUS SERA AT 20°C.
Fig. 3.8  INACTIVATION OF ROTAVIRUS (U.K. STRAIN) BY ONE HOMOLOGOUS SERUM AT 4°C AND 20°C.
Fig. 3.9 INACTIVATION OF ROTAVIRUS (U.K. STRAIN) BY HUMAN ANTIROTAVIRUS SERUM (Fig 111).
(Table 3.11 and Fig. 3.10). The maximum virus inactivation occurred in the first 2.5 minutes followed by a slow rate of inactivation up to 20 minutes.
Table 3.10  The inactivation of rotavirus (UK strain) by human antirotavirus serum (Pig III).

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>SERUM DILUTIONS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 : 10</td>
<td>1 : 20</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>550.4 ± 34.6</td>
<td>848.0 ± 19.7</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>494.0 ± 19.1</td>
<td>753.7 ± 11.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>410.7 ± 20.2</td>
<td>731.4 ± 17.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>272.7 ± 32.9</td>
<td>725.4 ± 12.7</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>332.0 ± 12.0</td>
<td>669.7 ± 14.7</td>
<td></td>
</tr>
</tbody>
</table>

* Mean plaque count/0.2 ml

Table 3.11  Inactivation of two strains of bovine rotaviruses (UK and NI strain) by heterologous sera

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>NI VIRUS</th>
<th>UK VIRUS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G199 (UK) 1 : 400</td>
<td>A/sera/37 (NI) 1 : 400</td>
</tr>
<tr>
<td>2.5</td>
<td>39.7* ± 6.0</td>
<td>40.7 ± 5.5</td>
</tr>
<tr>
<td>5</td>
<td>31.7 ± 1.6</td>
<td>41.0 ± 7.0</td>
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<tr>
<td>10</td>
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<td>40.0 ± 3.6</td>
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<tr>
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<td>25.4 ± 4.2</td>
</tr>
<tr>
<td>30</td>
<td>24.7 ± 2.5</td>
<td>17.7 ± 2.1</td>
</tr>
</tbody>
</table>

* Mean plaque count/0.2 ml.
Fig. 3.10 INACTIVATION OF TWO STRAINS OF ROTAVIRUSES (U.K. AND NORTHERN IRELAND STRAINS) BY HETEROLOGOUS SERA.
DISCUSSION

Sellers (1955) was first to report that strains of virus, closely related serologically, could be differentiated by plaque morphology. He was examining different strains of foot-and-mouth disease (FMD) virus in the primary calf kidney cells and also noted that the vesicular stomatitis virus produced different plaques to F.M.D. Hsiung and Melnick (1955, 1957) also reported that on the basis of plaque morphology, polio-, coxsackie- and echo- viruses could be differentiated from each other. At about the same time Dubes (1956) noted variation in the plaque size of type 1 and 2 poliovirus strains and he divided them into small, intermediate & large types. Since then, various other people have reported intratypic variations in viruses based on plaque morphology and sizes (Beare and Keast, 1971 and 1974; Roizman and Aurelian, 1965). Some of the most relevant examples relate to Herpes simplex virus Type I and II (Plummer et al., 1974). Indeed, the value of the observations tend to be greatest for these virus where serological or other methods for differentiation are inadequate. An interesting example of this is the apparent differentiation of rubella virus by plaque size (Gould et al., 1972).

The plaque formation by viruses are highly influenced by the test conditions and it is well known that not all viruses will form plaques even though they may be cytopathogenic (See review Takemoto, 1966). A wide variety of conditions may have to be tested in order to find those which facilitate plaque formation (See reviews by Cooper, 1961 and Takemoto, 1966). One of the most widely observed phenomenon has been the inhibition of plaques by factors in the agar (Takemoto and Liebhaber, 1961) and the most successful methods for counteracting the agar inhibition is the addition of polycations to the agar to bind and thus neutralise the sulfated polysaccharides which are thought to be the main inhibitor in agar. Liebhaber and Takemoto (1961) found that
polycations, DEAE-dextran and protamine were equally effective in permitting wild type of encephalomyocarditis (EMC) virus to form large plaques. In later work, DEAE-dextran at 1.0μg/ml was found to enhance both the number and size of the plaques produced by influenza A and B viruses (Takemoto & Fabisch, 1963) and the authors concluded that agar inhibitors functioned by interfering with the adsorption of the virus to the cell cultures rather than by affecting the virus themselves, since neither infectivity nor haemagglutinin were affected. However, DEAE-dextran has been shown to inhibit the attachment and therefore infectivity of the Japanese B encephalitis virus to host cells (Ozaki and Kumagai, 1972) and inhibit plaque formation with SV40 (McCutchan and Pagano, 1968).

Takemoto and Leibhaber (1962) found that, with the Mahoney strain of poliovirus although plaques were enlarged in dextran sulfate, they were suppressed by use of DEAE-dextran and this chemical also suppressed viral cytopathic effects. However, DEAE-dextran is more widely reported to enhance plaque formation than otherwise. Then, DEAE-dextran when present during adsorption of virus, increased the efficiency of plaque formation for rabies virus (Kaplan et al., 1967); respiratory syncytial virus (Nomura, 1968) rubella virus (Vaheri et al., 1967) and of focus formation by a sarcoma virus (Vogt, 1967). DEAE-dextran was also found to improve the plaque assay of malignant catarrhal fever virus in primary bovine testes cells (Russel, 1979) and increased the infectivity of herpes virus saimiri in a plaque assay (Ablashi, et al., 1971). Furthermore, DEAE-dextran has been shown to enhance the rate of attachment of fowl plaque virus to chick cells (Allison and Valentine, 1960). The enhancement here may result from interaction of polycations with receptors rather than the virions, since DEAE-dextran has found to increase ribosomal subunits in the cell and hence must interact with the cells (Breindl and Koch, 1972). It is possible that DEAE-dextran alters the spatial arrangement
of the subunits on the cell surface, or that it decreases the net charge of the cell surface and it is possible that it serves as a ligand between virus and the cell (Bengtson et al., 1964).

The enhancement of plaque formation by the presence of proteolytic enzymes has been widely reported. For example, chymotrypsin, trypsin and papain enhanced plaque formation by reoviruses (Spendlove and Schaffer, 1965; Spendlove et al., 1966) and trypsin and chymotrypsin were important for vaccinia virus plaque production (Gifford and Klapper, 1967 and 1968). Both influenza A and B plaque formation was affected by pancreatin and trypsin (Came et al., 1968; Appleyard and Maber, 1974) as was that of parainfluenza type 3 (Sabina and Munro, 1969) and type 4 (Morimoto et al., 1970). Spendlove and Schaffer (1965) suggested that the enhancement of plaque formation of reoviruses by proteolytic enzymes resulted from the action of enzyme on the virus particles themselves. On the other hand, Came et al., (1968) suggested that, the enhancement of influenza virus plaque formation by pancreatin resulted from the action of enzyme on the cells rather than directly on the virus particles or on a component of the overlay medium. However, according to Gifford & Klapper (1968), the enhancement of plaque number of vaccinèa virus was due to an effect of the enzyme on the virus and the enhancement of the plaque size was due to an effect on the cells. There is a possibility that, the proteolytic enzyme destroys interferon produced by infected cells and thereby allows the virus to form plaques.

Incorporation of trypsin in the agar overlay medium is reported as essential for plaque formation by rotaviruses (Matsuno et al., 1977b; Ramia and Sattar, 1979; Smith et al., 1979; Stuker et al., 1980). However, trypsin is not necessary for plaque production of rotaviruses in primary AGMK cells (Wyatt et al., 1978a). In contrast Ramia and Sattar (1979) could not develop plaque by SA11 in secondary
AGMK cells without incorporation of trypsin. But as noted elsewhere in the Chapter, Matsuno and Mukoyama (1979) reported that, rotavirus produced plaques in MA-104 cells in the absence of trypsin as efficiently as presence of it provided that the concentration of DEAE-dextran in the overlay medium was increased to 300μg/ml. It is not clear how the cells tolerated such a high concentration (300μg/ml) of DEAE-dextran, since Smith et al. (1979) found that DEAE-dextran at a concentration greater than 150μg/ml was destructive to such cells and a similar observation has been noted in the present study for calf kidney cells. In this study, incorporation of trypsin was not necessary but DEAE-dextran was required for the formation of the optimum number of plaques. Wyatt et al., (1978a) had earlier shown that, neither enzymes nor other chemical fascilitators were necessary for the plaque production by calf virus (U.K. strain) and SA11 in primary AGMK cells. In this regard it is well worth recalling that primary cells contain up to 10 times more Ca²⁺ than transformed cell cultures (Swierenga and Whitfield, 1978) so the development of plaques in such cells and indeed, calf kidney diploid cells grown in Earle's base medium (high Ca²⁺ concentration) might have been predicted. It is very interesting that, Ramia and Sattar (1979) could not produce plaques in secondary AGMK cells without the incorporation of trypsin, presumaby because the concentration of Ca²⁺ was low as it is known that the concentration of Ca²⁺ in the cells decreases on subculture (Swierenga and Whitfield, 1978).

The influence of divalent cations on adsorption of viruses to host cell has been reported occasionally, as, for example, the attachment of specific phages to E. coli (Puck et al., 1951), Pasteurella (Rifkind & Pickett,1954) or Salmonella (Tucker, 1961). The same is true for attachment of Japanese B encephalitis virus to hamster kidney cells (Rhim & Hammon, 1963) and the dengue virus to Rhesus monkey kidney cells (Georgiades et al., 1965). However, cation
requirement are not necessarily uniform within a large virus group such as the picornaviruses. For instance, while added divalent cations enhanced the attachment of coxackie A9 virus to human amnion cells (McLaren et al., 1960), the divalent cations were not essential for optimal attachment of poliovirus to HeLa cells (Holland and McLaren, 1959). The nature of the cation was also important. For instance, the attachment of Venezuelan equine encephalomyelitis virus to McCoy cells was optimum with 0.1 - 0.15M NaCl while low levels of Ca$^{2+}$ or Mg$^{2+}$ inhibited it (Hohan and Cooke, 1967). Matsumura et al. (1970) postulated that increased dengue yield of type 2 virus in vero cells was as a result of promotion of migration of new viral nucleocapsid to the cell membrane in presence of higher level of Mg$^{2+}$ in the medium. In the present study optimum attachment of the rotavirus to the calf kidney cells was apparently also dependent on the concentration of the divalent cation present in the medium during virus adsorption, however, the process was also pH dependent.

The optimum attachment of virus to a cell and its replication therein is known to be pH dependent (Allison and Valentine, 1960) and there is an interesting example where related strains of virus have different pH optima. For instance, different virulent strains of rabbit myxoma virus have an optimum pH range for plaque production in the rabbit kidney (RK13) cells (Ross and Sanders, 1979). They also reported that, the optimum pH for plaque production of the grade I strain was 6.9 - 7.5 and for grade V strain 7.5 but there was complete inhibition of plaques by grade V at pH 6.9 and 50% inhibition of grade III at pH 6.9. So, the fact that SA11 adsorbed and formed plaques at low pH in MA-104 cells (Estes et al., 1979a; Ramia and Sattar, 1979; Smith et al., 1979) is not necessarily in contrast with observations in the present work that U.K. calf rotavirus required a high pH. Another point worth commenting on is what levels of correlation exists between the pH within the cells, at the cell surface and that in the medium (Cooper, 1961) is not known.
In the present experiment plaques were only formed when the pH of the medium in the incubator was 7.8 ± 0.2 but no measurement of the pH of the agar overlay was attempted.

It is interesting to note that in the present study both high pH and high salt concentration were required during virus adsorption for optimum plaque formation. In relation to this it has been reported that optimum activation of the RNA-dependent-RNA polymerase of bovine rotavirus was dependent on the presence of 10 mM magnesium ions at the pH 8.0 and a temperature of 45 - 50°C (Cohen et al., 1979). It is also known that efficient adsorption of Venezuelan equine encephalitis (VEE) to McCoy cells is dependent on high salt concentration in the medium and is pH and temperature dependent (Hohan and Cooke, 1967).

It has been reported that, the bovine rotavirus (U.K. strain) has an optimum plaquing efficiency at 38°C in primary AGMK cells and plaques poorly at 32°C (Wyatt et al., 1978a). However, Estes et al., (1979a) could not find any difference of plaquing efficiency irrespective of temperature of incubation (32 - 39°C), when they did plaque assay of SA11 in MA-104 cells. But in the present study bovine rotavirus (U.K. strain) produced plaque at 38°C more efficiently than at 35°C. Such apparent discrepancies might be due to strain variation and the cell culture system used for the study. However, even when all the conditions for plaque development were standardized, the virus did not develop plaques when the cells were grown in Hank's base medium presumably because of the low Ca^{2+} concentration. This phenomenon has been discussed in detail in Chapter 2.

Generally neutral red is incorporated in the overlay medium to visualise the plaques. However, it is also known to have a toxic effect on certain cells (Cooper, 1961). In the present study, when neutral red (0.003% concentration) was added to the second overlay after three days of incubation
better defined plaques were developed but Welch and Twiehaus (1973) found that incorporation of neutral red (0.01%) in second overlay inhibited plaque formation. This may have been due to the fact that they used a high concentration (0.01%) which might have had a toxic effect on the cells. The fact that lamb serum (1.5%) appeared to be slightly inhibitory to plaque formation in the present work is not altogether surprising. It has already been reported that, foetal bovine serum contains some rotavirus inhibitors at the level sufficient to reduce titers of virus by 80 - 90% (Neal et al., 1978; cited by Clark et al., 1979). It is also interesting that Welch and Twiehaus (1973) failed to obtain plaques in primary bovine embryonic kidney and this might have been due to the incorporation of a relatively high concentration (5%) of bovine serum in their overlay medium.

Even in standardised conditions, there is some evidence for plaque variation amongst the different rotavirus subgroups. Thus, Estes and Graham (1980) reported that in MA-104 cells, SA11 usually yielded the largest plaques, the bovine rotavirus produced intermediate sized plaques and the porcine rotavirus formed the smallest plaques. So it is interesting in the present study that the U.K. and N.I. strains of bovine rotaviruses can be differentiated because this is the first evidence for serologically similar rotaviruses having different plaque morphology.

It has often been reported that one cell system but not another will support plaque formation by SA11 (Estes et al., 1979a; Smith et al., 1979) and with the present work, again some cells were sensitive whereas others were not.

In order to interpret the results of neutralisation on kinetics, it is in fact necessary to have an appreciation of the phenomenon of serum neutralisation test. Burnet et al., (1937) observed that when bacteriophage serum was used at low concentrations the reaction was multi-hit in contrast
to single-hit when used at three-fold higher concentration. Because of a short lag, Kalmanson et al., (1942) estimated that the neutralisation of bacteriophage required two or three molecules of antibody. Similarly, Lafferty (1963) observed single-hit and multi-hit kinetics when influenza virus was neutralised by serum at twenty or forty-fold dilutions respectively. A multi-hit reaction was also seen with rabbitpox virus (Lafferty, 1963). Philipson (1966) reported that the interaction of poliovirus with IgM antibody showed a time lag. The interpretation for a time lag is the requirement for multiple interactions, for example, Sagik (1954) reported that bacteriophage T₂ showed a brief rise in titer on reacting with T₂ antiserum preceding the linear decline. He also showed that an inhibitor was present that could mark viral infectivity and was removed by the antiserum. So a multi-hit hypothesis of neutralisation for most of the vertebrate viruses has been suggested (reviewed by Della-Porter and Westaway, 1978). In the present study when higher dilutions of serum were used to neutralise virus a multi-hit phenomenon was observed, which agreed to the observations of the above workers.

All rotaviruses are apparently closely related biochemically and they shared a common complement fixing (CF) antigen in addition they are also morphologically indistinguishable and show a high degree of cross infection between mammals (Woode, 1978). However, there may be certain distinct antigenic differences and Flewett et al., (1974a) were first to report that human and bovine strains showed poor cross neutralisation. Subsequent workers confirmed the lack of a common neutralising antibody to rotaviruses isolated from children, calves, pigs, sheep, foal and monkeys (Schoub et al., 1977; Snodgrass et al., 1977a; Theil et al., 1977; Thouless et al., 1977; Woode et al., 1976b) and further investigation of serological differentiation were made. For instance, an enzyme-linked immunosorbent assay (ELISA) has been employed to distinguish
the rotaviruses isolated from different animal species by a post infection serum blocking test (Yolken et al., 1978d). They were able to differentiate isolates of children from those of calves, piglets, foal, monkeys and infant mice. However, they failed to differentiate intratypic variations of 33 human rotavirus specimens collected from three different parts of the world or 4 bovine strains recovered from calves. In the same year three different groups of workers using various serological methods (complement fixation and immunoelectronmicroscopy, Zissi and Lambert, 1978; immunofluorescent foci serum neutralisation test, Thouless et al., 1978; ELISA, Yolken et al., 1978c) reported that there were at least 2 serotypes of rotaviruses affecting children. Furthermore, Flewett et al., (1978) reported two more serotypes of human rotaviruses by fluorescent foci serum neutralisation test. Recently, Zissi and Lambert (1980) developed five different ELISA test systems and found that when antidoby sandwich consisted of two type specific hyperimmune sera of different origin were used instead of one type specific and other group specific hyper immune sera, both sensitivity and ease of visual reading of the typing procedure were much increased. On the basis of their work, they differentiated 330 human rotavirus stool specimens from children with gastroentiritis to type 1 (15%) and type 2 (85%) in Brussels. A haemagglutination inhibition (HI) test has been developed to differentiate rotaviruses from different species and within the serotypes (Spence et al., 1978). However, the test works well to detect differences between bovine virus isolates especially if early immune serum is employed but not with hyperimmune serum as the degree of cross reactivity increased, so that serological differences are not detectable. It is also known that, U.K. strain of bovine rotavirus lacks haemagglutinin (Spence et al., 1978), so HI test cannot be used for the differentiation of these strains which do not possess haemagglutinin.
The neutralisation test may perhaps indicate a different serological reaction from the reaction by ELISA or by a complement fixation test; the later tests simply indicate a reaction between serum and whole virus particles. However, the neutralisation tests indicate a reaction on that particular part of the virus which causes neutralisation and the protein involved in the reaction may not be the same protein responsible for reaction by CF or ELISA tests (Flewett et al., 1979). The polypeptide present in the outercapsid layer thought to be responsible for neutralisation reaction but there is no proof yet of which polypeptide (Flewett et al., 1979). Recently, Matsuno et al., (1980) reported that major neutralizing antigen may reside in VP7 polypeptide.

In the present preliminary study the U.K. strain of rotaviruses neutralises homologous antiserum more efficiently than that of heterologous (human) antiserum which agrees with the work of others (Thouless et al., 1977; Woode et al., 1976b) and also the two bovine rotaviruses (U.K. and N.I. strain) vary on the basis of their rate of neutralisation to homologous than heterologous antisera. Thus the two strains of rotaviruses are apparently differentiated on the basis of their plaque morphology and size and also on the rate of neutralisation by homologous and heterologous antisera.
CONCLUDING REMARKS

Apart from serological differentiation of intratypic variation of rotaviruses, the differences in the relative electrophoretic mobility of corresponding genomic segments in polyacrylamide gel electrophoresis has been used to differentiate variations between the species and within the same species. So the genetic variations have been found among rotaviruses from single species (Espejo et al., 1977 and 1980; Kalica et al., 1978 a and b; Rodger and Holmes, 1979; Verley and Cohen, 1977). Rodger and Holmes (1979) found that in comparison between rotaviruses originating from three different animal species, RNA segment three varies in each case but this segment was invariable amongst eight bovine strains from Australia. They also found differences in segment four of Northern Ireland strain and eight Australian strains but not within all strains from Australia. So they suggested that segment '3' may code for a species specific component. On the contrary Kalica et al., (1978b) have shown that two rotaviruses isolated from pigs differed electrophoretically with respect to RNA segments 3, 4 and 7 which argue against this hypothesis. These authors suggested that RNA segment 5 may be responsible for serological specificity (Kalica et al., 1978a) since RNA analysis of calf rotavirus and the 'O' agent which are serologically distinct (Yolken et al., 1978d) showed they differed only in segment 5 and between 'O' and SA11 in segment 5 and 7 (Kalica et al., 1978 a and b).

The gel electrophoresis technique is a method for distinguishing rotaviral strains and their value in identifying rotaviruses following in vitro and in vivo passage, however, differences in migration do not necessarily reflect an antigenic difference (Espejo et al., 1980; Kalica et al., 1978b). Since there is a conflicting report about differentiation of intratypic variations on the basis of electrophoretic mobility of RNA from rotaviruses (Espejo et al., 1980; Kalica et al., 1978a), it is unlikely to be
used for differentiating large numbers of homologous strains. So serum neutralisation tests appear to be the best at present for differentiation of intratypic variations of rotaviruses. The rotavirus genome is segmented, new rotavirus strains may emerge through formation of recombinant viruses in a manner analogous to the reassortment of influenza virus RNA (Hightower and Bratt, 1977) and similar to the high recombinant rate achieved between different reovirus serotypes in mixed infections (Cross and Fields, 1977). The oligonucleotide mapping as has been used for differentiation of influenza viruses (Young et al., 1979) may be tried for intratypic variation of the rotaviruses.

The isolation of human rotaviruses in human foetal kidney, intestinal cells and foetal bovine kidney cells were unsuccessful (Albrey and Murphy, 1976; Purodham et al., 1975; Wyatt et al., 1976a). The explanation for this could be that the embryonic cells were nonfunctional and it is known that in babies effective renal blood flow rises between 12 and 48 hours after birth (Oh et al., 1966), so Ca$^{2+}$ concentration of kidney cells was also low. So human rotavirus isolation should be tried in primary monkey kidney cells which also has endogenous proteases (Baron and Barnett, 1960) with higher concentration of Ca$^{2+}$ in the medium and the other conditions as reported elsewhere (Chapter 2). Treatment with trypsin will facilitate uncoating of the virus. Since humans are close to apes, monkey kidney cells could be expected to have receptors for human viruses.

It is well known that bovine parvoviruses (Storz and Leary, 1979) and feline panleucopenea (FPL) (Johnson, 1967) virus replicate only if cells are dividing rapidly. The present study indicates similar conditions necessary for the replication of rotavirus and this correlates to the rapid rate of turnover of intestinal absorptive cells. So the conditions for cultivation of all new enteric viruses should be tried as suggested for rotavirus in the present study.
## CONTENTS OF APPENDICES

| Appendix I |  
|---|---|
| 1. Test for fungal and bacterial contamination | 179 |
| 2. Preparation of media for cultivation of mycoplasma | 179 |
| 2.1 Inoculation of media | 180 |
| 3. Direct test for demonstration of mycoplasma. | 181 |

| Appendix II |  
|---|---|
| Staining of coverslip cultures in haematoxylin and eosin. | 183 |

| Appendix III |  
|---|---|
| Photography | 184 |
| (a) Light microscopy | 184 |
| (b) Electronphoto micrograph | 184 |

| Appendix IV |  
|---|---|
| Preparation of grids for electron-microscopy of viruses. | 185 |

| Appendix V |  
|---|---|
| Preparation of borate Kcl buffer | 186 |

| Appendix VI |  
|---|---|
| Immunofluorescence titration of rotavirus in microtiter plate. | 187 |
APPENDIX I

1. TESTS FOR FUNGAL AND BACTERIAL CONTAMINATION.

Preparation of media:—

(a) Solid Media: Malt agar and blood agar plates were obtained from the Media Preparation Section of the Department of Microbiology.

(b) Liquid Media: Bacto nutrient broth (dehydrated, Difco Laboratories) and Thioglycollate medium, USP (Oxoid Ltd.,) were prepared in distilled water and sterilised by autoclaving (15 lbs/15 minutes) when cold the media was distributed in 10 ml volumes to sterile tubes and stored at 4°C until use.

Inoculation of cell culture fluid to media:—

A loop full of cell culture fluid inoculated to both solid media plates by streak plate method. One ml of cell culture fluid was inoculated to both liquid media. Along with uninoculated controls, the plates and broths were incubated at 20°C and 37°C for 15 days and 21 days respectively. Subcultures of 1 ml of broths were made to fresh medium and incubated as above. Three successive cultures were made and broths were examined for development of turbidity in the medium.

2. PREPARATION OF MEDIA FOR CULTIVATION OF MYCOPLASMA.

Liquid medium base was prepared by adding 2.1% (W/V) of Bacto PPLO broth (dehydrated, Difco Laboratories) to 1 litre of distilled water. This was distributed in 70 ml volumes in 4oz. medical flat bottles and autoclaved (15 lbs for 15 minutes)

Solid medium base was prepared by adding 0.75 g of Ionagar No.2 (Oxoid Ltd.,) to 70 ml liquid medium base before autoclaving. Both media were stored at 4°C
Yeast extract was prepared from active dried yeast (The Distillers Co (Yeast) Ltd.,) by adding 25 gms to 25 ml of distilled water by constant stirring. The mixture thus prepared was boiled for 5 minutes in a boiling water bath. The solution was centrifuged (4,000 Xg for 30 minutes) and the supernatant was autoclaved (10 lbs for 10 minutes). The sterile solution was again centrifuged (8,000 Xg for 30 minutes) and the supernatant was stored in 20 ml volumes at 4°C.

A stock solution of 10% (w/v) of thallous acetate (B.D.H. Laboratory Reagents) was prepared in distilled water, autoclaved (10 lbs for 10 minutes) and stored at 4°C.

A stock solution of penicillin (1000,000 units/ml) was prepared by dissolving the contents of 2 vials (Benzyl penicillin (sodium) B.P., Glaxo laboratories) in 10 ml of sterile deionised water and stores at 4°C to be used within 3 weeks.

(a) Preparation of liquid growth medium.

To liquid base unheated horse serum No. 3. (Wellcome Reagents Ltd.,) 20% (v/v), Yeast extract stock solution, 10% (v/v), and penicillin stock solution, 0.2% (v/v), were added.

(b) Preparation of solid growth medium.

To the molten solid base at 45°C, horse serum, yeast extract, thallous acetate and penicillin were added in the quantities described for liquid growth medium. The medium was held at 45°C and 5 ml volumes were poured onto 50 mm Petri dishes (ESCO (Rubber) Ltd.,). The Petri dishes were stored at 4°C and used within 10 days of preparation.

2.1 INOCULATION OF MEDIA

(a) Culture in liquid media: 1 ml of cell culture fluid was inoculated into 15 ml of liquid medium and the culture was incubated at 37°C for
6 days. Subculture of inoculated broth was achieved by inoculating 1 ml of broth to fresh liquid media and after 3 or 6 days of incubation at 37°C were inoculated to fresh liquid media as described above. From inoculated liquid medium from each transfer a few drops of broth was inoculated onto three plates of solid media.

(b) A few drops of cell culture fluid were inoculated directly onto three plates of solid media.

(c) A few drops of scraped cells were placed onto three plates of solid medium.

All plate cultures were inoculated at 37°C either aerobically or anaerobically in anaerobic jars containing 5% CO₂ nitrogen mixture.

The liquid broth medium was examined for development of turbidity. The inoculated plates were examined in an inverted light microscope for development of mycoplasma colonies.

3. DIRECT TEST FOR DEMONSTRATION OF MYCOPLASMA

The method of Fogh and Fogh (1964) was followed. Coverslip cultures in Leighton's tubes were washed twice with PBS after which 0.75 ml of 0.6% (W/V) sodium citrate (B.D.H. Chemicals Ltd.,) solution was added to each tube and to that, 0.25 ml of distilled water was added dropwise, making 0.45% final concentration of sodium citrate. The tubes were left for 10 minutes at room temperature and 1 ml of Carnoy's fixative was added drop by drop to each tube. The mixture was poured off and replaced by 2 ml of Carnoy's fixative alone. The fixation of cells continued for 10 minutes at room temperature. At the end of fixation, the coverslip cultures were removed, air dried and stained with Orcein (G.T.Gurr) for 5 minutes. The cultures were washed three times with absolute alcohol, mounted in DPX mountant, examined in oil immersion objectives of microscope and photographed.

Carnoy's fixative was prepared by mixing 1 part of glacial acetic
acid (B.D.H. Chemicals Ltd.,) to three parts of absolute ethyl alcohol (James Burrough).

Orcein stain: A 2% solution was prepared by dissolving natural orcein in boiling glacial acetic acid. The solution was allowed to cool to room temperature and was adjusted to 60% glacial acetic acid by addition of distilled water. Before use, the solution was filtered twice through Whatman No. 1 filter paper.
APPENDIX II

STAINING OF COVERSILIP CULTURES IN HAEMATOXYLIN AND EOSIN

The coverslip cultures in Leighton tubes were washed twice with PBS and were fixed in a 10% solution of formalin in PBS for at least 24 hours. The cultures were then washed twice with PBS, dehydrated by treatment for two minutes each in methyl alcohol (70%, 80%, 90%, 100%) and stained by haematoxylin for four minutes. The preparation was dipped in acid alcohol (1% hydrochloric acid in 70% methyl alcohol) to remove excess stain and the coverslips were blued in tap water before staining in 1% alcohol soluble eosin (B.D.H.Reagents Ltd.) for five minutes. The slides were dehydrated in an ascending series of alcohol (80%, 90% and absolute methyl alcohol). The cultures were cleansed by two changes is xylene and mounted onto clean slides in DPX mountant. The slides were examined by either ordinary or phase contrast microscopy and photographed.
APPENDIX III

PHOTOGRAPHY

(a) **Light microscopy**: All photographs except immunofluorescence were taken in 35 mm Ilford Pan F, 50 ASA films. Films were loaded to developing tank and developed with Unitol developer (Photo Technology Ltd., ) 1 : 10 dilution in tapwater at 20°C. The developing continued for five minutes with agitation at the end of each minute. Films were washed twice in running tap water and were fixed for 15 minutes in Kodafix (Kodak Ltd., ) 1 : 3 dilution in water with agitation at the end of each minute, then washed in running tap water for 30 minutes, followed by washing in distilled water. The films were air dried at room temperature.

Photographs were printed onto Veribrom 3F (Kodak Ltd., ) printing paper in an enlarger, followed by developing in an Ilford automatic printer, photographs were fixed in Kodafix (1 : 7 dilutions in water) for two minutes. After washing in running tap water for five minutes, the photographs were air dried at room temperature.

(b) **Electron photo micrograph**: All electronmicroscopy photographs were processed by the Structural Studies Unit of the University of Surrey and printed as described for light microscopy photograph.
PREPARATION OF GRIDS FOR ELECTRONMICROSCOPY OF VIRUSES:

These were prepared based on the method of Howatson (1969). Copper grids (3.05mm, 400 mesh, Agar Aid) were prepared as follows:

A precleaned dry slide was immersed in a solution of 0.3% (W/V) polyvinyl formal (Formvar) in chloroform at an angle of 45° and were removed immediately. The slide was air dried and a rectangular area was marked on the slide with a scalpel blade. The marked slide was immersed slowly at an angle of 45° into a beaker of distilled water. A thin film of Formvar membrane was floated on the surface to which copper grids were placed gently one by one. A rectangular filter paper was placed gently on the grid and was allowed to trap the grids between the filter paper and Formvar. The filter paper containing grid was removed and air dried in a Petri dish with grids at the top of the filter paper. The Formvar film was subsequently stabilised by evaporating onto it a thin layer of carbon (approximately 45Å) in vacuum.
APPENDIX V

Preparation of borate KCl buffer.

1) Borate KCl buffer pH 8.6 was prepared by adding 0.7456 g of potassium chloride (AR Hopkins and William Ltd) and 0.618 g boric acid crystals (LR, BDH) to 50 ml of deionised water in a volumetric flask to which 12 ml of 0.2M sodium hydroxide (AR, BDH Chemical Ltd.) was added and diluted to 200 ml in deionised water to give a pH 8.6 when 0.01M MgSO$_4$·7H$_2$O (AR, BDH) was added the pH of the buffer dropped to 8.4.

2) Borate KCl buffer pH 8.0 was prepared exactly as above but 3.97 ml of 0.2M sodium hydroxide was added to give pH 8.0. When 0.01M MgSO$_4$·7H$_2$O was added the pH of the buffer dropped to 7.9

The buffers were sterilised by filtration (0.22μm membrane filter, Milipore Inc.) and stored at room temperature (20°C)
**APPENDIX VI**

**Immunofluorescence Titration of Rotavirus in Microtitre Plate.**

The method of Thouless *et al.*, (1977) was followed with modification for titration of rotavirus in microtitre plate by indirect immunofluorescence test.

LLCMK$_2$ cells, a continuous cell line of monkey cells (obtained from ARC Institute for research on animal diseases, Compton) was used for microtitration of virus. The cells were grown in 10% (V/V) Eagle's MEM (10X, Wellcome Reagents Ltd.), 10% (V/V) Foetal Bovine serum (Flow Laboratories Ltd.); 0.132% (W/V) sodium bicarbonate (Wellcome Reagents Ltd.) and 1% (V/V) of penicillin (100 IU/ml) and streptomycin (100 µg/ml) to which sterile deionised water was added to make up to 100 ml of growth media.

A 0.1 ml of LLCMK$_2$ cells ($1 \times 10^6$ cell/ml) was added to each well of microtitre plate (Linbro), the plates were sealed with plate sealer (Titertek) and incubated at 37°C until confluency. The cell sheet was washed with Eagle's MEM. Decimal dilution of virus were made in Eagle's MEM and 0.04 ml of each dilution was added to four replicate wells to which 0.1 ml of maintenance medium was added. The plates were sealed and centrifugated (1200 Xg for 1 hour) at room temperature in an MSE Magnun centrifuge swingout platform after which the plates were incubated for 22 - 24 hours at 37°C. The medium was discarded and the cell sheet was washed once with PBS, then fixed with cold 80% acetone for 15 minutes. The plates were throughly air dried at 37°C. A drop of a 1/20 dilution of gnotobiotic calf antirotavirus serum (G199) was added to each well, incubated in a humidified chamber at 37°C for 1 hour, then washed three times with PBS. A drop of a 1/40 dilution of fluorescin-conjugated rabbit antibovine gamma-globulin (Nordic Immunological Ltd.) was added to each well,
incubated at 37°C for 1 hour as above and washed three times with
PBS. Uninfected control cells were also treated as infected
cells. The cell sheets were observed through the floor of the well
of inverted plates and using an incidence ultraviolet light in a Zeiss
Orthomat microscope. Specific fluorescence was observed only in
cytoplasm of infected cells. The fluorescence was recorded either
+ve or -ve.
ABBREVIATIONS

Cell Culture
AGMK = African green monkey kidney primary
Balb/C-3T3 = Mouse cell line
BEL-2 = Bovine embryonic lung
BGM = Buffalo green monkey kidney
BHK-21 = Baby hamster kidney
BSC-1 = African green monkey kidney
CHO = Chinese hamster kidney
CV-1 = African green monkey kidney
FCDC-2 = Foetal calf diploid cells
FL = Human amnion cell line
HAE-70 = Human amnion cell line
HEL = Human embryonic lung
HRT 18 = Human rectum adenocarcinoma cells
KSV/XLV NRK - infecting uncloned normal rabbit kidney (NRK) cells with Kristen Sarcoma virus (KSV)/xenotropic leukemia virus pseudotype
L-cells = Mouse fibroblast
LLCMK$_2$ = Rhesus monkey kidney cells
MA-104 = Embryonic rhesus monkey kidney cells
MDBK = Madin and Darby bovine kidney cells
MDCK = Madin and Darby canine kidney cells
MRC-5 = Medical Research Council - 5
PK-15 = Porcine kidney
PS cells = Porcine kidney stable line
PK$_{13}$ = Rabbit kidney cell line
SMBE-3 = Skin muscle of bovine embryo
Vero = African green monkey kidney
WI-38 = Wistar Institute - 38

Miscellaneous
Bacl$_2$ = Barium chloride
BHV = Bovine herpes virus
B UdR = 5-bromo-deoxyuridine
BVDV = Bovine virus diarrhoea virus
Ca = Calcium
CF = Complement fixation
CIEOP = Counter-immunoelectroosmophoresis
DNA = Deoxyribonucleic acid
E = Eosin
EDIM = Epizootic diarrhoea of infant mice
ELISA = Enzyme-linked immunosorbent assay
EM = Electronmicroscopy
ETEC = Enterotoxigenic E. coli
FA = Fluorescent antibody
FPV = Feline panleukopenia virus
FS = Family study
FVPT = Fluorescent virus precipitation test
G-banding = Giemsa banding
H = Haematoxylin
HA = Haemagglutination
HI = Haemagglutination inhibition
IAHA = Immune adherence haemagglutination assay
IEM = Immune-electronmicroscopy
IgA = Immunoglobulin A
IgG = Immunoglobulin G
IgM = Immunoglobulin M
InvEC = Invasive E. coli
IUDR = 5-ido-2-deoxyuridine
Kcl = Potassium chloride
Mc = Montgomery county agent
MEV = Mink enteritis virus
MgCl2 = Magnesium chloride
MgSO4 = Magnesium sulfate
Na = Sodium
NCDC = Neonatal calf diarrhoea coronavirus
NCDV = Nebraska calf diarrhoea virus
'O' agent = Offal agent
Q-banding = Quinacrine banding
RIA = Radioimmuno assay
RNA = Ribonucleic acid
SA11 = Simian agent 11
TCID50 = Tissue culture infective dose 50


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