STUDIES ON VIRAL INFECTIONS OF THE RESPIRATORY TRACT IN CATTLE

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Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh, in the Faculty of Science.

August, 1968.

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INTRODUCTION

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Starvation and malnutrition in this world, with a rapidly increasing population poses an immense problem. A prime concern therefore has been to encourage as well as foster crop and livestock husbandry both by national governments and international agencies such as F.A.O. Livestock husbandry plays a vital role in this drive for increased food production by the efficient conversion of feeds utilizable only by livestock into proteins which are of high biological value. However, a prerequisite for maximal conversion rates is a high standard of animal health, for, a diseased animal can only produce a fraction of the output of a healthy animal as determined by parameters such as diminished milk production and loss of weight.

A particular concern in recent years has been that of respiratory infections of cattle kept under intensive conditions of husbandry. Morbidity statistics published by the Ministry of Agriculture, Veterinary Investigation Centres in Britain for the years 1961 to 1963 indicate that 9.5% to 10.8% of calves examined at post mortem had died of pneumonia (Report 1964, 1966). It has been estimated that for every calf that dies, there is a loss in the region of £20 to the individual farmer (Thompson 1966). Beveridge (1967) states that £1,100,000 is lost from the animal industries in the developed countries of Britain, North America and France. He attributes a major portion of this loss to the effects of insidious infections of the respiratory and gastrointestinal tracts.

Clinically viral infections of the respiratory tract have essentially a similar symptomatology. An elevated temperature with a mild rhinitis, a degree of anorexia and conjunctivitis are the initial manifestations. Sometimes a cough develops. With progression of infection, however, bacterial involvement occurs and a more severe disease results with death in a percentage of cases as a consequence of pneumonia or fades into a chronic form with considerable weight losses and diminished milk production (Blood and Henderson 1961).

Historical Review of Literature of Bovine Respiratory Disease

The occurrence of "colds" and influenza-like diseases in cattle have been observed for over 120 years. White (1825) and Youat (1834) described clinical conditions affecting the respiratory tract of cattle in England. These authors described epizootics of catarrh with coughing or "Hoose" involving whole districts in England. Epidemiologically these outbreaks occurred during the winter and early spring months, when the weather was wet and cold as the "wind is easterly" (White 1825), in crowded and overheated byres or "when oxen had been driven over considerable distances to fairs and markets". These epizootics of cold and coughing affected young animals and cows after calving. White also states that "sometimes it comes on without any perceptible cause and prevails in such a degree as to appear contagious". Similar outbreaks of cattle influenza occurring on the continent in 1889 and 1892 have

also been reported, either in association with prevailing epidemics of influenza in man or independently (Hutyra, Marek, Manninger 1938). In Britain and America a particular respiratory infection was seen, following transportation of cattle over long distances either by rail or sea. The animals became sick within about 10 days after arrival at their destinations. The clinical symptoms presented by these animals varied from a mild upper respiratory infection to a more severe and chronic pneumonia. Since this syndrome was associated with movement of animals, the condition was called "transit" fever in Britain and "shipping fever" in North America (Anderson 1939, Brandenburg 1917). Since the most consistent feature in these diseases was the occurrence of pulmonary consolidation in a percentage of the affected stock, veterinary workers began to study the causes of these pneumonias.

The application of bacteriological techniques in the study of "pneumonias" in cattle resulted in the isolation of different types of bacteria. The most constant of the bacteria that were isolated were members of the Pasteurella Spp: (Brandenburg 1917, Farley 1932, Carter et al 1955). Scott and Farley (1932) in a preliminary bacteriological report on shipping fever found that in an investigation of 125 herds of cattle affected with this condition. Pasteurella boviseptica was the commonest bacterium isolated. Hoerlein et al (1961) found that nearly 60% of cattle with clinical shipping fever carried Pasteurella Spp in their nasal passages.

Although members of the Pasteurella group were frequently

isolated from the pneumonic lung, the acceptance of this bacterium as the primary cause of pneumonia was not total. Jorgensen (1925) studied the bacterial flora of the nasal passages of 250 apparently normal cattle and found that P. boviseptica could be isolated from 37 cases (15%). After feeding experiments on cattle, one cow was chilled and driven for one hour and then sprayed on the nose with cultures of P. boviseptica. This treatment resulted in congestion of lungs and diarrhoea. A second cow was chilled, driven for 7 miles and then fed P. boviseptica. This animal died of clinical shipping fever. From these experiments, Jorgensen concluded that P. boviseptica could be pathogenic when animals were exposed to stress. Bosworth and Lovell (1944) were able to recover 9 Pasteurella Spp from the upper respiratory tract of 113 apparently normal cows and calves in three different herds. Scott and Farley (1932) isolated P. boviseptica from four lungs which had no visible lung lesions and they concluded that although P. boviseptica may be the primary bacterial factor of shipping fever, the causative factor in the production of the disease must be another agent.

Another body of evidence presented against these bacteria being the primary agents of pneumonia, was that, experimental inoculation of calves with cultures (18 to 24 hours old) did not consistently produce clinical disease or pneumonia (Gale and Smith 1958). Further, in vaccine studies Farley (1932) using bacterial vaccines and aggressins prepared from P. boviseptica failed to prevent the occurrence of shipping fever. In his investigation of 100 to 200 outbreaks of shipping fever a loss of 3.58% occurred

amongst 566 vaccinated animals, whilst among 411 untreated animals the loss was only 1.02%. Whether or not this increased loss was due to other factors such as, for example, anaphylactic shock is not discussed.

Although these studies suggested that Pasteurella Spp were not the primary cause, their role in the development of clinical pneumonitis was still considered likely. Treatment of sick animals with antibiotics brought about clinical improvement. Prophylactic use of "Terramycin" in feeds within 3 to 5 days after shipment of cattle to feedlots reduced the incidence of shipping fever (Hawley 1957). In a more recent vaccine trial using Pasteurella vaccines obtained from commercial sources, Palotay et al (1963) observed a reduction in the incidence of respiratory disease in the vaccinees (9.38%) as compared to the non-vaccinated group (21%). This study indicated the importance of Pasteurella Spp and also suggested that other agents might be involved in bovine respiratory disease.

The first demonstration that a virus was involved was provided by the Irish workers, Lamont and Kerr (1939). These investigators found that in calves 2 to 9 weeks of age, many died as a result of pneumonia with a morbidity of nearly 50%. In many animals killed and examined immediately after death by bacteriological techniques, no bacteria were isolated. However, when impression smears were made from these cases, Gram -ve coccobacillary forms were found which would only grow with difficulty in heated blood agar. These workers also demonstrated that Seitz filtered suspensions of lungs when inoculated intranasally would induce nasal discharge and

coughing from the 7th day onwards. From these experiments they concluded that calf pneumonia was infectious and probably the exciting cause was a virus.

About this time, Waldman and Kobe (Hutyra, Marek, Manninger 1938) demonstrated that bacteria-free filtrates of lung tissue from a racehorse involved in an outbreak of epidemic catarrh could transmit the disease to cattle. Further, they demonstrated that foals artificially infected with the horse virus could transmit the disease to cattle and thereby give rise to an enzootic affecting 82% of these animals. Jennings and Glover (1952) recovered a virus from calves in an outbreak of pneumonia in England. Pooled turbinates and lung suspensions from infected calves were passed through a gradacol membrane of 0.67 mμ porosity. This filtrate when inoculated into calves intravenously, intranasally and intratracheally produced turbinitis, mild diarrhoea and pneumonia. This agent did not grow in embryonated eggs or infect mice, ferrets, hamsters and guinea pigs.

In 1954, Schroeder and Moys described a respiratory disease affecting cattle in California and Colorado. The predominant clinical signs were nasal discharge, explosive cough, excessive salivation and grinding of teeth. When antibiotic-treated pooled sputum and nasal material from sick cows in two dairies were inoculated intranasally into 4 to 6 month old heifers, they developed an elevated temperature accompanied by nasal discharge and coughing. An uninoculated calf placed in contact with the inoculated calves also developed an upper respiratory tract

infection within three days (McIntyre 1954).

The tool which made possible the study of the virus aetiology in the respiratory tract infections of cattle was the application of tissue culture techniques. In 1949, Enders, Weller and Robbins showed that poliomyelitis virus multiplied in fibroblasts which grew out from explants of human embryonic tissue in culture with the production of cytopathic effects. The use of trypsin to disperse cells from tissue fragments by Rous and Jones (1916) and Scherer et al (1953) effectively led to the development of tissue culture monolayer techniques. The application of these techniques in veterinary medicine to the investigation of respiratory infections in cattle has led within the past eight years to the discovery of a great number of viruses.

Bovine Respiratory Viruses

Infectious bovine rhinotracheitis virus (IBR)

In 1956, Madin, York and McKercher reported the isolation of a virus in bovine embryonic kidney from cattle with acute respiratory disease from Colorado and California. This is now known as the IBR virus. The clinical signs are referable to an upper respiratory tract infection. Considerable work has been done by McKercher and his colleagues in elucidating the aetiology and epidemiology. The disease is characterised by elevated temperature (40°C to 41°C), rhinitis and tracheitis and runs a course of about ten days. In severe cases the disease enters into a more chronic phase with the deposition of diptheritic pseudomembranes in the anterior third of

the trachea and as a consequence of bacterial extension secondary pneumonia occurs. The disease occurs mainly in feedlots and is more severe in beef cattle than in dairy cattle, occurring within one to three weeks after the introduction of cattle from an outside source. The morbidity in infected herds is high and varies from 30 to 100% whilst the mortality is low (2 to 10%). Although prevalent throughout the year the disease was more severe during the winter months (McKercher et al 1955, 1957 and McKercher 1959). The clinical disease occurs in a more severe form in farms where the stocking rate is high and the cattle are in close contact. It is postulated that in such situations, due to rapid spread, the virulence of the virus is enhanced. The disease has also been known to occur in farms where no recent introduction of cattle has been made. In these instances it is presumed that following infection, the virus may persist in sites such as the conjunctival sac and be intermittently excreted (McKercher et al 1963). The virus has been isolated from field cases of conjunctivitis and mild respiratory disease in North America (Abinanti et al 1961a, Quinn 1961) and in England (Darbyshire et al 1962, Darbyshire and Shanks 1963).

IBR virus is a herpes virus and shares many of the properties of other members of this group. Incorporation of nucleic acid analogues such as 5-bromodeoxyuridine into the growth medium during virus replication in a continuous line of calf kidney cells results in a decrease of infectivity by approximately five hundred fold in the virus yield as compared to the controls (Stevens and Groman 1964).

This metabolic inhibitor acts by blocking the conversion of deoxyuridylic acid to thymidylic acid and thus inhibits viral DNA synthesis. This effect is, however, reversed by the addition of thymidine.

Virus particles when examined under the electron microscope have shown a cubic symmetry with 162 capsomeres having a diameter of 125 to 150 mμ (Cruickshank et al 1965, Watrach et al 1966). The virus grows in a variety of renal cells of bovine, caprine, ovine and porcine origins (Andrewes 1964). In calf kidney cell cultures, IBR virus produces a cytopathic effect consisting of cells with inclusions and rounded cells with pyknotic nuclei. These inclusions are situated centrally, within the nucleus, are acidophilic when stained with haematoxylin and eosin and are similar to Cowdry type A inclusions (Cheatham and Crandell 1957). Margination of the chromatin is a characteristic feature in virus infected cells. Orsi and Cabasso (1958) have demonstrated that the inclusion material was Feulgen negative whereas the marginated chromatin was strongly Feulgen positive. It is believed that these inclusions represent coagulated protein formed after the mature virus particle has left the nucleus (Cowdry 1934). Stevens and Chow (1959) have shown that the demonstration of inclusions requires a highly acidic fixative containing acetate ions at pH 1.3 and a mordanting agent such as picric acid. Ultrathin sections of IBR virus-infected cells examined by electron microscopy have shown the developmental cycle to be similar to that of the herpes virus hominis. Development commences in the nucleus where particles consisting of a nucleoid

and a single outer membrane can be demonstrated. At the next stage, these virus particles appeared in the cytoplasm and were found to have a double outer membrane. These particles apparently represent mature virus. (Armstrong *et al* 1961, Grinyer *et al* 1962, Wildy and Watson 1962). The virus is sensitive to ether and other lipoidal solvents and is acid labile (Griffin *et al* 1958).

In cattle the virus of IBR has been associated with a variety of diseases. Australian workers were the first to associate this virus in outbreaks of meningoencephalitis in a herd of four to six-month-old calves, where the mortality was nearly 50% (Johnston *et al* 1962). IBR virus designated strain N569 was recovered in calf kidney and bovine adrenal cell cultures following inoculation with infective brain suspension from these calves (French 1962 a, b). Meningoencephalitis associated with IBR infections has also been reported by Barenfus et al (1963) and Gardiner *et al* (1964).

The other important clinical syndrome associated with IBR virus in cattle is infection of the genital tract (Kendrick *et al* 1958) and abortions (McKercher and Wada 1964).

Comparative studies to establish or refute the existence of strain differences between isolates of IBR virus causing different clinical syndromes have as yet not been made. Opinion at present is that all isolates are antigenically homogenous.

Bovine para-influenza 3 (PI_3)

Chanock and his colleagues in their investigations of respiratory tract infections in children isolated several viruses in

monkey kidney monolayer cultures. These viruses grew poorly or not at all in eggs and did not produce a cytopathic effect in tissue cultures, but were recognised by their ability to adsorb guinea pig erythrocytes on to the cells in which they multiplied (Chanock et al 1958, Vogel and Shelekov 1957). This group of apparently new viruses were called the para-influenza viruses (Andrewes et al 1959). Four types of para-influenza viruses are recognised, Types 1, 2, 3 and 4.

The basic properties that characterise these viruses are similar to the other members of the myxovirus group. Structurally they are spherical particles which are pleomorphic, having a diameter varying from 150mμ to 800mμ. Unlike the influenza viruses they are extremely fragile when prepared for electron microscopy. The virion consists of an internal coiled helical nucleo-protein component which when lying free on the grid has a characteristic "herring bone" appearance with a diameter of 18mμ. This constitutes the soluble (S) or the nucleo-protein antigen. Surrounding the nucleo-protein is the envelope which is believed to be of host material and consists of lipid and protein and essential for infectivity (Waterson et al 1961, Waterson and Hurrell 1962, Waterson 1962). It is ether sensitive, acid labile, heat-sensitive and haemagglutinate guinea pig erythrocytes (Andrewes 1964). These viruses possess haemolytic activity and neuramidase activity (Chanock and Coates 1964). Antigenically they are distinct from influenza A, B and C with which they show no sharing of complement fixing antigens. Each type of para-influenza virus,

however, though antigenically distinct when tested by complement fixation, haemagglutination-inhibition and neutralization tests show some heterologous reactions amongst themselves (Cook et al 1959). Further, the para-influenza viruses have been shown to be antigenically related to the mumps virus and Newcastle disease virus, when adult human sera are used. These heterologous rises in titres occur as a consequence of an anamnestic reaction due to early experiences with mumps or parainfluenza viruses.

In 1959 Reisinger et al in their investigations of the shipping fever syndrome in Maryland, U.S.A., reported the isolation in calf kidney cell cultures of an haemagglutinating and haemadsorbing virus, which they designated the SF-4 virus. This virus was isolated from the nasal mucus of two Angus feeder steers approximately 8 months of age. On primary isolation, this agent induced cytopathic effects consisting of rounding of cells with subsequent detachment from the glass surface. The cytopathic effects were observed 48 hours after inoculation and were complete by the fifth day. The virus possessed the ability to haemagglutinate guinea pig, bovine and human type O erythrocytes, the reaction occurring both at $4^{\circ}C$ and room temperature. It was sensitive to ether and had the same structural details as human para-influenza viruses when viewed under the electron microscope (Waterson and Hurrell 1962, Dawson 1964).

The antigenic relationship of the SF-4 strain and other members of the para-influenza viruses was first shown by Abinanti and Heubner (1959). Using hyperimmune rabbit and rooster sera and sera from three naturally infected cattle, these workers found that the

SF-4 strain was serologically indistinguishable from the human prototype HA-1 strain of para-influenza 3. However, Ketler et al (1961) reported that when hyperimmune guinea pig sera prepared against the human and bovine strains were tested by the complement fixation and haemagglutination-inhibition tests, the inhibiting titre of these sera was 4 to 8 times higher when the homologous virus was used as compared to the heterlogous virus. Further, they found that the bovine SF-4 antigen was broader in reactivity than the human strain. Abinanti et al (1961) extended these studies to include 7 human and 5 bovine strains of para-influenza 3. By the haemagglutination-inhibition test and the use of pooled immune guinea pig sera, these workers confirmed the findings of Ketler et al (1961). Although these workers suggest very distinct antigenic differences between the human and bovine strains, these viruses appear to be closely related. Jensen et al (1962) immunised ferrets by intranasal inoculation with a human and the bovine strain (10068 Phillips) of para-influenza 3 viruses. Convalescent immune serum samples when subjected to cross haemagglutination-inhibition tests, showed only a two to eight fold difference in inhibiting titres when the homologous and heterlogous viruses were used as antigens. Similar two to four fold differences have also been observed using immune sera prepared from hamsters and guinea pigs (Churchill 1963).

In addition to this very close similarity in their antigenic constitution, Inaba et al (1963) demonstrated haemolysin activity with their strain of para-influenza virus isolated in Japan.

Recently Drzeniek et al (1967) and Dawson and Patterson (1967) have shown that bovine para-influenza strains which they examined had low but definite neuramidase activity.

Since the first isolation of the SF-4 virus, para-influenza viruses type 3 have been isolated from several incidents of shipping fever in the U.S.A. and from respiratory conditions of cattle in several countries of the world. Bakos and Dinter (1960) isolated a strain of bovine para-influenza called the Umea strain from an epizootic in cattle occurring in Northern Sweden in 1958-1959. In England, PI_3 virus has been isolated from the tonsils of a healthy calf by Dawson (1964) and from an outbreak of pneumonia and respiratory disease by Betts et al (1964) and Dawson et al (1964). In Australia, St. George and French (1966) have reported the isolation of PI_3 from lung tissue of a calf dying of pneumonia.

Although PI_3 virus appears to be essentially a respiratory tract pathogen, virus has also been isolated in other clinical syndromes of cattle. Deas et al (1966) in Edinburgh have reported the isolation of a strain of PI_3 virus from the testicles of an infertile bull, whilst Sattar et al (1965) have recovered the virus from an aborted foetus in Ohio, U.S.A. Hamdy (1966) isolated strain 1750 of bovine para-influenza 3 from the large intestines of a 36 hour old Jersey calf which had died of pneumoenteritis.

Bovine adenoviruses

In 1965, Aldasy, Csontos and Bartha (1965) published their virological investigations of an outbreak of pneumoeneritis in young calves two weeks to four months of age which was assuming epizootic

proportions in Hungary in 1962. From 21 of 52 affected calf herds these workers reported the isolation of 78 strains of virus which were recovered from nasal and conjunctival discharges, pneumonic lungs, bronchial exudates and faeces samples from the affected animals.

The isolates examined were all ether resistant and grew with difficulty only in calf testis monolayer cultures forming intra-nuclear inclusions, but not in calf kidney monolayer cultures. One of the isolates S102/2 possessed a common group complement fixing antigen when tested against a human anti-adenovirus serum, whilst convalescent calf sera fixed complement in the presence of an antigen prepared from human adenovirus types 1, 4, 7 and 15.

In England, Darbyshire et al (1965) have reported the recovery of a bovine strain of adenovirus WBR 1 from a swab taken from the eye of an apparently healthy cow, in calf kidney monolayer cultures. The possession of common group antigens to human adenovirus type 5 was demonstrated both by complement fixation and gel diffusion tests.

In addition, two isolates of bovine adenoviruses had been made in 1959 and 1960 in the U.S.A. by Klein and his co-workers, isolations being made in calf kidney cell cultures from faeces samples obtained from two apparently normal cattle.

These bovine strains of adenoviruses are distinct from each other when tested by the neutralization test (Klein, 1962, Darbyshire et al 1965, Bartha and Áldásy 1965).

Reoviruses

Rosen et al (1960, 1962) in the course of their studies

on the natural history of reoviruses in animals were the first to isolate these viruses from cattle aged 1 to 24 months. The reoviruses of cattle were isolated from 3 herds in Maryland from faecal specimens, recoveries being made only in monkey kidney monolayer cultures. All three types of reoviruses were isolated, the commonest being types 1 and 3. By haemagglutination-inhibition tests using specific sera from convalescing children and calves undergoing a primary infection, the cattle strains were indistinguishable from the human strains. No respiratory illness attributable to these viruses were observed in these three herds.

Rhinoviruses

In 1962, Bögel and Böhm reported the recovery of a virus from a calf 4½ weeks of age. The earliest symptom presented by this animal was an elevated temperature (40.7°C) and a clear seronasal discharge with conjunctivitis. The virus was recovered from the nasal passages in calf kidney cells but was not isolated from the lungs, blood, internal organs or faeces. This virus was resistant to the action of chloroform and trypsin but was acid-labile, infectivity decreasing by approximately 2 $\log_{10}$ TCD_{50} on exposure for half hour at 37°C to a pH of 5.2. Wizigmann and Schiefer (1966) have also reported the isolation of strains of rhinoviruses from the upper respiratory tract of 12 calves approximately 1 month of age.

Bovine enteroviruses

As in human virology, bovine enteroviruses have been associated

with respiratory disease in cattle. However, very little work appears to have been done in delineating their role in such infections. Moll and Davies (1959) and Huck and Cartwright (1964) have reported the isolation of bovine enteroviruses from young calves two weeks to six months of age showing symptoms of nasal discharge, coughing, pneumonia and mild enteritis, isolations of virus being made from faecal specimens; whilst Omar (1966) mentions their recovery from pneumonic lungs.

Objectives

At the commencement of this investigation within the Edinburgh area, although respiratory illnesses were known to occur in several farms no virus recoveries from the respiratory tract had been made. Therefore, as a first step such an investigation was undertaken. The work presented in this thesis consists of the investigations into two outbreaks of respiratory disease in cattle as it occurred in the Edinburgh area in 1966/67 and the studies on the biological properties of the isolates.

MATERIALS AND METHODS

General methods and experimental procedures used in parts I and II of this thesis are described in this section

Tissue Culture Media

All media used were prepared according to methods described by J. Paul in "Cell and Tissue Culture" (1959) unless otherwise stated.

(a) Growth medium for primary and second generation calf kidney cells

This contained Hank's balanced salt solution (HBSS) with 0.25% lactalbumin hydrolysate (HLA) and 10% calf serum (Hank's and Wallace 1949, Melnick and Riordan 1962).

(b) Maintenance medium for cultures

In most experiments, the maintenance medium used was medium 199 (Glaxo) supplemented with 0.5% lactalbumin hydrolysate and 2% inactivated horse serum. Medium 199 was obtained from Glaxo Laboratories, Stokes Poges, England, as 10 x solution. This was diluted in sterile distilled water to give a 1 x solution. Sodium bicarbonate (4.4%) was added to give a final concentration of 0.165% bicarbonate before use.

In some experiments, Earle's lactalbumin yeast extract (ELY) was used (Robertson et al 1955). This medium supplemented with 2% inactivated horse serum was employed as maintenance medium.

(c) Eagle's medium

For the determination of the pH sensitivity of the viruses

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(c) Eagle's medium

For the determination of the pH sensitivity of the viruses

under study, Eagle's medium (Eagle 1955) was used as recommended by Hamparian et al (1963).

(d) Virus diluent

All virus and serum dilutions were made in M/15 Sorensen's phosphate buffered saline containing 0.75% bovalbumin (Armour) at a final pH of 7.1 ± .1.

(e) Transport medium

Nasal swabs collected from cattle in the field were transported to the laboratory by immersing them in 3 to 4ml of HBSS supplemented with 2% bovalbumin (pH 7.0) in bijoux bottles. To reduce bacterial and fungal growth, this medium was supplemented with 500 units of penicillin, 200 µg of streptomycin and 50 units of mycostatin per ml.

(f) Erythrocyte suspension

For haemadsorption and haemagglutination tests, 0.5% guinea pig red blood cells in saline solution were used. Guinea pigs were bled by heart puncture and the blood stored in an equal volume of Alsever's solution at 4°C. Prior to use cells were spun down in a 10ml graduated centrifuge tube and washed three times in saline solution. For the final washing the red blood cells were sedimented at a speed of 1500 r.p.m. for 10 minutes. The packed cell volume was read off and a 10% cell suspension was made. This was diluted further to give an 0.5% suspension just before use. For removal of natural agglutinins for guinea pig erythrocytes in sera to be tested by haemagglutination-inhibition test, 0.1ml of a 50% erythrocyte suspension was added to 1ml of diluted sera. In some

instances an equal volume of a 10% cell suspension was used instead of the 50% suspension.

(g) Penicillin-streptomycin solution

A solution of penicillin and streptomycin was made up in normal saline for addition to tissue culture media as an aid in controlling contamination. The concentration of penicillin and streptomycin in the stock solution was such that 0.25ml of this solution when added to 100ml medium gave a final concentration in the media of 250 units of penicillin and 100 µg of streptomycin per ml of medium.

(h) Erythrocin solution

A stock solution of erythrocin was prepared in distilled water so as to contain 60mg per ml. One ml of this solution was added to 100ml of growth medium. This antibiotic was considered essential for the prevention of bacterial contamination of primary cell cultures.

(i) Mycostatin solution

A stock solution of mycostatin was prepared in distilled water so as to contain 5000 units per ml. One ml of this solution was added to 100ml of medium to prevent fungal contamination of monolayer cultures.

Cells for use in Tissue Culture

Calf kidney cells

Kidneys were obtained from the abattoir from calves aged about

4 to 6 months for the preparation of calf kidney monolayer cultures. The method employed was essentially based on Younger's (1954) method for the preparation of monkey kidney cell cultures. The cortical tissue was separated from the medulla, minced finely with scissors and repeatedly washed with phosphate buffered saline (PBS) (Dulbecco and Vogt 1954) at pH 7.2 to remove erythrocytes. The minced cortical tissue was trypsinized for 30 minutes at 37°C in a 500ml indented flask containing approximately 200ml of a 0.25% trypsin in PBS. The contents were agitated by means of a magnetic stirrer. The supernatant fluid was then discarded and fresh trypsin added. Ten trypsinization cycles, each of 7 minutes duration were carried out at 37°C. Cells released from each trypsinization cycle were collected in growth medium containing 20% calf serum and kept at 4°C. On the completion of the trypsinization cycles, the cell harvests were packed by centrifugation at 200 r.p.m. for 30 minutes, the packed cells were resuspended, pooled, and diluted in growth medium when a cell count was made. The final cell suspension usually used to initiate monolayer cell cultures was 3×10^5 cells per ml unless otherwise stated.

Preparation of Monolayer cell cultures

Roux flasks

Roux flasks were seeded with 100ml of suspensions of primary renal cells in growth medium. On the third day of incubation at 37°C, the medium was removed and fresh growth medium was added. Complete monolayers formed in 6 to 8 days.

Primary cells grown in Roux flasks were used for the preparation of second generation renal cells. Secondary cell cultures were used extensively in the work reported in this thesis unless otherwise stated.

For the preparation of second generation cell cultures, growth medium from previously monolayered Roux flasks was removed and the monolayer washed with PBS. Five to 10ml of trypsin-versene mixture was added and gently pipetted up and down until all the cells were detached from the glass. Cell suspensions obtained in this way were used to initiate second generation cell cultures in 15 x 150mm pyrex tubes.

Tube cultures

Second generation monolayer calf kidney cell cultures were grown in 15 x 150mm pyrex tubes with white rubber stoppers. The cell concentration used to initiate such cultures was 5×10^6 cells per ml, and the usual inoculum of cell suspension per tube was 1 ml. When seeded at this concentration monolayers formed within 24 to 48 hours on incubation at 37°C in a stationary position.

Tube coverslip cultures

For studies of virus cytopathic effects in second generation calf kidney cells, monolayer cultures were prepared on coverlips in pyrex tubes. An inoculum of 1.5ml of cell suspension per tube was used. Monolayers formed after 24 to 48 hours of incubation, when they were used.

Bottle cultures

Monolayer cultures of primary calf kidney cells grown in 4oz.

medicine "flats" were used for a limited number of plaque formation experiments. The bottles were seeded with 12ml of cell suspension. The growth medium was replaced after 4 to 5 days incubation at 37°C with ELY supplemented with 5% calf serum. The bottle cultures were ready for use on the 6th or 7th day.

Petri plates

Primary monolayer cultures of calf kidney cells grown in plastic Petri plates (Esco Grade AA) were used in plaque formation experiments. Cell suspensions containing 5 x 10^5 cells per ml were filtered twice through one layer of gauze and dispensed in 8ml amounts into each Petri plate. The plates were then incubated at 37°C in a humidified atmosphere containing 4 to 5% carbon dioxide. On the fourth day the medium in the plates was replaced by ELY containing 5% calf serum. Cultures were ready for use on the 6th or 7th day.

Viruses

Viruses used in this study could be grouped into:-

I. Viruses isolated from the respiratory tract of cattle from field cases of respiratory infections.

II. Reference viruses.

I. Viruses isolated from field cases

B/MOR1/66

This virus was isolated from the nasal swab of calf 75 aged about 7 months at a farm in Edinburgh, the details of which are

reported in Part I of this thesis. It was used at the 2nd, 3rd, 7th, 8th and 9th passage levels in calf kidney cell cultures.

7F73/1

This virus was isolated from swab material from the nasal turbinate passage from a steer 7F73. The animal was brought in for neuropathological examination to this Institute. The details of the population of cattle from which this and two subsequent strains of virus were isolated are given in the results section of Part II of this thesis. It was used in its first calf kidney pass.

C2/66

This was an encephalitogenic strain of virus which was originally isolated from the brain of a dead yearling steer in December 1966 in Scotland. It was used in its second and third calf kidney pass.

C1/66

This strain was isolated from a nasal swab collected from a sick animal in the same affected herd from which C2/66 was obtained. It was used in its second and third calf kidney pass.

Reference Viruses

1. Human para-influenza 3

This strain was obtained from the Central Public Health Laboratory, Colindale, at the fifth passage level in human embryo kidney. It was passed once more in human embryo kidney cell cultures and then in primary calf kidney monolayer cultures. It was used after one, two and three passes in calf kidney.

2. Bovine para-influenza 3

This strain was isolated in England from the tonsils of a healthy calf (Dawson 1964). It was obtained in the form of infective tissue culture fluid at the 13th passage level in calf kidney cultures. It was used at the 14th to 17th passage levels in calf kidney cell cultures.

3. Bovine para-influenza 3 (SP_1 strain)

This virus was isolated from the testicles of an infertile bull (Deas et al 1966). It was received at the 5th passage level in calf kidney cultures and was used at the 6th passage level in calf kidney cell cultures.

4. Infectious bovine rhinotracheitis virus (IBR)

The "Oxford" strain of IBR virus (Darbyshire et al 1962) was obtained as an ampoule of freeze-dried material from Dr. J.H. Darbyshire of the Central Veterinary Laboratory, Weybridge, England. It was used to infect a Roux flask of second generation calf kidney cells for the purpose of preparing a virus pool. It was used at the 16th to 18th passage levels in calf kidney cell cultures.

5. Bovine mammilitis virus

A strain of bovine mammilitis virus isolated from the teats of a cow (Deas et al 1966a) was used to determine any antigenic relationship to the above isolates. The virus had been passed five times in calf kidney cell cultures and once in calf testis monolayer cultures. Infective tissue culture fluid from this passage was used for the preparation of anti-serum in rabbits and in

neutralization tests described in part II of this thesis.

Preparation of stock virus pools

Stock virus pools of the above strains were prepared in primary or second generation calf kidney monolayer cultures grown in 4oz. medicine "flats" or Roux flasks. Infective tissue culture fluids were inoculated either undiluted or at a dilution of 10^{-1} or 10^{-2} in 0.5ml or 1ml amounts on to previously washed (with HBSS) monolayer cultures. After an adsorption period of one hour at 37°C, maintenance medium (12ml per bottle culture; 100ml per Roux flask) was added and the cultures were incubated stationary at 37°C. Cultures were examined daily for cytopathic effects. When approximately 90% of the cell sheet was involved infective tissue culture fluids were harvested and spun at 500 r.p.m. for five minutes. In the preparation of pools of para-influenza viruses, the monolayers were tested for adsorption of 0.5% guinea pig erythrocytes at room temperature.

Infective tissue culture fluids were stored in 0.2ml amounts in sealed ampoules or in bijoux bottles (2ml) at -65°C or at -20°C. Tissue culture fluids were usually harvested on the fourth, fifth or sixth day with the para-influenza viruses and on the second or third day with the IBR viruses.

Antisera

All antisera used against the indicated viruses were prepared in this laboratory in normal rabbits, unless otherwise stated.

1. Antiserum against isolate B/MOR1/66

Hyperimmune sera against this isolate was prepared in rabbits R1 and MR1.

i. Rabbit R1 was given intravenously 2ml of infective tissue culture fluid of isolate B/MOR1/66 at the second passage level in calf kidney cell cultures. A sample bleed was made six days later and designated $R1_{(A)}$. A second intravenous inoculation of 2.5ml infective tissue culture fluid was then given and 46 days later a third intravenous inoculation of 1ml of infective tissue culture fluid was given. The animal was bled 14 days later.

ii. "Early" phase rabbit antierum MR1 was also prepared. 2.0ml and 2.5ml of isolate B/MOR1/66 at the 7th tissue culture pass in calf kidney cell cultures were inoculated intravenously at a seven day interval. The rabbit was bled 7 days later.

2. Antiserum against isolate C2/66

For the preparation of this antiserum, isolate C2/66 was grown in calf testis cultures. Two inoculations of 1.5ml infective tissue culture fluid were given intradermally at multiple sites on day 0 and day 6. Two further intravenous inoculations of 1.0ml and 1.5ml of infective tissue culture fluids were then given at intervals of six days. The animals was bled, 17 days after the last inoculation.

3. Antiserum against T1 strain of bovine para-influenza 3

Rabbit AS1 was given 2.0ml of infective tissue culture fluid intravenously and 14 days later a further 2.0ml was given by the same route and 3.0ml intraperitoneally. Ten days after this the

animal was bled.

Rabbit DR1 was given two intravenous inoculations of 3ml infective tissue culture fluid seven days apart and was bled seven days after the second inoculation. The serum obtained from this bleed constituted the "early" phase serum.

4. Antiserum against SP1 strain of bovine para-influenza 3 (bovine testicular strain)

Rabbit SR1 was inoculated according to the schedule as for rabbit DR1 and bled seven days after the last inoculation.

5. Antiserum against human para-influenza 3

A human strain of para-influenza 3 received from the Central Public Health Laboratory, Collindale, was passed once in calf kidney monolayer cultures. 2.0ml of infective tissue culture fluid was inoculated intravenously into a rabbit PR4. Fourteen days later the animal was bled and the immune serum so obtained was designated $PR4_{(A)}$. A second intravenous inoculation of 2.0ml and an intra-peritoneal inoculation of 3.0ml of infective fluid was then given. The animal was then bled ten days after this.

6. IBR antiserum

Rabbit RS was given five weekly intravenous inoculations of 1.0ml infective tissue culture fluid. The animal was bled one week after the last inoculation.

7. Bovine mammilitis virus antiserum

Rabbit R19 was inoculated according to the schedule shown in Table I. The animal was bled on day 71.

TABLE I

DOSAGE SCHEDULE USED FOR THE PREPARATION OF ANTISERUM IN RABBIT R19 AGAINST BOVINE MAMMILITIS VIRUS

DAY	DOSE AND ROUTE OF INOCULATION
0	Prebled
0	1.5ml intradermal (multiple sites)
7	1.5ml intradermal (multiple sites)
14	2.0ml intravenous
47	2.0ml intravenous
54	3.0ml intravenous
61	1.0ml intravenous
71	Bled, serum separated and stored at -20°C

8. Colindale antisera

For initial typing of isolate B/MOR1/66 studied in part I of this thesis, lyophilized neutralizing antisera were obtained from the Central Public Health Laboratory, Colindale, England. These antisera had been prepared against the following members of the myxovirus group.

i. Influenza A

ii. Human strains of para-influenza 1, 2 and 3.

iii. Simian strain of para-influenza 2 (SV5).

These were reconstituted in distilled water (0.2ml) and used according to the instructions given on the label. The neutralizing titre of the antisera were not given, but the final dilution at which the sera were to be used was given. This varied according to the preparation.

Antisera prepared against influenza A and para-influenza 2 simiae were to be used at a final dilution of 1 in 400. Antisera against human strains of para-influenza 1 and 2 were to be used at a final dilution of 1 in 200, whilst that against human para-influenza 3 was 1 in 50.

For neutralization tests these sera were diluted 1 in 8 or 1 in 10, inactivated at 56°C for half an hour and serial doubling dilutions then made up to or beyond the stated dilution.

9. IBR "Oxford" antiserum

Hyperimmune serum prepared against the "Oxford" strain of IBR virus was obtained from Dr. J.H. Darbyshire, Central Veterinary Laboratory, Weybridge, England. This serum was obtained as a

freeze-dried ampoule and was reconstituted in 0.5ml of distilled water prior to use. The neutralizing titre (ND_{50}) of this serum was given as 1 in 518.

Collection and storage of sera

Blood samples obtained from the heart or ear vein from immunised rabbits and jugular vein from cattle were allowed to stand at room temperature to clot. The clot was freed from the sides of the bottle and left overnight at 4°C. Serum was separated from the clot, centrifuged at 3,5000 r.p.m. for 30 minutes. The supernatant serum was then dispensed into ampoules or bijoux bottles and stored at -20°C.

Detection and Infectivity Titrations of Para-influenza 3, IBR and bovine mammilitis viruses

Detection and titrations of infectivity of para-influenza 3 viruses for calf kidney monolayer cultures were determined by the haemadsorption technique of Vogel and Shelekov (1957). IBR and mammilitis viruses were, however, detected by the occurrence of cytopathic changes in these cell cultures.

Before infection of the cell sheet, monolayer cultures grown in tubes were washed twice with HBSS to remove any inhibitors which might be present in the growth medium. Virus-containing material, either undiluted or in serial log dilutions were then inoculated on to the cultures and virus was allowed to adsorb on to the cells for one hour at 37°C, the cultures being kept in a stationary position. At the end of this period, maintenance medium was added and the

cultures were re-incubated as before. Infectivity determinations were then made on the third or fifth day post inoculation by the haemadsorption technique or on the fifth day by the cytopathic changes in the monolayer cultures.

i. Haemadsorption and haemadsorption-inhibition test

Tissue culture fluids from infected monolayer cultures were removed and 0.2ml of an 0.5% guinea pig erythrocyte suspension was added to each tube. After addition of red cells, 1ml of HBSS was added to the tubes and the cultures were incubated horizontally at room temperature for 30 minutes. At the end of this period the cultures were shaken, the cell suspension tipped off and examined under the microscope for adsorption of the red cells on to the cell sheets. In instances where the adsorption of red cells was questionable the tube cultures were washed with two changes of HBSS and re-examined under the microscope. Uninfected tube cultures were similarly treated and served as controls. Infection was indicated by adsorption of the erythrocytes to the cell sheet.

For haemadsorption-inhibition tests, hyperimmune serum was diluted 1 in 8 in saline and inactivated at 56°C for half an hour. Tissue culture fluids were removed and the cell cultures were washed twice with HBSS. 0.2ml of the antiserum and o.8ml of HBSS were added to each tube rolled periodically to ensure the spread of the inoculum over the cell sheet and the tubes left at room temperature for about 45 minutes. At the end of this period, the tubes were washed once with HBSS and 0.2ml of a 0.5% guinea pig erythrocyte suspension was added as before. Infective tube

Cultures treated similarly but with saline were set up as controls and the tubes were read for adsorption of erythrocytes as before.

ii. IBR viruses

The infectivity of the IBR viruses described in part II were determined by their cytopathic effects in tissue culture. The cytopathic changes consisted of foci of rounding of cells which progressively increased until there was complete destruction of the monolayer, with detachment of the cells from the glass surface. Final readings were made on the fifth day.

iii. Bovine mammilitis virus

The infectivity of bovine mammilitis virus was also determined by its cytopathic effect in calf kidney cultures. These changes consisted of typically "ballooned" cells and formation of syncytial masses which subsequently sloughed off the glass surface. In experiments where this virus was used, final readings were made on the seventh day.

From the results of the titration of these viruses, the 50% end point (TCD_{50}) per 0.1ml of virus suspensions were determined by the Kärber method (Rhodes and Van Rooyen 1962).

Plaque Assay

The infectivity of strains of bovine para-influenza 3 and IBR viruses were also measured by the plaque assay procedure (Dulbecco and Vogt 1954).

Serial 1.0 log, 0.5 log or 0.3 log dilutions of virus were made in virus diluents and mixed with an equal quantity of virus diluent. 0.2ml of each virus was inoculated on to three or four confluent monolayer cultures of

calf kidney grown in plastic Petri plates. Before virus inoculation, the cultures were washed twice with HBSS. Virus was allowed to adsorb on to the cells for 2 hours at 37°C in an atmosphere of 4 to 5% carbon dioxide. To prevent drying of the monolayers, they were periodically tilted. At the end of the adsorption period, the monolayers were washed once with 5ml of HBSS and overlaid with nutrient medium containing 0.9% agar for the para-influenza viruses and 0.7% agar for the IBR viruses. Seven ml of overlay was poured per plate. The overlay was allowed to harden at room temperature, following which cultures were incubated at 37°C in an humidified incubator containing 4-5% CO_2. On the fourth day, a second overlay containing 0.001% neutral red was added, 6ml per plate and the plaques were counted 1, 2 or 3 days later, depending on the virus under study.

The agar overlay was prepared by mixing equal quantities of 1.8% Noble agar (Difco) solution and double strength nutrient medium. 1.8% agar was prepared in ion exchange water, sterilized at 10lbs pressure for 10 minutes and kept at 43°C.

The nutrient medium used was that recommended by Porterfield for plaque assay of arboviruses in chick embryo fibroblasts (Porterfield 1960, 1964) with slight modifications and is shown in Table II. It was kept at 37°C, and mixed with agar solution before use.

The titre of the virus was expressed as the number of plaque-forming units per ml of the undiluted virus suspension (PFU/ml).

TABLE II

CONSTITUTION OF MEDIA FOR PLAQUE ASSAY OF BOVINE STRAINS OF PARA-INFLUENZA 3 AND IBR VIRUSES IN CALF KIDNEY MONOLAYER CULTURES

INGREDIENTS*	NUTRIENT MEDIA I (ml)	NUTRIENT MEDIA II (ml)
Gey A (x 10)	20	20
Gey B	10	10
Gey C	10	10
5% lactalbumin hydrolysate	10	10
5% proteose peptone	10	10
Inactivated horse** serum	10	10
Neutral red (1:1000)	-	10
Ion exchange water	33	23
Antibiotics	1	1

* prepared without phenol red

** concentration of horse serum was reduced to 6% in experiments with IBR virus.

In vitro Neutralization Tests

The neutralization tests used in this work were carried out either in tube cultures or as plaque neutralizations. In both methods an estimate of the infectivity titre (TCD_{50} or PFU) per 0.1ml of each virus suspension used was determined by previous titration in calf kidney cell cultures, grown in tubes or Petri plates, by methods described previously. The viruses were then used at a dilution estimated to contain approximately 100 TCD_{50} or 100 to 200 PFU per 0.1.

Tube culture neutralization tests

Two procedures were used in tube culture neutralization tests.

(a) Constant virus, varying-serum method.

(b) Constant serum, varying-virus method.

Of these two methods, the former was the more frequently used whilst the latter was used in typing of isolates C1/66 and C2/66 where the amount of antiserum available was limited.

In method (a) the titres of neutralizing antibody in sera were determined by mixing serial two-fold or four-fold dilutions of antiserum with an equal quantity (0.5ml) of virus diluted to contain approximately 100 TCD_{50} of virus in 0.1ml of suspension. The mixtures were incubated at room temperature for 1 hour. However, in the neutralization tests carried out in part II of this work, the serum-virus mixtures were held at room temperature for 2 hours or overnight at 4°C. At the end of this period, 0.2ml amounts of serum-virus mixtures were inoculated on to three or four tube cultures. After an adsorption period of half an hour at 37°C in a

stationary position, 1ml of maintenance medium was added to each tube. The tubes were then incubated stationary at 37°C. On the third or fifth day the neutralizing end point was determined by either examination for haemadsorption or occurrence of cytopathic effects. Absence of haemadsorption or cytopathic effects indicated neutralization.

From the results, a Kärber estimate of the 50% neutralizing end point, the ND_{50} was obtained. Controls included an internal virus titration to determine the actual dose of virus used, tubes inoculated with serum only at the lowest dilution used to determine cell toxicity, uninoculated tissue cultures tubes and tubes inoculated with virus plus pre-inoculation serum.

In some tests, a limited "chequer board" neutralization test was carried out. In these tests, equal quantities of serial ten-fold dilutions of virus were incubated with serial two or four-fold dilutions of serum at room temperature for 2 hours and then inoculated in 0.2ml amounts on to 3 or 4 tube cultures as before.

In testing for antigenic relationships reciprocal cross neutralization tests were performed. The procedure was as described above.

In the constant serum, varying-virus method, hyperimmune IBR virus antiserum and bovine mammilitis virus antiserum R19 were used. For these tests, the antiserum was diluted to contain an estimated 20 ND_{50} in 0.1ml.

Serial ten-fold dilutions of the unknown isolates and the known IBR "Oxford" strain or bovine mammilitis virus were made in virus

diluent. Equal quantities of each virus dilution (0.5ml) were mixed with a constant dilution of hyperimmune antisera. The mixtures were incubated at room temperature for 2 hours. 0.2ml aliquots of serum-virus mixtures were then inoculated into each of four previously washed monolayer culture tubes. Virus was allowed to adsorb for half an hour after which 1ml of maintenance medium was added and the cultures incubated stationary at 37°C. The cultures were examined daily for cytopathic changes and a final reading was made on the fifth day or on the seventh day in the case of bovine mammilitis virus. Controls included:-

1. Tubes which contained serial ten-fold dilutions of virus with normal rabbit serum at the same dilution as the test serum.
2. Tubes which contained serial ten-fold dilutions of virus in virus diluent only.
3. IBR, "Oxford" and bovine mammilitis viruses were also treated in the same manner as the unknown isolates with IBR or bovine mammilitis antisera containing 20 ND_{50} per 0.1ml.
4. Uninoculated tissue culture tubes.

From the results, the log neutralization index was calculated. The log neutralization index = titre of virus in the presence of normal control serum - the titre of the virus in the presence of hyperimmune serum.

Plaque neutralization tests

In plaque neutralization tests, antisera were diluted in serial two-fold or four-fold dilutions and incubated with an equal quantity

(0.5ml) of virus suspension diluted to contain approximately 100 to 200 PFU per 0.1ml for 2 hours at room temperature. At the end of this period, 0.2ml aliquots of serum-virus mixtures were inoculated on to two previously washed monolayer cultures grown in Petri plates. The plates were then incubated as previously described. In order to estimate the actual virus dose used in the test, a virus titration as described in the plaque assay section was done. In addition, 0.2ml of diluent only was inoculated to serve as tissue culture controls. 0.2ml of the lowest dilution of sera used were inoculated as above to test for cell toxicity. Subsequent procedures followed is as described in the section on plaque assay. The 50% neutralizing titre of the sera (ND_{50}) tested were calculated by the Kärber method.

Neutralization rate constant determination

When two closely related viruses are neutralized by antiserum produced against one, the rate of neutralization of the homologous virus is faster than the heterologous virus (McBride 1959). Dulbecco et al (1956) and Mandel (1961) using a poliomyelitis virus-antibody system have shown that when the logarithm of the proportion of virus survivors after treatment with a fixed dose of antiserum are plotted against time on the arithmetic scale, a straight line is obtained indicative of a first order reaction until more than 99% of the virus population has been neutralized. This reaction, between virus and antibody, has been expressed mathematically by the equation

$$K = \frac{2.3\ D}{t}\ \text{Log}\ \left(Po/Pt\right)$$ where

K = fractional rate of inactivation per unit time and is a characteristic for a particular virus under a given set of experimental conditions

D = dilution of antiserum

t = time of inactivation

Po = plaque count at zero time

Pt = plaque count at time t.

Since Po and Pt can be determined experimentally, K values can be calculated for homologous viruses as well as heterologous viruses after reaction with antiserum. To compare the K values obtained with different sera, the values are "normalized" on the assumption that the rate of neutralization of the homologous virus is 100 or unity.

The method which was adopted was based on the method described by Furezs et al (1964) for differentiation of poliovirus strains.

Homologous (B/MOR1/66) and heterologous (SP1 and T1) strains of bovine para-influenza virus in their seventh and ninth, sixth and sixteenth to seventeenth tissue culture passages in calf kidney were used. They were diluted to contain approximately 2.5×10^4, 1.7×10^4 and 5.3×10^3 PFU per 0.1ml respectively. A quantity of 0.5ml of these virus suspensions were mixed with an equal volume of rabbit antiserum MR1. The rabbit antiserum was used at a dilution of 1 in 2.5. This dilution of antiserum neutralized approximately 1 to 2 logs of the homologous virus when equal quantities of serum-virus mixtures were incubated for 10 minutes at 37°C.

Serum-virus mixtures were incubated for 10 minutes at 37°C in a water bath, after which 0.1ml of these mixtures were diluted in 10ml of diluent kept in McCartney bottles in an ice bath. As controls, both homologous and heterologous virus suspensions were treated similarly, but with diluent only instead of antiserum. Further dilutions, 1:2 or 1:4 of antiserum treated and untreated virus were made. 0.2ml aliquots of these dilutions were then inoculated on to previously twice washed calf kidney monolayer cultures grown in Petri plates. After an adsorption period of 2 hours, the monolayers were washed once with 5ml of HBSS and overlaid with nutrient medium containing 0.9% agar using 7ml per plate. The rest of the procedure is as described in the section on plaque assay. From the plaque counts obtained on the 6th or more usually

the 7th day after infection of the cell sheets, the following counts were obtained for each of the virus strains examined:-

i. Control plaque count of untreated virus at time zero and time 10 minutes. From these two counts any heat inactivation of virus that may have occurred was determined. The value Po was obtained from the count at time 10 minutes for substitution in the above formula. No heat inactivation of virus was observed during the 10 minute period.

ii. Plaque counts of serum treated virus at time 10 minutes. From this the value Pt was obtained.

Homologous as well as heterologous virus strains were tested on the same day using the same batch of kidney cells.

To determine the exponential fall of homologous virus when treated with serum in relation to time, the procedure was as described above, except that samples of serum-virus mixtures (0.1ml) were removed and diluted in cold diluent at regular timed intervals.

To determine the presence or absence of excess antibody, experiments were done as described above except that the homologous virus was used at two different concentrations, i.e. diluted to contain a concentration of virus 1 $\log_{10}$ higher than was used in the usual experiments.

Haemagglutination and Haemaggutination-Inhibition Test (HI)

The titration of haemagglutinin in infective tissue culture fluids were done with slight modification, according to methods

described by Salk (1944) and the Expert Committee on Respiratory Virus Diseases (W.H.O. Report, 1959) in W.H.O. perspex plates.

Serial doubling dilutions of infective tissue fluids were made in saline in bijoux bottles. 0.2ml of saline was added to each well followed by 0.2ml of each diltuion. The mixtures were shaken and 0.4ml of a 0.5% guinea pig erythrocyte suspension added. As controls, non-infective tissue culture fluid (in some tests only) and saline were included. The mixtures were shaken and left at room temperature. The test was read when the cells in the control wells had settled into a "button". Usually this occurred in about 90-120 minutes. The haemagglutinin titre was the highest dilution which produced agglutination of the erythrocytes. From this result the amount of haemagglutinin for use in the HI test was determined.

In haemagglutination-inhibition (HI) tests, sera to be tested were diluted initially 1:8 or 1:10 in saline and inactivated at 56°C for half an hour. Natural agglutinins for guinea pig erythrocytes which may be present were removed by adsorbtion with 50% guinea pig erythrocytes for 4 hours at room temperature or overnight at 4°C. The cells were sedimented and the supernatant was tested for haemagglutinin-inhibition antibodies.

Serial two-fold dilutions of serum in saline were made such that each well would contain 0.2ml of serum at different dilutions. Antigen to be used was diluted to contain 4 haemagglutinating units per 0.2ml in saline. 0.2ml of this diluted antigen was added to each well containing an equal quantity of serum. The

plates were shaken and allowed to incubate at room temperature for 1 hour. At the end of this period 0.4ml of a 0.5% guinea pig erythrocyte suspension was added to the serum-virus mixtures, shaken and re-incubated at room temperature. Controls included,

i. a titration of the antigen to determine the amount of antigen used in the test.

ii. Saline cell controls.

iii. Serum cell controls.

iv. Pre-inoculation serum.

The test was read when the cells in the control wells had settled into a "button". The HI titre of the serum was the highest dilution of serum which completely inhibited agglutination of the erythrocytes.

Tests for non-specific inhibitors in serum

The presence or absence of non-specific inhibitors in calf and rabbit sera to the para-influenza viruses were determined by one of two methods, treatment of sera with receptor-destroying enzyme (RDE) or trypsin (Difco 1:250) at a concentration of 4µg per ml and M/90 potassium periodate. The procedure for the use of the latter has been described in the chapter on influenza viruses in "Diagnostic Procedures for Viral and Rickettsial Diseases" - Lennette and Schmidt - 3rd Ed: p 465. RDE was used according to the instructions given by the manufacturers (Burroughs-Wellcome).

As controls, saline was added to an aliquot of the sera under test, in similar proportions to the above chemicals. Treated and untreated samples of sera were then simultaneously "run" on the

same day. By comparing the results of the H.I. titre obtained with the treated and untreated sera, the presence or absence of non-specific inhibitors were determined.

Tween-ether treatment of infective tissue culture fluid

The effect of tween-ether on the haemagglutinating activity of bovine para-influenza 3 (T1 strain) was tested by the method described by Norrby (1962).

Haemagglutinin was produced by infecting calf kidney cell cultures grown in Roux flasks with the T1 strain of bovine para-influenza 3. When cytopathic effects were maximal, aliquots of tissue culture fluid was centrifuged in McCartney bottles at 1,000 r.p.m. for 10 minutes at 4°C. The supernatant fluid was transferred to bijoux bottles (1-2ml) which were kept cold by immersing them into plastic bags containing ice. 10% tween was then added to this cold mixtures to give a final concentration of 0.125% tween. The bijoux bottles were shaken in a mechanical shaker for a few minutes. Ether was next added in the proportion of 0.5cc for every 1cc of tissue culture fluid. The mixtures were then well shaken in the shaker for 20 minutes with the bijoux bottles still being kept within the plastic bags. At the end of this period ether-tween tissue culture fluid mixtures were transferred to Wasserman tubes and centrifuged at 2,500 r.p.m. for 20 minutes at 4°C. The aqueous bottom phase was removed, transferred to fresh Wasserman tubes or bijoux bottles and nitrogen was slowly bubbled through to remove the excess of ether. This preparation constituted the tween-ether treated haemagglutinin.

Cationic Stabilization to Heat

In 1961 Wallis and Melnick showed that divalent cations such as Mg^{++} had the property of stabilizing poliovirus against heat inactivation. Since then other workers (Tyrrell and Chanock 1963, Melnick 1962) have utilized this property in the characterisation of virus isolates.

Equal quantity of infective tissue culture fluid was mixed with an equal quantity of 2M $MgCl_2$ so that the final concentration was 1M $MgCl_2$. The mixture was heated in a water bath at 50°C for 1 hour. Virus containing fluid was similarly treated, but with distilled water. A further control was kept at room temperature. At the end of 1 hour the infective titre of these preparations were determined in monolayer cultures of calf kidney grown in tubes as before.

Chloroform Sensitivity

Infective tissue culture fluid was treated with 20% chloroform and left for half an hour at room temperature in a mechanical shaker (Hamparian et al 1963). The mixture was then centrifuged at 1,000 r.p.m. for 10 minutes at 4°C. The supernatant was removed and serial ten-fold dilutions were inoculated into monolayer tube cultures. As control, virus from the same pool was prepared in the same way as treated virus but saline instead of chloroform was added.

Sodium Deoxycholate Sensitivity

Infective tissue culture fluid was mixed with equal volumes (0.5ml) of 1:500 sodium deoxycholate, so that the final concentration of sodium deoxycholate in the mixture was 1:1000 (Theiler 1957). The mixture was allowed to stand at room temperature for 1 hour. Saline controls were set up instead of deoxycholate. After one hour at room temperature, serial ten-fold dilutions of virus treated and untreated were made and titrated in tube cultures.

pH Sensitivity

Infective tissue culture fluid was diluted 1:10 in Eagle's medium without sodium bicarbonate (pH 3:1 - 3:6). As controls infective tissue culture fluid was also mixed with Eagle's medium containing 0.01M Tris buffer at pH 7.2 (Ketler et al 1962a, Hamparian et al 1963). The mixtures were allowed to stand at room temperature for 4 hours. At the end of this time, the infectivity of the virus preparations at the two pH values were determined in tube cultures, dilutions being made in Eagle's medium (pH 7.2).

Electron Microscopy

Infective tissue culture fluids were examined under the electron microscope to determine the approximate size and structure of virus particles.

Isolate B/Morl/66 was inoculated at a dilution of 1:10 on to monolayer cultures of second generation calf kidney grown in a Roux

flask. When complete destruction of the cell sheet had occurred, infective tissue culture fluids were lightly centrifuged (500g) for five minutes at room temperature, to deposit gross cellular particles. The supernatant fluid was then centrifuged at 144,000g for one hour at 3°C to deposit the virus particles. The supernatant was discarded and the deposit was resuspended in 0.1M ammonium acetate and again subjected to light centrifugation at 500g for five minutes.

Drops of the supernatant fluid were applied to carbon coated grids, surplus fluid was then removed and the preparation stained with 2% sodium phosphotungstic acid for two minutes. The preparation was then examined with a Siemens Elmiskop I at an instrumental magnification of 40,000X.

Light Microscopy

The cytopathic effects caused by the multiplication of the viruses used in this study were examined by staining coverslips with haematoxylin and eosin.

Monolayer cultures of calf kidney grown in coverslips were infected with approximately 100 TCD_{50} of virus. Infected and non-infected cultures were stained daily until cytopathic effects were maximal in the cultures. The cultures were fixed in Bouins fixative for 30 minutes, after which the cultures were rinsed in three changes of 70% ethyl alcohol, leaving the cultures for 15 minutes at each change. The cultures were carried through decreasing strengths of ethyl alcohol from 70% to 50% to 25%, then stained for 8 minutes

in an aqueous solution of Mayer's Haematoxylin. They were then "Blued" in running tap water for 5 minutes, rinsed in distilled water and stained with 0.5% aqueous eosin for 2 to 3 minutes. The cultures were rinsed quickly in distilled water and rapidly dehydrated in acetone, acetone-xylol (2:1), xylol-acetone (2:1) and cleared in xylol. The cultures were then mounted in DePeX and examined under the light microscope.

PART I

Investigations of a Mild Upper Respiratory Tract Infection of Cattle

INTRODUCTION

In attempting to isolate viruses from the respiratory tract of cattle, several nasal swabs (103) collected from such animals with histories of nasal discharge or pneumonia were examined in calf kidney monolayer cultures, but with no apparent success.

However, during the latter part of May 1966, a population of calves with respiratory illness was investigated. These calves (Friesians and Ayrshires) were kept under intensive conditions of husbandry at a farm in East Lothian. The practice followed by this farmer was to buy calves at farm sales when they were three months of age. The calves were brought to the farm and housed with the older calves in adjacent pens which were separated by tubular railings. The animals were moved from pen-to-pen with increasing age and weight gain. When the animals were approximately nine months of age, they were sent to slaughter and a new batch brought in. On the first visit to this farm, some animals showed nasal discharge and coughing whilst one was showing pneumonic symptoms. According to the owner, the occurrence of "pneumonia" had been going on for about a week and he had attributed this outbreak to the clearance of hay which was being done at the farm during this time. Symptoms of respiratory disease were still present in the herd on three subsequent visits three and fourteen days later.

A total of 162 nasal swabs collected during the three visits, from 53 of these animals were examined for viruses and bacteria. Nasal swabs for virological examination were transported to the laboratory in transport medium (pH 7.1) in the cold and stored

frozen at -65°C until ready for inoculation. Blood samples were taken from 11 animals, 10, 20 and 30 days after the first swabs were taken. A blood sample was obtained from one animal (calf number 85) on the day the nasal swab was collected which yielded virus. Serum was stored at -20°C.

RESULTS

Nasal swabs kept in transport medium at -65°C were rapidly thawed and the fluid was centrifuged at 3,000 r.p.m. for 45 minutes at 4°C. 0.2ml of the supernatant from each specimen was inoculated on to four, five or six tube cultures of 24 hour old second generation calf kidney cell cultures. Prior to inoculation with such material, the monolayers were washed twice with HBSS. After an adsorption period of 1½ to 2 hours at 36°C, maintenance medium was added and the cultures incubated in a stationary position at 36°C. Cultures were examined at the end of 24 hours and subsequently every two days for the presence of any cytopathic agents. On the seventh day, supernatant fluids from each specimen were pooled and cultures tested for adsorption of 0.5% guinea pig erythrocytes at room temperature. Any specimen yielding a positive result was passed to a fresh set of calf kidney cell cultures both undiluted and diluted 1:10 to establish transmissibility or were stored at -65°C until cells were available.

Table 1/1 gives details regarding the nasal swabs yielding haemadsorping agents. On passage, three of the isolates B/MOR5/66, B/MOR7/66 and B/MOR11/66 were accompanied by granularity of the cell cultures and weak haemadsorption when tested on the fourth day. Infective tissue culture fluids of these isolates were subsequently centrifuged at 10,000 r.p.m. for 1 hour at 4°C and inoculated on to further calf kidney monolayers. After such treatment, with the exception of B/MOR11/66, the other two suspected isolatesdid not show cytopathic effects or haemadsorption. These two specimens

TABLE 1/1

NASAL SWABS FROM CALVES WHICH YIELDED AN HAEMADSORBING AGENT IN SECOND GENERATION CALF KIDNEY CELL CULTURES AND THE BACTERIAL POPULATION RESIDENT IN THEIR NASAL PASSAGES

DATE OF COLLECTION	CALF NUMBER	DESIGNATION OF VIRAL ISOLATE	TYPE OF BACTERIA PRESENT
17. 5. 66.	75	B/MOR1/66	
20. 5. 66.	75	B/MOR2/66	Freidlander Spp: and Streptococcus viridans.
31. 5. 66.	85	B/MOR3/66	Streptococcus viridans.
	294	B/MOR4/66	Streptococcus viridans.
	22	B/MOR5/66	*P. multocida.
	13	B/MOR6/66	P. multocida.
	6	B/MOR7/66	P. multocida.
	288	B/MOR8/66	P. multocida.
	298	B/MOR9/66	P. multocida.
	300	B/MOR10/66	P. multocida.
	284	B/MOR11/66	P. multocida.

* P = pasteurella.

were then considered negative.

The original specimens were re-examined in calf kidney cell cultures after seven to eight weeks storage at -65°C. Three haemadsorping agents were reisolated.

Freshly harvested tissue culture fluid of isolate B/MOR1/66 after two passages in calf kidney cell cultures had an haemagglutinin titre of 1:256 per 0.4ml when tested against 0.5% guinea pig erythrocytes. The same pool of tissue culture fluid after storage for 1 month at -65°C in 1ml amounts showed an haemagglutinin titre of 1:1280 per 0.4ml. No elution of virus from guinea pig erythrocytes was observed after leaving overnight at room temperature.

Table 1/1 also shows the bacteria resident in the nasal passages of these calves yielding haemadsorping agents in cell cultures. The predominant bacteria were the Pasteurella species. Hoerlein et al (1961) in a study of the shipping fever found that 60% of clinically ill cattle carried this species of bacterium in their nasal passages.

Properties of the Isolate B/Mor1/66

Isolate B/MOR1/66 was selected for further study as a representative member of a group of isolates recovered in this outbreak. It was used at the second, third, fourth, seventh to ninth passage levels in calf kidney cell cultures throughout the work reported in this thesis.

The scheme of study of this isolate and of the unknown isolates (Part II) were to study their basic physico-chemical and structural

properties and thus place them within a well defined group. Once this was done, it was proposed to ascertain their antigenic relationships to other well defined members within these groups, consequently arriving at an antigenic identification of the isolates.

Chloroform sensitivity

By determining the effect of ether on the infectivity of viruses, Andrewes and Horstmann (1949) were able to separate viruses into two well defined groups, the ether sensitive and ether resistant viruses. Recently Hamparian et al (1963) have shown that chloroform functions as a more efficient lipid solvent than ether. The mechansism whereby lipid solvents bring about a loss in infectivity is not known. It is probable that they act by disrupting the lipo-protein envelope which surrounds the nucleo-capsid with consequent disorganisation of the capsomeres and thus a loss of infectivity.

Infective tissue culture fluid of isolate B/MOR1/66 when treated with chloroform reduced its infectivity to undetectable levels, indicating that the isolate was lipid sensitive.

pH sensitivity

Hamparian et al (1963) have suggested that lability or stability to acid pH is a basic property of viruses and is a useful criteria for sub-grouping of viruses. On the basis of their studies they have found a close correlation between sensitivity to lipid solvents and acid lability.

Exposure of infective tissue culture fluid of isolate B/MOR1/66 reduced its infectivity by at least $10^{4.8}$ TCD_{50} per 0.1ml suggesting

that the isolate was acid labile.

The results of the effect of chloroform and pH on isolate B/MOR1/66 is contained in Table 2/1.

Cationic stabilization to heat

Wallis and Melnick (1961) have shown that stabilization to heat by divalent cations is a property of enteroviruses and reo viruses. Members of all other virus groups showed enhanced inactivation by this treatment.

The effect of heating infectious tissue culture fluid at 50°C for 1 hour was investigated both in the presence and absence of 1M $MgCl_2$. These results are shown in Table 3/1. Heating diminished the infectivity of the isolate by approximately $10^{4.8}$ TCD_{50} per 0.1ml and no stabilization was conferred on the isolate by 1M $MgCl_2$. This showed that the isolate was not a member of the enterovirus or reo virus group.

Electron microscopy

Electron microscopic observations of infective tissue culture fluid of isolate B/MOR1/66 after two passages in calf kidney showed particles which were spherical in shape (Figure 1/1). The particles showed considerable variation in size, ranging in diameter from 2000Å to 8000Å with most of the particles falling within the range, 2000Å to 4000Å (Figures 1/1 and 3/1). The intact particles were composed of an internal coiled structure which was surrounded by an envelope carrying radially arranged spike-like projections (Figure 1/1 bottom left). Numerous threads or

TABLE 2/1

EFFECT OF CHLOROFORM AND ACID pH ON THE INFECTIVITY OF ISOLATE B/MORI/66 WHEN TESTED ON CALF KIDNEY MONOLAYER CULTURES

SOURCE	TREATMENT	INFECTIVITY TITRE ($Log_{10}TCD_{50}$/0.1ml)
Infective Tissue Culture Fluid B/MORI/66	20% chloroform for ½ hour at room temperature in a mechanical shaker	< 1.0
	SALINE CONTROL	5.8
	EAGLE'S '59, pH 3.1 4 hours at room temperature	< 2
	EAGLE'S '59, pH 7.2 with Tris buffer, 4 hours at room temperature (control)	6.8

TABLE 3/1

EFFECT OF DIVALENTCATIONS ON THE INFECTIVITY OF ISOLATE B/MORI/66 WHEN TESTED ON CALF KIDNEY MONOLAYER CULTURES

SOURCE	TREATMENT	INFECTIVITY TITRE ($Log_{10}TCD_{50}$/0.1ml)
Tissue Culture Fluid B/MORI/66	50°C for 1 hour in the presence of 1M $MgCl_2$	< 1.0
	50°C for 1 hour in the presence of aqua dest	2.0
	Room temperature for 1 hour (control)	6.8

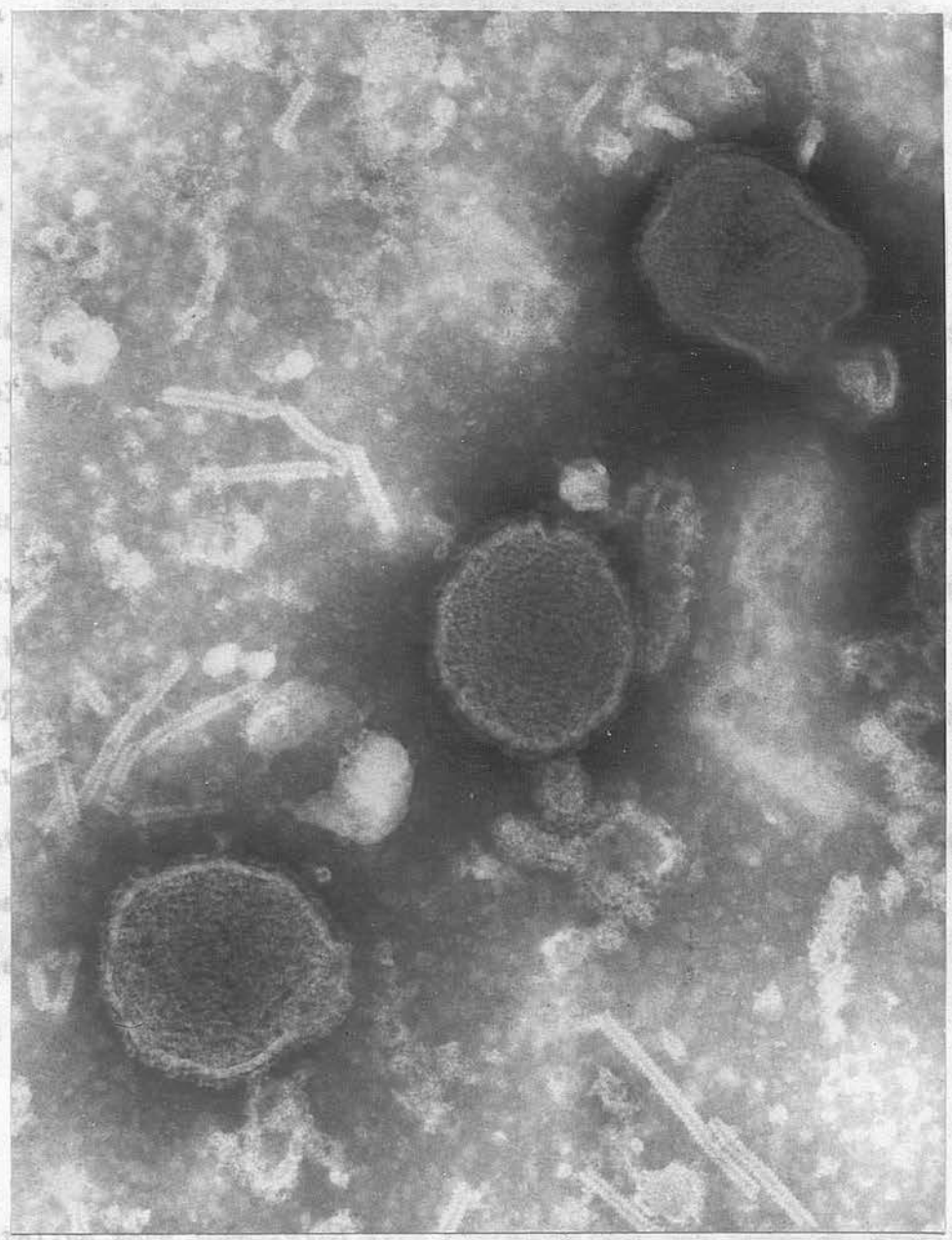

Figure 1/1 Intact Spherical Virus particles of isolate B/MOR1/66 illustrating:

1. Inner coiled structure.

2. Surrounding envelope with projections.

Negative contrast staining with phosphotungstic acid x 120,000.

strands which probably represent the inner helical structure released as a result of the rupture of the intact virus particle and lying free on the grid can be seen in Figures $^{1}/_{1}$, $^{2}/_{1}$ and $^{3}/_{1}$. These structures have a diameter of approximately 200Å with an internal core of 65Å.

The structure and measurements made of the virion of this isolate showed similarities to the structure of the larger myxoviruses namely the Newcastle disease virus - mumps - parainfluenza group; measles - rinderpest - distemper group and respiratory syncytial virus described by Horne et al (1960), Horne and Waterson (1960), Waterson et al (1961) and Waterson and Hobson (1962). These viruses exhibited considerable pleomorphism and their size varied from 1500Å to 6000Å as in the case of mumps virus. Further, unlike the influenza viruses which were smaller these viruses disintegrated spontaneously when specimens were prepared for electron microscopy, releasing their internal helical coiled structure which is the nucleo-capsid, the diameter of this component being 150Å to 170Å with a hollow core of 40Å.

Light microscopy

Confluent monolayer cultures of calf kidney cells grown in coverslips were inoculated with approximately 100 TCD_{50} of isolate B/MOR1/66. At successive 24 hourly intervals, infected and non-infected cultures were removed, fixed in Bouins fluid and stained with haematoxylin and eosin and examined under the light microscope.

In wet preparations, the first noticeable cytopathic effect as seen under the light microscope occurred five days after infection.

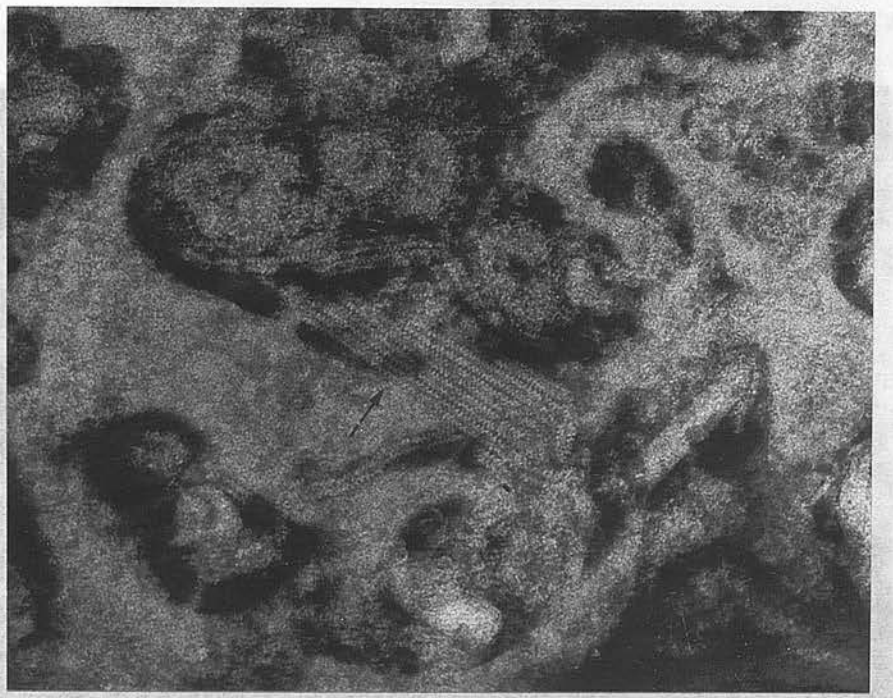

Figure 2/1 Arrow indicates strands having a "herring bone" like appearance lying free on the grid. Probably these strands represent the released inner helical structure as a result of the rupture of the intact virus particle.

Negative contrast staining with phosphotungstic acid x 120,000.

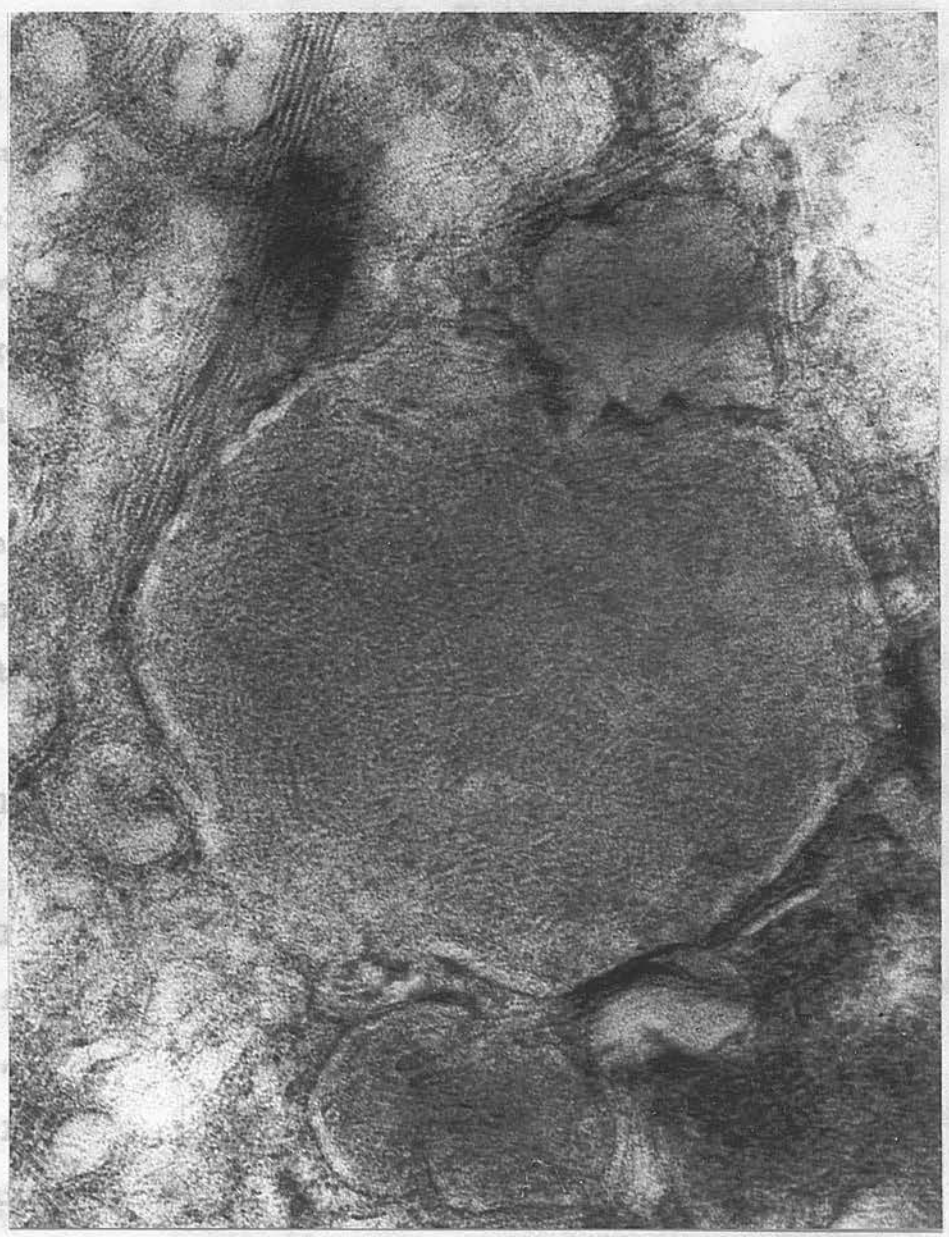

Figure 3/1 Highly pleomorphic virus particle of isolate B/MOR1/66 having a diameter of 8000Å and showing the inner helical structures lying in bundles free on the grid.

Negative contrast staining with phosphotungstic acid x 120,000.

At this time numerous gaps were seen in the infected cells, whilst the cytoplasm of some of the remaining cells were drawn out, thus giving the appearance of a "lattice work" like structure. Such an effect, however, was not evident in the control non-infected cultures. Within the next 48 hours, infection progressed to involve greater areas of the cell sheet and by the seventh day only a few cells were seen attached to the glass surface. At this stage cytoplasmic bridges connecting cells in one group to islets of neighbouring cells were seen.

In stained preparations, however, the earliest manifestation of a cytopathic effect was observed 48 hours after infection. Uninfected calf kidney cells exhibited the typical appearance of epithelial cells being polygonal during the early stages. However, due to confluency of the monolayer and pressure of the adjacent cells, the individual cells appeared to be laterally compressed. The cytoplasm was pinkish and appeared to be foamy with indistinct cellcular outline. Within occasional non-inoculated control cells, eosinophilic inclusions were observed in their cytoplasm. These were usually round and did not appear to distort the nucleus. The nucleus was basophilic, the nucleoli 2 - 6 in number deeply basophilic and the chromatin granular (Figures $^{4}/_{1}$ and $^{5}/_{1}$).

48 hours after infection, eosinophilic intracytoplasmic inclusions were seen in a few cells. These appeared as pin-point reddish masses with a distinct "halo" around them. They differed from the occasional cytoplasmic inclusions seen within a few cells of the non-infected cultures in that they were much smaller. At

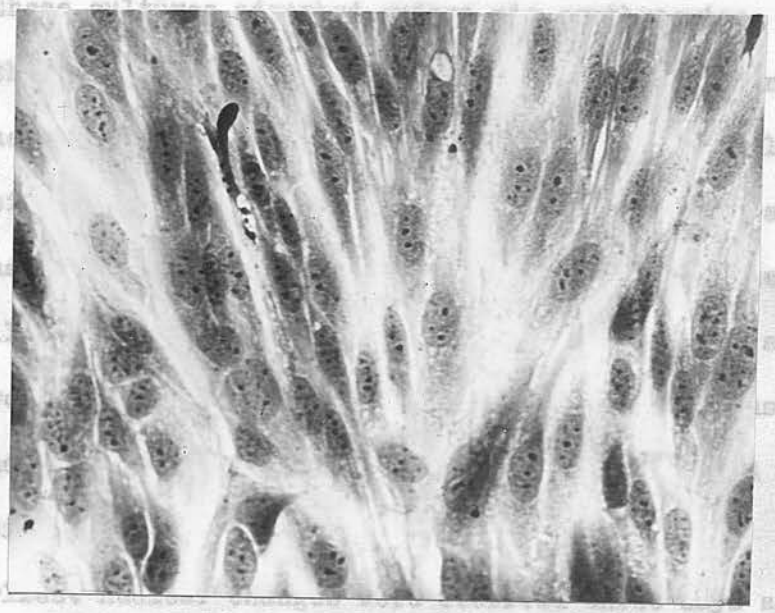

Figure 4/1 Normal 6 day old calf kidney monolayer culture.
Haematoxylin-eosin stained x 440.

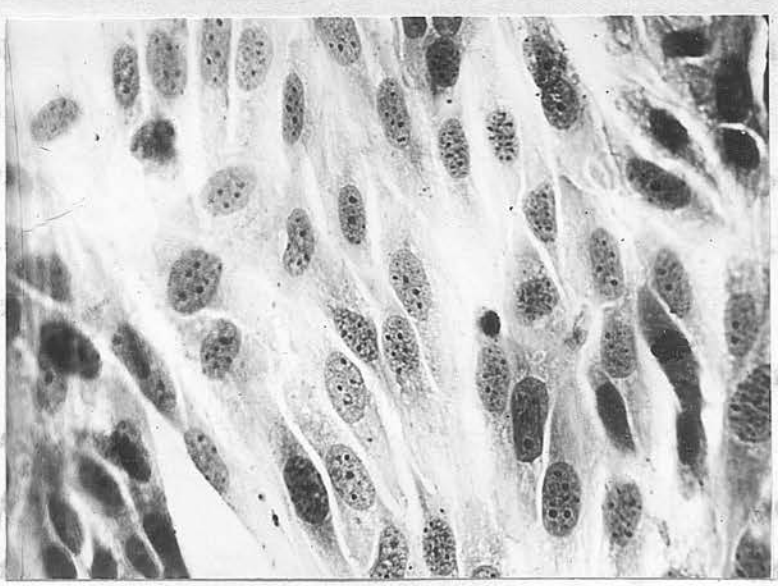

Figure 5/1 Normal 9 day old calf kidney monolayer culture.
Haematoxylin-eosin stained x 440.

this time, these cultures adsorbed guinea pig erythrocytes.

After the third day, changes within the cytoplasm and nucleus as a consequence of infection were more evident. The cytoplasmic changes consisted of the development of "bizzare" shaped eosinophilic inclusions and the coalescing of the cytoplasm of the adjacent cells with the formation of giant cells. Several such inclusions were observed within the same cell. These inclusions were surrounded by an unstained area of irregular shape and were frequently situated close to the nucleus.

The earliest nuclear changes were observed three days after infection. At this stage the chromatin material of the nucleus in a very few cells appeared to be reticulated giving a vacuolated appearance to the nucleus. Within such nuclei "pink" dots were seen. After the fourth day, however, the occurrence of distinct intranuclear inclusions were clearly seen. These inclusions were round, eosinophilic and surrounded by an unstained area. They occurred singly, in pairs or in groups of more than two within a single nucleus.

The occurrence of giant cells, bizzare shaped intra-cytoplasmic inclusions and intranuclear inclusions are shown in Figure $^{6}/_{1}$. As infection of these cultures progresses, these giant cells slough off the glass surface thereby leaving gaps or "holes" in the cell sheets (Figure $^{7}/_{1}$). By the seventh day following infection practically the whole cell sheet was destroyed, although a few islets of cells still remained attached to the glass surface (Figure $^{8}/_{1}$). These cells were intensely eosinophilic and

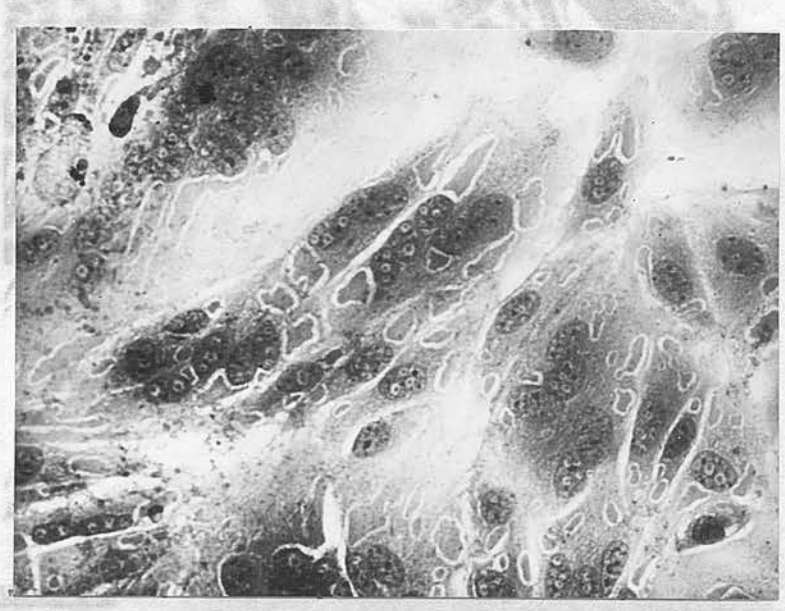

Figure 6/1 6 day old calf kidney monolayer cultures, 96 hours after inoculation with isolate B/MOR1/66 illustrating the occurrence of:

1. Giant cells.
2. Eosinophilic intracytoplasmic inclusion bodies.
3. Eosinophilic intranuclear inclusion bodies.

Compare with figure 4/1.

Haematoxylin-eosin stained x 440.

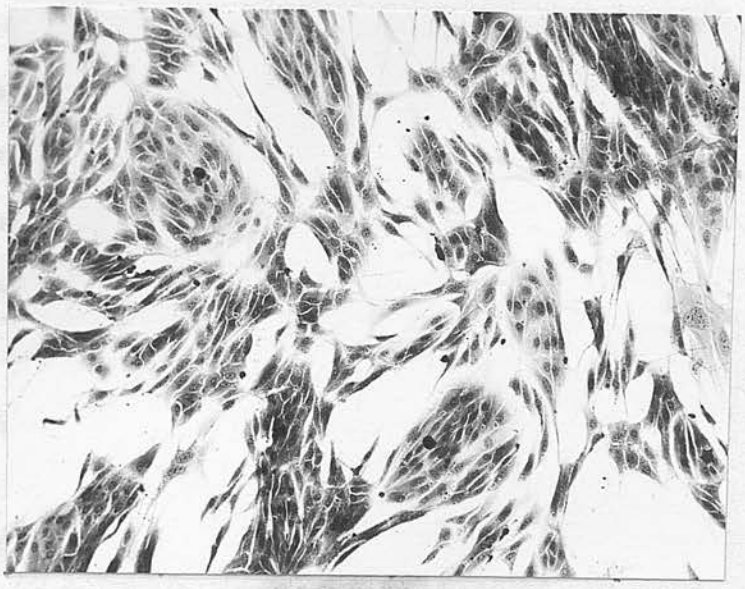

Figure 7/1 7 day old calf kidney monolayer cultures, 120 hours after inoculation with isolate B/MOR1/66 showing the gaps or "holes" in the cell sheet due to detachment of affected cells from the glass surface.

Haematoxylin-eosin stained x 110.

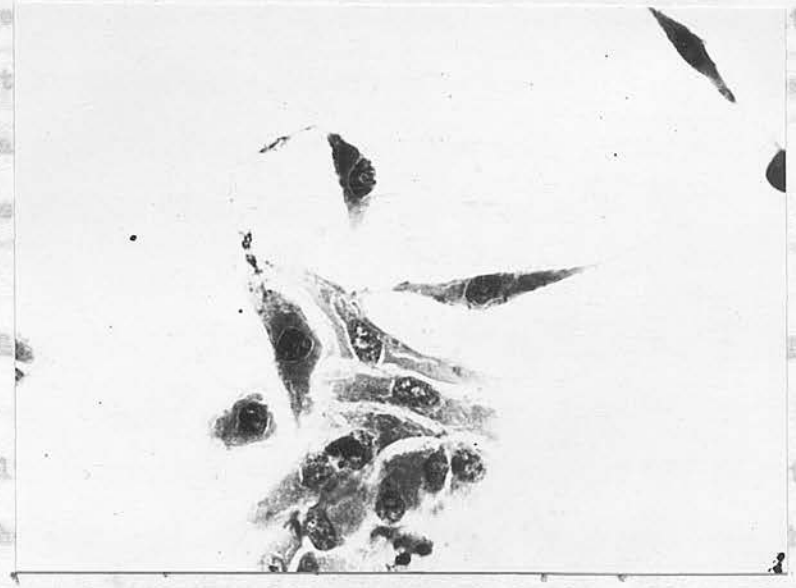

Figure 8/1 Late cytopathic effects seen in calf kidney monolayer cultures, 7 days post inoculation with isolate B/MOR1/66.

Note complete destruction of monolayer.

Compare with figure 5/1.

Haematoxylin-eosin stained x 440.

contained within them both intracytoplasmic and intranuclear inclusions.

When these same cultures were tested by the haemadsorption technique it was found that guinea pig erythrocytes were adsorped to the infected cells as early as 48 hours after infection. At this time, cytopathic effects, however, were not evident suggesting that the haemadsorption technique was a superior method for detecting this virus. This result is shown in Table 4/1.

Antigenic Relationship of Isolate B/MOR1/66 to the Para-influenza Group

The results previously described suggested that the isolate was a member of the myxovirus group. This isolate differed from the influenza viruses in its larger size and fragility, whilst unlike rinderpest - distemper viruses and respiratory syncytial virus showed both the property of haemadsorption and haemagglutination. Measles and mumps viruses induce clinical infections in humans only. Accordingly, antigenic relatedness to members of the para-influenza group of viruses was investigated by the HI and tube neutralization tests.

1. HI test

As stated in a preceding section infective tissue culture fluid of the isolate had the property of agglutinating 0.5% guinea pig erythrocytes at room temperature. In tables 5/1 and 6/1 are contained the results of experiments done to show:

a) the absence of common inhibitors for myxoviruses in

TABLE 4/1

TIME IN DAYS FOR THE OCCURRENCE OF ADSORPTION OF GUINEA PIG ERYTHROCYTES AND CYTOPATHIC EFFECTS IN CALF KIDNEY MONOLAYER CULTURES FOLLOWING INOCULATION WITH ISOLATE B/MORI/66

DAYS AFTER INFECTION	ADSORPTION OF ERYTHROCYTES	CYTOPATHIC EFFECTS IN PREPARATIONS	
		STAINED (Haematoxylin-eosin)	WET
1	ND	NIL	NIL
2	+*	Pinpoint intracytoplasmic inclusions in a few cells	NIL
3	+	Intracytoplasmic inclusions Giant cells	NIL
4	+	Intracytoplasmic and intranuclear inclusions Giant cells	Indistinct
5	+	As above	"Lattice" work like appearance
6	+	As above	> 50% of the cell sheet affected
7	+	As above	Complete destruction of sheet

VIRUS DOSE USED $100TCD_{50}$/0.1ml

+* = positive for adsorption of erythrocytes

Control non-infected cell cultures did not adsorp erythrocytes

hyperimmune rabbit serum R1 and calf sera.

b) the antigenic relationship of isolate B/MOR1/66 and human para-influenza 3.

c) the inverse two-fold relationship between serum titre and the amount of antigen used in the test. In the two experiments done when 1 unit of antigen was used a two or four-fold variation occurred in the serum titre.

The four-fold variation in titres in Experiment I and II may possibly have been due to some destruction in antibody due to overnight exposure at 37°C, followed by heating at 56°C for 1 hour. In experiment II the serum was inactivated at 56°C for ½ an hour. Both experiments were done on consecutive days, using the same batch of haemagglutinin and guinea pig erythrocytes.

The antigenic relationship of isolate B/MOR1/66 to human and two bovine (T1 and SP1) strains of para-influenza 3 were examined by reciprocal cross HI tests which were done as before. The antisera used in these tests were all prepared in rabbits. In addition, cross HI tests were done with calf sera obtained from four animals in the infected farm, where isolate B/MOR1/66 was obtained. The results of these experiments are shown in Tables $^{7}/_{1}$ and $^{8}/_{1}$. The results indicate that isolate B/MOR1/66 is a bovine strain of para-influenza 3 antigenically closely related to the human and bovine strains of para-influenza.

TABLE 5/1

THE RELATIONSHIP BETWEEN THE HAEMAGGLUTINATION-INHIBITION ANTIBODY TITRE OF HYPERIMMUNE RABBIT SERUM R1 AND VARIOUS DOSES OF HAEMAGGLUTININ OF ISOLATE B/MORI/66

SOURCE	UNITS OF HAEMAGGLUTININ USED	Haemagglutination-inhibition titre of rabbit serum R1	
		Experiment I	Experiment II
		ǂ*	
Tissue Culture fluid-isolate B/MORI/66	16	80	320
	8	160	640
	4	320	1280
	2	640	2560
	1	2560	5120
Tissue Culture fluid-human strain of Para-influenza 3	2	80	ND
	1	320	

ǂ Reciprocal of highest dilution giving complete inhibition of haemagglutination

* Serum inactivated at 56°C for 1 hour after standing overnight at 37°C

TABLE 6/1

THE EFFECT OF RECEPTOR DESTROYING ENZYME, TRYPSIN AND POTASSIUM PERIODATE ON THE HAEMAGGLUTINATION-INHIBITION TITRE OF HYPERIMMUNE RABBIT SERUM R1 AND CALF SERA WHEN TESTED AGAINST ISOLATE B/MOR1/66 AND HUMAN STRAIN OF PARA-INFLUENZA 3

SERUM	HAEMAGGLUTINATION-INHIBITION TITRES*	
	TREATED	UNTREATED
Rabbit serum R1•		
Preinoculation	< 10†	< 10
Postinoculation	320	320
Postinoculation	80‡	80
Calf sera••		
75	256†	128
85	128	128
77	2048	2048
78	128	128
88	1024	1024
79	1024	1024
284	128	128
294	128	128
300	512	512
282	512	512

* Reciprocal of the highest dilution of serum giving complete inhibition of haemagglutination.

• Serum treated with RDE (Burroughs-Wellcome) overnight at 37°C followed by heating for 1 hour at 56°C.

•• Serum treated with trypsin and potassium periodate

† Four units of antigen, B/MOR1/66 used.

‡ Two units of antigen, human para-influenza 3 used.

TABLE 7/1

RELATIONSHIP OF HUMAN AND BOVINE STRAINS OF PARA-INFLUENZA 3 AND ISOLATE B/MOR1/66 BY RECIPROCAL CROSS HAEMAGGLUTINATION-INHIBITION TESTS WITH RABBIT ANTISERA

VIRUS ANTIGEN USED IN THE TEST†	HAEMAGGLUTINATION-INHIBITION TITRES OF RABBIT ANTISERA *					
	R1(A)‡	PR4(A)‡	R1‡‡	PR4‡‡	MR1**	DR1**
B/MOR1/66	80	160	3200	320	320	640
Human para-influenza 3	20	640	400	640	80	NOT DONE
Bovine para-influenza 3						
T1 strain	40	80	3200	320	160	1280
Spl strain	NOT DONE	NOT DONE	3200	NOT DONE	320	1280

* Reciprocal of highest dilution giving complete inhibition of haemagglutination.

† 4 units of antigen used in the test.

‡ R1(A) and PR4(A) were immune sera obtained after one intravenous inoculation of isolate B/MOR1/66 and human para-influenza 3. The animals were bled on the 6th and 14th day respectively.

‡‡ R1 and PR4 were hyperimmune sera prepared against isolate B/MOR1/66 and human para-influenza 3 respectively.

** MR1 and DR1 were hyperimmune sera prepared against isolate B/MOR1/66 and bovine para-influenza 3 (T1) strain respectively.

All pre-inoculation sera < 1:10.

TABLE 8/1

RELATIONSHIP OF HUMAN AND BOVINE STRAINS OF PARA-INFLUENZA 3 AND ISOLATE B/MOR1/66 BY CROSS HAEMAGGLUTINATION-INHIBITION TESTS WITH CONVALESCENT CALF SERA

VIRUS ANTIGEN USED IN THE TEST*	HAEMAGGLUTINATION-INHIBITION TITRES OF CALF SERA			
	CALF NUMBER			
	75	85	300	294
B/MOR1/66	40	ND	80	40
Para-influenza 3 Human	20	40	80	20
Bovine - T1	40	80	80	40

* 2 - 4 units of antigen used in the test.

Effect of tween-ether treatment on the haemagglutinin of bovine para-influenza (T1 strain)

In some preparations the yield of haemagglutinin in the tissue culture fluid harvest was so low that it was not possible to obtain 4 units of haemagglutinin for the HI test. Hence a fresh batch of haemagglutinin had to be produced by infecting a further set of cell cultures.

The investigation of some conditions for the production of high titre haemagglutinin were carried out during the initial phase of this investigation with the T1 strain of bovine para-influenza 3 at the fourteenth and fifteenth passage levels in calf kidney cell cultures.

The following observations were made:

1. Infection of Roux flasks of calf kidney cell cultures with a virus dose containing $10^{4.5}$ TCD_{50} and $10^{3.5}$ TCD_{50} yielded haemagglutinin in the freshly harvested tissue culture fluid to a titre of 1:20 and 1:60 respectively. Tissue culture fluids were harvested when cytopathic effects were maximal (usually 3 to 4 days post infection). The slightly lower dose of virus appeared to increase the haemagglutinin titre in the tissue culture fluid.

2. Concentration of infective tissue culture fluids by ultra-centrifugation at 39,000 r.p.m. for 1 hour and resuspending the pelleted virus particles in a small quantity of PBS did not significantly alter the haemagglutinin titre when compared to

unconcentrated tissue culture fluid.

3. Treatment of infective tissue culture fluid with tween-ether (Hosaka et al 1959, Norrby 1962) increased the haemagglutinin titres.

Calf kidney cell cultures were infected with a dose of virus containing approximately $10^{3.0}$ TCD_{50}. On the 4th day after infection, when complete cytopathic effects were produced tissue culture fluids were harvested and centrifuged at 1,000 r.p.m. for 10 minutes at $4^{o}C$ to remove gross cellular debris. An aliquot of the supernatant was distributed into bijoux bottles and treated with 10% tween in the cold to give a final concentration of 0.125%. Ether was next added (proportion 0.5:1) and the mixture shaken for 20 minutes in the cold. At the end of this period, tween-ether mixture was centrifuged after transferring into Wasserman tubes at 2,500 r.p.m. for 20 minutes at $4^{o}C$, the aqueous bottom phase removed and nitrogen slowly bubbled to remove the excess ether. 10ml of infective tissue culture fluid was concentrated 10 x by centrifuging at 39,000 r.p.m. at $4^{o}C$ for 1 hour, to sediment the virus particles. The pellet was reconstituted in 1ml PBS and similarly treated with tween-ether. Non-infective control tissue culture fluids were also treated similarly as the infective tissue culture fluid with tween-ether. The haemagglutinin titre of these various preparations were then determined using 0.4ml of two-fold dilutions of antigen and 0.2ml of an 0.5% guinea pig erythrocyte suspension at room temperature.

The results are shown in Table 9/1. Tween-ether treatment caused an eight-fold increase in the haemagglutinin titre when

TABLE 9/1

EFFECT OF TWEEN-ETHER ON THE HAEMAGGLUTININ TITRE OF BOVINE PARA-INFLUENZA 3

PREPARATION	TREATMENT	HAEMAGGLUTININ TITRE PER 0.4ml*
INFECTIVE TISSUE CULTURE FLUID	0.125% TWEEN AND 33% ETHER IN THE COLD FOR 20 MINUTES IN A SHAKER	1280
	UNTREATED CONTROL	160
INFECTIVE TISSUE CULTURE FLUID CONCENTRATED 10 X BY ULTRA-CENTRIFUGATION	0.125% TWEEN AND 33% ETHER IN THE COLD FOR 20 MINUTES IN A SHAKER	2560
	UNTREATED CONTROL	320
NON-INFECTED CONTROL TISSUE CULTURE FLUID	0.125% TWEEN AND 33% ETHER IN THE COLD FOR 20 MINUTES IN A SHAKER	<10
	UNTREATED CONTROL	<10

* Reciprocal of the highest dilution causing complete haemagglutination of erythrocytes of the guinea pig at room temperature

compared to the untreated virus.

Tube neutralization test

The antigenic relationship of the isolate B/MOR1/66 to human and bovine strains of para-influenza 3 were further confirmed by tube neutralization tests. The method of doing these tests and the manner of reading them have been described in the materials and methods section.

The results presented in Table $^{10}/_{1}$ show that the isolate was neutralized by antiserum prepared against human para-influenza 3 when tested at the recommended dilution, but not by antisera prepared against the human strains of para-influenza 1, 2; simian para-influenza 2 and influenza A.

In Table $^{11}/_{1}$, the results of a further test using Collindale serum prepared against the human strain of para-influenza 3 is given. The plan of the test was that of a limited chequer-board neutralization. For this test, antiserum was diluted 1:10, 1:50 and 1:250 in virus diluent and mixed with equal quantities (0.5ml) of dilutions of virus containing $10^{4.5}$, $10^{3.5}$, $10^{2.5}$ and $10^{1.5}$ TCD_{50} per 0.1ml held at room temperature for 1 hour and inoculated on to monolayer cultures of calf kidney cells as before.

The results show that a dilution of 1:10 of serum neutralized $10^{4.5}$ TCD_{50}, whilst the 1:50 dilution of serum neutralized $10^{3.5}$, $10^{2.5}$ and $10^{1.5}$ TCD_{50} of virus.

In Table $^{12}/_{1}$ are shown the results of a reciprocal cross neutralization test between the human para-influenza 3 virus and isolate B/MOR1/66.

TABLE 10/1

NEUTRALIZATION OF ISOLATE B/MORI/66 BY ANTISERA PREPARED AGAINST VARIOUS STRAINS OF PARA-INFLUENZA AND INFLUENZA A VIRUSES USING CALF KIDNEY CELL CULTURES

ANTISERUM PREPARED AGAINST INDICATED VIRUSES‡	HIGHEST DILUTION OF SERUM INHIBITING HAEMADSORPTION OF ERYTHROCYTES
Human para-influenza 1‡‡	< 1:8
Human para-influenza 2‡‡	< 1:8
Human para-influenza 3*	1:64
Simian para-influenza 2‡‡	< 1:8
Influenza A‡‡	< 1:8

‡ Antisera obtained from Collindale

‡‡ Recommended final dilution for use 1:200

‡‡‡ Recommended final dilution for use 1:400

* Recommended final dilution for use 1:50

TABLE 11/1

NEUTRALIZATION OF VARIOUS INFECTIVE DOSES OF B/MORI/66 BY VARYING DILUTIONS OF ANTISERUM PREPARED AGAINST HUMAN PARA-INFLUENZA 3 USING CALF KIDNEY MONOLAYER CULTURES

VIRUS USED	DOSE OF VIRUS NEUTRALIZED ($Log_{10}TCD_{50}$/0.1ml)	DILUTION OF ANTISERUM INHIBITING HAEMADSORPTION*		
		1:10	1:50	1:250
B/MORI/66	4.5	+**	NOT DONE	NOT DONE
	3.5	+	+	NIL***
	2.5	+	+	NIL
	1.5	+	+	NIL

† Titre of the virus pool was $10^{6.5}$ TCD_{50}/0.4ml

* Recommended dilution for use of this antiserum was 1:50

**+ Negative for haemadsorption of guinea pig erythrocytes on calf kidney cell cultures

***NIL Positive for haemadsorption

TABLE 12/1

RECIPROCAL CROSS NEUTRALIZATION BETWEEN PARA-INFLUENZA 3 (HUMAN STRAIN) AND ISOLATE B/MORI/66 USING CALF KIDNEY MONO-LAYER CULTURES

VIRUSES USED IN TEST	NEUTRALIZING TITRE OF HYPERIMMUNE RABBIT SERUM (ND_{50})†	
	R1*	PR4
Parainfluenza 3 Human	1:2371	1:1189
B/MORI/66	1:4732	1:335

† Dilution of antiserum containing IND_{50} when tested against 30 - 100 TCD_{50} per 0.1ml of virus. Kärber Estimate.

* R1 = Hyperimmune serum prepared against isolate B/MORI/66

** PR4 = Hyperimmune serum prepared against human para-influenza 3.

These results, all show the close antigenic relationship of isolate B/MOR1/66 to human para-influenza 3, and indicates that the isolate is a bovine strain of para-influenza 3.

Table 13/1 contains the results of a reciprocal cross neutralization test done between the T1 strain of bovine para-influenza 3 isolated in England and a cross neutralization test between this isolate and the SP1 strain isolated from the bovine testicle. The results of these tests show the close antigenic similarity of the three virus strains used in these tests and confirm the results obtained from HI tests.

Antibody Response in Clinically Infected Calves

The HI and serum neutralizing antibody levels in the sera of calves, on the farm where isolations were made, were determined.

The results in Table 14/1 indicate that all calves had HI titres of 1:40 or over. The HI titres obtained with the same sera in a previous test (Table 6/1) showed an approximate four-fold increase. This probably reflects the inherent variation of the HI test done on different days.

In Table 15/1 sera from 8 calves were examined for neutralizing antibody. No acute phase sera were available. It was hoped that a rise of neutralizing antibody would be detected. Neutralizing antibody develops later in the course of infection (Hamparian et al 1961). No significant rise in neutralizing antibodies was observed. Acute phase serum was available from calf 85. The neutralizing antibody titre of serum samples collected from this animal on Day 0, 10 and 28 were 1:32, 1:150 and 1:90 respectively. The corresponding HI titres were 1:40, 1:80 and 1:80.

TABLE 13/1

RECIPROCAL CROSS NEUTRALIZATION AND CROSS NEUTRALIZATION TESTS BETWEEN BOVINE STRAINS OF PARA-INFLUENZA 3 (T1 AND SP1) AND ISOLATE B/MOR1/66 IN CALF KIDNEY MONOLAYER CULTURES

VIRUS USED IN TEST	NEUTRALIZING TITRE OF HYPERIMMUNE RABBIT SERUM (ND_{50})‡	
	R1*	AS1**
Bovine para-influenza 3		
T1 strain	1:2818	1:320
SP1 strain	1:2985	NOT DONE
B/MORI/66	1:3350 1:3548	1:128

‡ Dilution of antiserum containing IND_{50} per 0.1ml when tested against 30-300 TCD_{50} of virus. Kärber estimate.

* R1 = Hyperimmune rabbit serum prepared against isolate B/MORI/66.

** AS1 = Hyperimmune rabbit serum prepared against bovine para-influenza 3, T1 strain.

TABLE 14/1

HAEMAGGLUTINATION-INHIBITION TITRES IN THE SERA OF CALVES FROM THE INFECTED FARM

CALF NUMBER	HAEMAGGLUTINATION-INHIBITION TITRE OF CALF SERUM††		
	Bleed I*	Bleed II**	Bleed III***
† 75	1:80	1:80	1:80
77	1:640	1:320	1:320
78	1:160	1:80	1:80
71	1:40	1:80	1:80
79	1:160	1:160	1:80
88	1:80	1:80	1:80
† 284	1:40	1:80	NC
† 294	1:80	1:80	NC
† 300	1:160	1:160	NC
282	1:160	1:80	NC
281	1:320	1:640	NC

†† Highest serum dilution which completely inhibits haemagglutination of guinea pig erythrocytes at room temperature. Four units of antigen B/MOR1/66 used in the test.

* 1st serum specimen obtained 10 days after collection of nasal swabs.

** 2nd serum specimen obtained 10 days after collection of first serum specimen.

*** 3rd serum specimen obtained 18 days after collection of second serum specimen.

† Virus isolated.

NC Not collected.

TABLE 15/1

NEUTRALIZING ANTIBODY TITRES IN SERA OF CALVES FROM THE AFFECTED FARM

CALF NUMBER	NEUTRALIZING ANTIBODY TITRES OF CALF SERUM†		
	Bleed I*	Bleed II**	Bleed III***
• 75	1:16	1:36	1:36
77	1:125	1:128	1:89
78	1:32	1:32	1:32
• 284	1:32	>1:40	NC
• 294	1:28	1:40	NC
• 300	1:28	1:28	NC
282	1:72	1:190	NC
281	>1:64	1:151	NC

† Dilution of antiserum containing $1 ND_{50}$ per 0.1ml when tested against approximately 50 - 500 TCD_{50} of challenge virus. Kärber estimate.

* 1st serum specimen obtained 10 days after collection of nasal swab.

** 2nd serum specimen obtained 10 days after collection of first serum specimen.

*** 3rd serum specimen obtained 18 days after collection of second serum specimen.

• Virus isolated.

In Table 16/1 calf sera collected from two groups of calves were examined for HI antibodies. Calves belonging to group A were approximately 4 months of age. Two of the animals in this group had "pneumonia" during the past few weeks, whilst the others appeared to be apparently normal. Although no para-influenza 3 virus was isolated from nasal swabs collected from these animals, one of the animals which had "pneumonia showed a distinct four-fold rise of haemagglutinin antibodies against isolate B/MOR1/66. Calves in group B were approximately 3 months of age and had been brought into the same farm as group A calves. These calves had been on the premises for about one week. This result shows the limitation of the laboratory in diagnosing para-influenza 3 in the absence of a clear four-fold difference in serum titres by serological tests.

Serial samples of sera from a batch of calves (18) brought into this institute were examined for antibodies against bovine para-influenza 3 by the HI test. The animals were 1 week of age at the time of arrival and with the exception of one, the others did not show signs of a respiratory tract infection during the period they were here. Attempts were made to isolate para-influenza 3 virus from these animals on two occasions but with no success. In Table 17/1 HI levels in the sera are tabulated against the age of the calves.

Plaque Formation

The infectivity of isolate B/Morl/66 for primary calf kidney monolayer cultures was determined by the plaque assay procedure.

TABLE 16/1

DISTRIBUTION OF THE NUMBER OF CALVES HAVING INDICATED HAEMAGGLUTINATION-INHIBITION ANTIBODIES IN THEIR SERA AGAINST ISOLATE B/MOR1/66

GROUP	HAEMAGGLUTINATION-INHIBITING TITRES			TOTAL NUMBER OF ANIMALS
	10 - 20	40 - 80	160 - 320	
GROUP 1 †	0	6	6	12
GROUP A ‡	6	12	7	25
GROUP B ‡‡	7	6	1	14

† Group 1 = Herd from which bovine para-influenza 3 was isolated.

‡ Group A = Herd from which bovine para-influenza 3 was not isolated, but one animal showed a distinct four-fold increase in haemagglutination-inhibition titre when a paired serum sample was tested

‡‡ Group B = Herd apparently normal.

TABLE 17/1

DISTRIBUTION OF HAEMAGGLUTININ ANTIBODY

IN CALF SERA ACCORDING TO AGE

AGE OF CALVES	HAEMAGGLUTINATION-INHIBITION TITRES IN CALF SERA		
	< 10 - 20	40 - 80	160 - 320
1 week	4	8	6
2 months	13	5	NIL
3 months	18	NIL	NIL
4 months	18	NIL	NIL
5 months	18	NIL	NIL
6 months	18	NIL	NIL
7 months	18	NIL	NIL

Serial two-fold, three-fold or ten-fold dilutions of virus were made in diluent. Five-tenths ml of each virus dilution was mixed with an equal volume of diluent (0.5ml) and inoculated on to monlayer cultures grown in plastic Petri plates using three or four plates per dilution. The plaque counts observed after 6 days incubation at $37^{\circ}C$ in an atmosphere of 4 to 5% carbon dioxide are plotted against the relative virus concentration (Figure 9/1). A linear relationship within the limits of the dilutions tested was observed between virus concentrations and number of plaques indicating that each plaque was initiated by a single virus particle or an aggregate of particles indivisible on dilution.

A pool of the T1 strain of bovine para-influenza 3 virus was titrated both by plaque assay and tube cultures in second generation calf kidney monolayer cultures. It was observed that when this pool was titrated by the plaque assay procedure the titre of the preparation was 5.72×10^6 and 4.5×10^6 PFU per ml in two experiments. The same pool when titrated in tube cultures gave a titre of $10^{6.5}$ and $10^{6.08}$ TCD_{50} per ml.

The plaques of isolate B/MOR1/66 were round, with irregular edges and turbid having a diameter of 1.4mm when measured on the 7th day. Plaques formed by the T1, and SP1 strains were approximately 2.3 to 3mm in diameter, also with irregular borders but much clearer.

The specificity of the plaques formed following infection of the monolayers of calf kidney cells was tested by "picking" a single plaque into 1ml of virus diluent and sub-inoculating into monolayer cultures. On the fourth day the cultures were tested by the

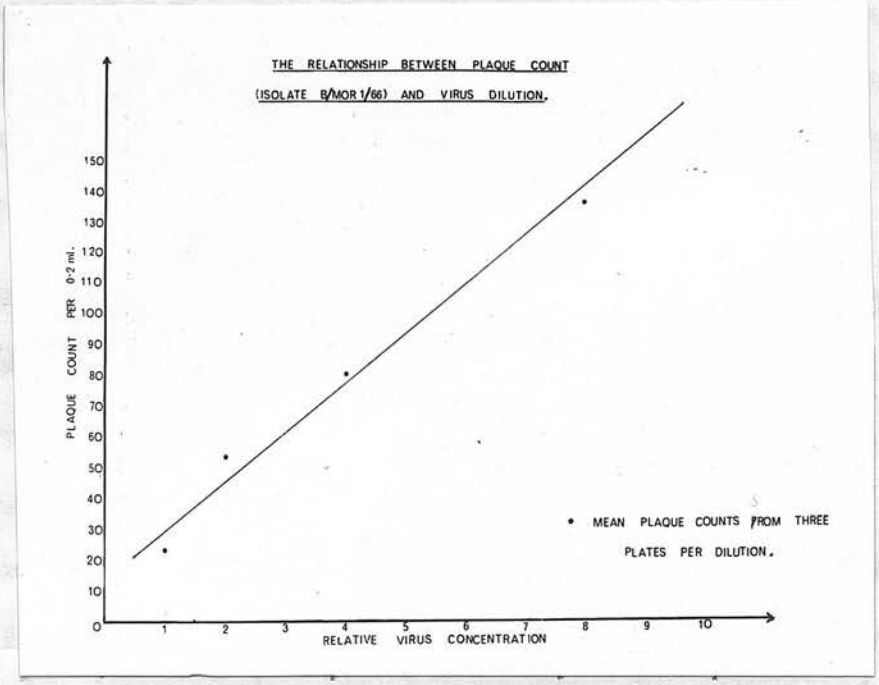

Figure 9/1 The relationship between plaque count (isolate B/MOR1/66) and virus dilution.

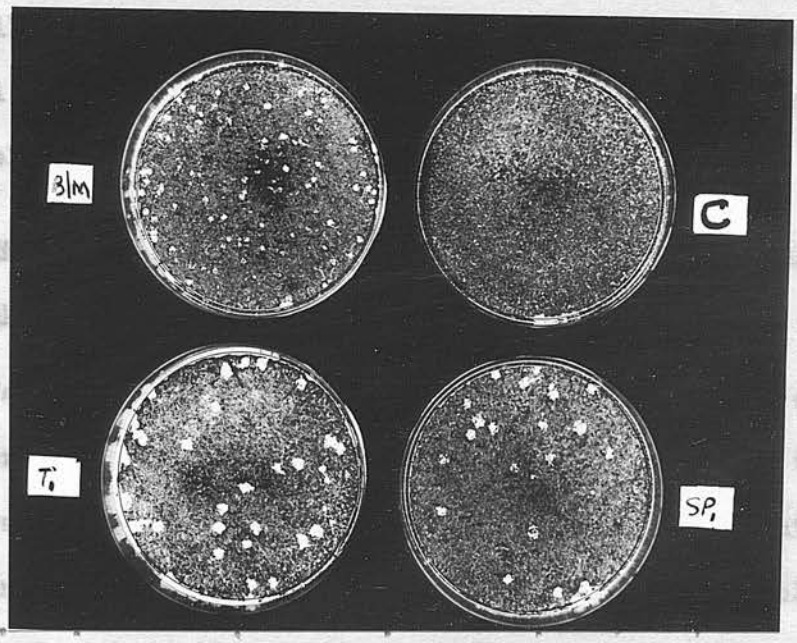

Figure 10/1 Plaques on calf kidney monolayer cultures by bovine strains of para-influenza virus.

B/M - Isolate B/MOR1/66

C - Control

T_1 - T_1 strain isolated in England from the tonsils of an apparently normal calf.

SP_1 - Bovine testicular strain, isolated in Scotland.

Stained by the method of Holland and McLaren. (1959).

haemadsorption and haemadsorption-inhibition technique. Tubes which were treated with hyperimmune antiserum R1 for half an hour before the addition of 0.5% guinea pig erythrocytes did not show haemadsorption, whilst the untreated tubes showed adsorption. Absence of bacterial contamination was demonstrated by inoculation into fluid thioglycollate medium.

Intratypic sero-differences between strains of bovine para-influenza viruses

In Tables $^{7}/_{1}$ and $^{13}/_{1}$ were presented the results of cross HI and serum neutralization tests. The results indicated that all three strains of para-influenza 3 of bovine origin were antigenically similar and could not be distinguished by these tests. McBride (1959) faced with similar problems applied the principles of the neutralization reaction advanced by Dulbecco et al (1956) and developed a technique whereby he could differentiate strains of type 1 and 3 poliovirus. He found that a virus with its own homologous antiserum was neutralized faster than the heterologous virus, under a given set of experimental conditions. This principle and the technique of McBride's have been used by others (Ozaki et al 1963, Furez et al 1964). Ashe and Scherp (1963) employed the method to characterize strains of herpes simplex virus into 4 serological groups and 3 heterogenous strains. Rubins and Franklin (1957) have shown that the principles of the kinetics of neutralization applied by Dulbecco et al (1956) to poliovirus and western equine encephalomyelitis virus was equally applicable to the neutralization of Newcastle disease virus (a member of the myxovirus group).

The findings of these workers and the fact that a plaque assay procedure had been developed for the direct assay of infectivity of the bovine para-influenza viruses (see preceeding section) suggested that this method may be profitably employed for the differentiation of these three strains (B/MOR1/66, SP1 and T1) of virus.

"Early" phase rabbit antiserum MR1 prepared against B/MOR1/66 was used. McBride (1959) has shown that early antiserum was more specific than late antiserum.

A preliminary experiment was done to ascertain what dilution of serum MR1 would inactivate 1 to 2 $\log_{10}$ of homologous virus B/MOR1/66 in 10 minutes at 37°C. Antiserum was diluted 1:2.5, 1:5 and 1:10. These dilutions of serum were mixed with equal quantities of virus (0.5ml) containing 3.2×10^4 PFU per 0.1ml and held for 10 minutes at 37°C. At the end of this period, 0.1ml of each serum-virus mixture was pipetted into 10ml of ice cold diluent. These $^1/_{100}$ dilutions were further diluted in two-fold series. 0.2ml of each of these various dilutions were then inoculated on to twice washed calf kidney monolayer cultures. After 2 hours adsorption, monolayers were washed once with HBSS and overlaid with nutrient agar. As controls, virus was similarly treated but with diluent only. Plaques were counted on the 7th day after infection. The results are contained in Table $^{18}/_1$. They show:

a) There is no heat inactivation of virus during the 10 minute period.

b) The amount of virus neutralized in a given period of time was proportional to the concentration of antibody, being greater with the higher concentration of antiserum.

Dulbecco and his colleagues (1956) and Taylor-Robinson and Tyrrell (1962) with the viruses which they have worked with, have also shown a similar result.

The kinetics of neutralization of animal viruses is dependent on the presence of excess antiserum. In these experiments, there was excess antiserum when homologous virus B/MOR1/66 was reacted with serum MR1 diluted 1:2.5 for 10 minutes at 37°C. This was shown by the following experiment. An ampoule of virus containing approximately 4.8×10^7 PFU per 0.1ml was diluted 10^{-2} and 10^{-3}. These dilutions were mixed with equal quantities of antiserum (0.5ml), incubated for 10 minutes at 37°C and inoculated on to calf kidney monolayer cultures as before. Controls consisted of the dilutions of virus treated with diluent only. The following result was obtained. When the concentration of virus was 4.8×10^5 PFU per 0.1ml, the fraction of surviving virus was 0.04. When the virus concentration was 1 $\log_{10}$ less, the fraction of surviving virus was 0.03. This variation parallels the variation in control plaque counts at the same dilutions of virus.

The exponential decline of homologous virus B/MOR1/66 when inactivated with antiserum MR1 at a dilution of 1:2.5 for 10 minutes at 37°C were observed in the following two experiments. When 3.2×10^4 PFU of virus per 0.1ml was incubated with antiserum MR1 for 5 minutes, the fraction of surviving virus was 0.17. During

TABLE 18/1

EFFECT OF HEAT* AND VARYING DILUTIONS OF RABBIT ANTISERUM MR1 ON THE INACTIVATION OF ISOLATE B/MOR1/66 WHEN TESTED ON CALF KIDNEY MONOLAYER CULTURES

DILUTION OF VIRUS + DILUENT CONTROL	MEAN PLAQUE† COUNT (Po)		SERUM** DILUTION	DILUTION OF VIRUS + SERUM	MEAN † PLAQUE COUNT (Pt) (TIME 10')	% INACTIVATION
	TIME 0'	TIME 10'				
2×10^{-5}	146	148	1:2.5	2×10^{-5}	14	94
				4×10^{-5}	6	
				8×10^{-5}	3	
4×10^{-5}	80	95	1:5.0	2×10^{-5}	32	79
				4×10^{-5}	20	
8×10^{-5}	53	54		8×10^{-5}	9	
16×10^{-5}	23	25	1:10.0	2×10^{-5}	37	75
				4×10^{-5}	25	

* Incubation of $\simeq 3.4 \times 10^4$ PFU/0.1ml virus + diluent (equal quantities, 0.5ml) for 10 minutes at 37°C.

** Incubation of virus with equal quantity of indicated serum dilution, for 10 minutes at 37°C

† Mean plaque count obtained using 3 plates per dilution.

the next 5 minute period it was 0.24. This indicates that approximately equal quantities of virus were inactivated in equal periods of time. The inactivation curve of B/MOR1/66 when incubated with serum MR1 for a period of up to 10 minutes is shown in figure 11/1; the surviving fraction $^{Pt}/_{Po}$ on a logarithmic scale and time on the arithmetic scale. The graph shows an exponential decline of virus with time.

It can be concluded that the neutralization kinetic method is applicable to the bovine para-influenza viruses.

In comparing the rate of inactivation of homologous and heterologous strains of virus, all were tested together at a single observation point of 10 minutes at 37°C. It was hoped by this means to eliminate variations in susceptibility of different batches of calf kidney cells and any variations in environmental and technical factors. Such variations would be reflected equally on the reaction rates of homologous and heterologous viruses.

In Table 19/1, the K values, i.e. the rate of inactivation per unit time and the normalized K values obtained from three experiments, are shown. The results indicate that isolate B/MOR1/66 differs from the SP1 and T1 strains.

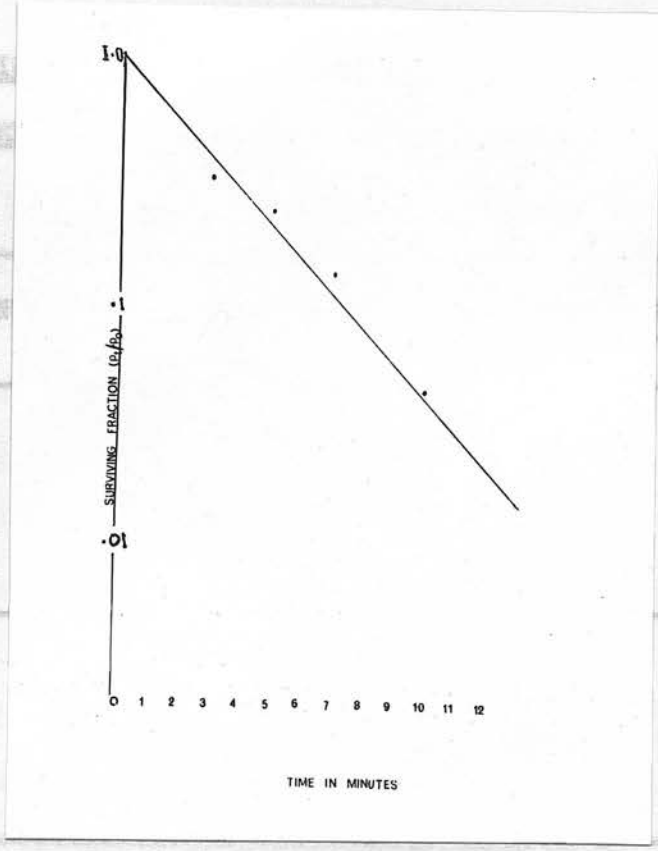

Figure 11/1 Inactivation curve of isolate B/MOR1/66 by rabbit antiserum MR1 diluted 1:2.5. Plot of the surviving fraction ($Pt/_{Po}$) on a logarithmic scale against time in minutes on an arithmetic scale.

TABLE 19/1

A COMPARISON OF THE NEUTRALIZATION RATE CONSTANT (K) AND NORMALISED CONSTANT (NK) OF THREE BOVINE STRAINS OF PARA-INFLUENZA 3

EXPERIMENT	VIRUS STRAIN	RABBIT ANTISERUM MR1	
		K	NK
I*	B/MOR1/66	0.93	100
	SP1	0.3	33
	T1	0.56	60
II	B/MOR1/66	1.1	100
	SP1	0.54	49
	T1	0.55	50
III	B/MOR1/66	0.78	100
	SP1	0.31	39
	T1	0.44	55

* Experiments I to III = dilution of antiserum 1:2.5

DISCUSSION

DISCUSSION

The similar symptoms induced by different viruses precludes an aetiological diagnosis. The only satisfactory means of establishing the aetiology is by undertaking a virological investigation.

In this investigation such an approach was made. In this herd, comprising of about 100 animals, symptoms of nasal discharge and coughing were particularly noticeable in one of the pens. The animals within this pen were approximately 7 months of age and had been introduced onto the farm on 23.1.66. when 3 months of age. The behaviour of the animals within this herd suggested that some form of an infectious agent was circulating amongst them. From several of these animals a total of 9 isolates of virus was made. Experience had indicated that for virus isolation, the selection of animals with frank pneumonia or definite clinical symptoms only was futile, unless careful clinical observations were made from the onset of the disease. For successful virus isolation especially with the myxoviruses, a relatively short excretion period occurs and consequently as many animals as possible were swabbed.

The recovery of 9 isolates of para-influenza 3 from this herd was not surprising. In Britain, Dawson and Darbyshire (1964) have reported that nearly 88% of the cattle population have antibodies to this virus at titres of 1:32 or greater when tested by the haemagglutination-inhibition test, indicating widespread distribution of the virus.

Some observations of the course of natural para-influenza virus infection were possible in these calves. In humans, following natural infections of para-influenza 3, virus excretion is relatively short depending upon the antibody status of the individual. Chanock et al (1961) in their studies on such infections in nursery children observed that children without pre-existing antibody would shed virus for up to about 8 to 10 days in a majority of cases. In the present study it was observed that in one animal (calf No. 75) virus could be recovered from the nasal passages on day 1 and day 3, but not on day 14. Following experimental aerosol infection of young calves, Hamparian et al (1961) observed that virus excretion occurred up to 10 days but not on the 14th day post-infection. Dawson et al (1965) in their series of experimental infections of colostrum deprived calves found that virus-shedding occurred up to the 12th day after infection at which time the HI antibody titre was 1:64 to 1:128. From a second animal (calf No. 85) a blood sample was obtained on the day of initial swabbing. A three to four-fold increase in neutralizing antibody was observed in this animal in the two subsequent blood samples, but only a two-fold rise in HI antibodies.

The mechanism whereby circulating antibody is brought to the epithelial surface of the respiratory tract to assist in recovery of the animal is not known. Anderson et al (1962) who had made an immuno-electrophoretic study of changes in the protein composition of human nasal secretions before and during the course of respiratory infections, postulates that following infection an

inflammatory response occurs within 48 hours after infection with breakdown of capillaries. Consequently seepage of γ globulin occurs and this bathes the lining cells of the respiratory mucosa. It is most likely that a similar operational mechanism also occurs in calves.

Epidemiologically, outbreaks of bovine respiratory disease occur in farms usually as a consequence of the introduction of new stock into the herd. However, outbreaks of such diseases have also occurred when there have been no introduction of stock (Abinanti et al 1960). In these outbreaks it is possible that latent virus may have been reactivated as a consequence of an adverse environment which may result in physiological stress. The evidence of isolation of viruses such as the T1 strain of para-influenza 3 from a normal animal lends credibility to this view (Dawson 1964). The circumstantial evidence is that the origin of this outbreak may have been the introduction of new stock into the farm. Approximately 3 to 4 weeks after their introduction the present outbreak occurred. Acquisition of infection in calves commonly occurs at market sales where animals originating from diverse sources are brought together, then are dispersed to various farms. The level of colostral antibody may be an important factor in the susceptibility or resistance of such stock to para-influenza 3 viruses.

An opportunity presented itself of examining sera of young calves which had been brought into this institute and whose colostral status was not known. It was felt that this was a good

random sample which perhaps would reflect what would be happening at market sales. The study revealed that 22% of the calves did not possess any colostral immunity ($\leq$ 10 - 20) when they were 1 week of age, whilst by the third month all of the calves tested had very low anitbody levels. On the basis of Sinha and Abinantis' observations (1962) on a natural outbreak of shipping fever in which cattle with HI anitbody titres of 1:40 or higher did not develop clinical signs, the results suggest a considerable risk of infection with para-influenza 3 virus at this age.

Dawson (1966) has shown that by the time the calves are 10 weeks of age they have lost their colostral immunity. In the same paper he states that 25% of the outbreaks of respiratory disease in calves have been associated with PI3 infection, on the basis of serological rises of antibody.

On primary isolation, the isolates were detected by the haemadsorption technique (Vogel and Shelekov 1957). This technique was found to be more reliable and sensitive for the detection of bovine strains as is true with the human strains. Inoculation of 100 TCD_{50} of virus on to coverlsip preparations of calf kidney monolayers showed that after 48 hours such cultures adsorbed erythrocytes of the guinea pig although no cytopathic effects were seen.

A study of the basic properties of one of the isolates B/MOR1/66 revealed it to be a myxovirus, whilst its antigenic relationship to the para-influenza 3 viruses were established by cross HI and neutralization tests.

A close similarity of the Edinburgh isolate to the human strain of para-influenza 3 was observed, although by cross HI and serum neutralization tests, the two viruses could be distinguished. The present results are at variance with those observed by Ketler et al (1961), who showed that rabbit antiserum prepared against the human strain (HA-1) neutralized the homologous virus to a titre of 25 x or greater than the heterologous bovine virus. Unfortunately in Ketler's paper no mention of the immunizing schedules for the rabbits are given and it is unlikely though possible that the rabbit HA-1 was given a single immunizing dose. In the present study antiserum against the human and bovine viruses were prepared from pools grown in calf kidney cell cultures. It is possible that if, as in the other myxoviruses, the surface antigen which is the immunizing antigen incorporates host cell material during its developmental phase, a closer antigenic relationship than really exists may be observed.(It has been estimated that Newcastle disease virus incorporates host cell material up to 40% of its mass Rott 1964). Another possibility, apart from individual animal variation, is the prior sensitisation of the animals to a closely related antigen, though pre-existing antibodies may not be detectable in the pre-inoculation sera (Hsuing et al 1965). The para-influenza viruses are ubiquitous organisms and have been recovered from a variety of animals, horses, sheep, monkeys and mice. Abinanti et al (1961) in studying the antigenic relationship between the human and bovine strains found that when the sera of convalescing children from a primary infection with human para-

influenza 3 were subjected to cross HI tests, 2 of the 5 sera inhibited the bovine antigen, in one case to the same titre as the human antigen (1:1024) and in the other to sixteen-fold less. This complex relationship may perhaps be resolved by the use of immune serum prepared after a single immunizing inoculation. Possibly a plaque method as has been described for the differentiation of Semiliki Forest virus and Mayaro virus complexes within the group A arboviruses (Porterfield 1964) could be used.

The close association of man with his domestic animals leads one to speculate as to whether the para-influenza viruses can cross the species barrier. Recently, Sibinovic et al (1965) has reported that their equine strain of para-influenza 3 was more closely related to the bovine strain than the human. Waldman and Kobe (see Introduction) were able to infect calves placed in contact with a young foal infected with a horse virus. This then is a real possibility. However, Abinanti et al (1961) mention that they were unable to infect a calf with the human strain and this animal did not subsequently withstand challenge with the bovine SF-4 virus.

The object of attempting to differentiate the three strains of bovine para-influenza virus was that the SP1 strain was from an unusual site of recovery for a respiratory tract pathogen namely the testicles of the bull.

A technique which has yielded useful information with regard to antigenic differences between strains has been the neutralization kinetic method. McBride (1959) was the first to show that strains

of poliovirus type 1 could be characterized on their rates of inactivation. Strains which differed would be inactivated at a lesser rate when reacted with antiserum prepared against the homologous virus. Several workers have used this method and recently, Ashe and Scherp (1963) characterized strains of herpes simplex virus by this method.

In the present study by utilizing this method minor antigenic differences between B/Morl/66 isolated in Edinburgh and the SP1 and T1 strains of para-influenza 3 virus were observed. The K value of the serum employed was very low. Since it was essential to have specific serum no more than two inoculations were administered to the rabbit.

Chanock et al (1961) have stated that human strains of para-influenza 3 viruses were antigenically homogenous. However, the experiences with the bovine strains appear to be contrary. Hamdy (1965) studied 13 isolates of bovine para-influenza 3 strains from naturally occrring cases of shipping fever in Ohio by cross HI tests and found that there were major antigenic differences; isolate 57 titred 8 times more with the homologous serum as compared to the SF-4 prototype strain, whilst the SF-4 antiserum titred 256 times less when compared with the SF-4 strain. Similar major antigenic differences were observed with two further isolates 118 and 133. Dinter et al (1966) found that the virus population of the Umea strain of para-influenza 3 was composed of 3 variants, 22, 33 and 196 which though varying in plaque morphology were indistinguishable antigenically. However, they comment that sera

prepared against the three variants gave significantly lower HI titres against strain 196 than against strain 22 and 33. Inaba et al (1963) on plaquing their Japanese isolate BNL1 obtained two plaque variants, a small (1.8mm) and a large (3.1mm) plaque variant. These two variants were antigenically indistinguishable by the HI tests.

On plaquing isolate B/MOR1/66 it was regularly observed that the plaques were pinpoint and hazy on day 5 and by day 7 was approximately 1.4 mm, whilst that of the T1 and SP1 strains were much clearer and approximately 2.3 to 3mm in size.

The significance of para-influenza 3 in bovine respiratory disease has as yet not been fully assessed. In England, where a large part of the epidemiology of this infection has been worked out, Dawson et al (1964) have implicated this virus in approximately 21% of the outbreaks of respiratory disease based on serological rises in antibody titres. Omar (1966) on the basis of his histopathological studies on pneumonic lungs collected from outbreaks has attributed 15% of the lesions seen to PI3 infection The widespread distribution of antibodies to this virus and its recovery from apparently normal cattle necessitates the simultaneous sampling of a representative, apparently healthy population during the time of these disease outbreaks.

PART I

- SUMMARY -

1. Investigation of a mild upper respiratory infection of cattle in East Lothian, Edinburgh, resulted in the recovery of 9 haemadsorbing agents.

2. One of the isolates B/MOR1/66 was characterized by a study of its:-

   1. Physico-chemical
   2. Structural
   3. Antigenic properties and shown to be a bovine strain of para-influenza 3 virus.

3. The serologic response as a consequence of para-influenza infection in cattle sera from this farm was examined by the HI and neutralization test. HI titres ranged from 1:40 to 1:640 whilst the serum neutralizing antibody levels varied from 1:16 to 1:160.

4. The isolate B/MOR1/66 induced plaques in calf kidney monolayer cultures. The plaques were small and turbid with irregular border and on day 5 were pinpoint. In contrast the plaques produced by the T1 and SP1 strains of bovine para-influenza were much clearer and larger measuring 2.5 to 3mm on day 7.

5. The method of neutralization kinetics was applied to the study of three strains of bovine para-influenza and minor antigenic differences were observed.

6. Treatment of infective tissue culture fluid of bovine para-influenza 3 with tween-ether effectively increased the haemagglutinating titre eight-fold when compared with the untreated control.

PART II

PART II

Virological Investigations of Upper Respiratory Tract Infection in Cattle Accompanied by Neurological Symptoms

INTRODUCTION

In mid-December 1966 an outbreak of respiratory disease affecting cattle, occurred on a farm located at Pathhead, twelve miles from Edinburgh. The herd was composed of nearly 700 yearling bullocks and heifers all kept in one shed. The animals were herded in pens within this shed each being separated from the next by wooden partitions.

The initial clinical manifestation noticed was an outbreak of coughing. On the first visit several animals, from between twenty to thirty, were showing severe respiratory embarrassment, discharge from the nasal passages and lachrymation. After about one week, some animals showed nervous symptoms, became recumbent and subsequently died (Johnston 1967).

Four to six weeks after the initial phase of the outbreak, four animals were brought into this institute for neuropathological examination. The predominant clinical symptoms exhibited by these animals were those of central nervous system involvement. Two of them 7F42 and 7F73 had been ill and recumbent for 7 to 14 days previously the remaining two animals 7F75 and X had shown nervous symptoms previously and four weeks after this attack had become recumbent remaining so for 7 days.

Of the four animals, one animal 7F42 at post mortem exhibited necrosis of the vocal cords with local diptheric deposit, a frothy exudate in the trachea and severe gangrenous pneumonia involving nearly two-thirds of lung tissue.

Dr. R.M. Barlow, who carried out the neuropathological

examination of these four cases, has kindly provided the following report:

"Histopathological examination of these four cases revealed, with the exception of one animal, no lesion in the central nervous system. In this animal 7F73, the respiratory tract was clear at post mortem. There was gross thickening of the leptomeninges of the cervical spinal cord by an organising fibrinous exudate. This was associated with extensive softening of the grey matter of the cervical spinal cord. Many vessels of the central grey matter and meninges showed perivascular mononuclear cell cuffing 8 to 10 cells thick. No inclusion bodies were found."

RESULTS

Isolation of Virus

Samples of nasal and tracheal secretions and secretions from the nasal turbinates were taken by swabbing with sterile cotton wool swabs on wooden applicator sticks from four chronically ill animals 7F42, 7F75, 7F73 and X brought into Moredun Institute for post mortem examination. All four swabs were immersed in 3ml of HBSS containing 2% bovalbumin (pH 7.1) to which had been added 500 units of penicillin and 200µg of streptomycin. After the animals were killed, brain material, portions of the cervical spinal cord and cerebrospinal fluids were also collected. All materials were stored at -65°C until inoculated on to calf kidney cell cultures.

Prior to inoculation on to tissue culture, swabs were twirled in the collecting fluids and centrifuged at 3,5000 r.p.m. for 45 minutes in a refrigerated centrifuge at 4°C. Portions of brain material were ground with fine sterile sand and a 20% suspension made. This suspension was centrifuged at 3,500 r.p.m. for 45 minutes at 4°C. Cerebrospinal fluids were also centrifuged as above.

Supernatant fluids were then inoculated in 0.2ml amounts on to 24 hour old tube cultures of previously washed calf kidney monolayers. After an adsorption period of 1 hour at 37°C, maintenance medium was added and the cultures incubated in a stationary position at 37°C. Cell cultures were examined daily for cytopathic effects. The period of observation was up to 13 days

post inoculation. At the end of this period, tissue culture fluid plus cells from those tubes showing no cytopathic effects were passed to a fresh set of second generation calf kidney cell cultures and observed for 7 days. If no cytopathic effects occurred the cultures were considered negative and discarded.

The results of the examination of these pathological materials in calf kidney monolayer cultures are presented in Tables $^{1}/_{2}$ and $^{2}/_{2}$. Table $^{1}/_{2}$ indicates the animals yielding cytopathic agents and the source from which they were isolated. Table $^{2}/_{2}$ indicates the minimum time required for detection of the cytopathic agents on primary isolation.

Properties of the Isolates

Three isolates, (i) C1/66 recovered from the nasal passage of a steer showing marked nasal discharge and lachrymation; (ii) C2/66 recovered from brain suspension material collected at post mortem from a steer showing nervous signs during the early phase of the outbreak and, (iii) 7F73/1 recovered from the mucosa of the nasal turbinates from a steer showing predominantly nervous symptoms during the late phase of the outbreak were characterized.

Chloroform, sodium deoxycholate sensitivity and acid lability

Treatment of tissue culture fluid containing $10^{4.3}$ TCD_{50} per 0.1ml of isolate C1/66 with 20% chloroform reduced the infectivity titre for calf kidney monolayer cultures to undetectable level.

TABLE 1/2

ISOLATION AND SITE OF RECOVERY OF CYTOPATHIC AGENTS FROM FOUR FIELD CASES IN CALF KIDNEY MONOLAYER CULTURES

ANIMAL NUMBER	NASAL SWAB	TRACHEAL SWAB	NASAL TURBINATE	20% BRAIN SUSPENSION	20% CERVICAL CORD	CSF
7F42	+**	+	+	-	ND*	-
7F73	+	-	+	-	-	-
7F75	-	-	-	-	-	-
X	-	-	-	-	ND	-

*ND = Not done

**+ = Cytopathic agent isolated

TABLE 2/2

TIME IN DAYS REQUIRED FOR THE DETECTION OF CYTOPATHIC AGENTS FROM TWO FIELD CASES IN CALF KIDNEY MONOLAYER CULTURES

ANIMAL NUMBER	TIME IN DAYS FOR CYTOPATHIC EFFECTS TO OCCUR IN MONOLAYER CULTURES					
	1	2	3	4	5	6
7F42						
1. Nasal swab	-	-	-	±*	±	+**
2. Nasal turbinate	-	-	-	±	±	+
3. Tracheal swab	-	-	-	±	±	+
7F73						
1. Nasal swab	-	±	+			
2. Nasal turbinate	-	±	+			

*± = Small foci of rounded cells in an otherwise intact monolayer sheet.

**+ = Foci of rounding seen in more than 50% of the intact monolayer sheet with sloughing off of the cells from the glass surface.

Treatment with 1:1000 sodium deoxycholate of tissue culture fluid containing $10^{5.3}$ TCD_{50} per 0.1ml of isolate 7F73/1 reduced the infectivity titre to undetectable level, when tested in the same system. This showed that the two isolates C1/66 and 7F73/1 were sensitive to lipid solvents.

It was found that treatment of infective tissue culture fluid of isolate 7F73/1 at acid pH diminished its infectivity by at least $10^{4.5}$ TCD_{50} per 0.1ml.

The results of these three tests are shown in Table 3/2 and clearly show that the two isolates C1/66 and 7F73/1 were lipid sensitive and acid labile.

Cationic stabilization to thermal inactivation

The stability of isolate C1/66 to heating at 50°C for 1 hour in the presence and absence of 1M $MgCl_2$ was tested. The results are shown in Table 4/2. This isolate was somewhat resistant to heat. In the presence of 1M $MgCl_2$, however, there was enhancement of loss in infectivity.

Electron Microscopy

Concentrated infective tissue culture fluid of isolate C2/66 was examined with the electron microscope using the negative contrast technique of Brenner and Horne (1959). When examined with the electron microscope, spherical particles of diameter ranging from 960Å to 1200Å were seen. These particles consisted of three main structural components, (i) an inner centrally situated core presumably containing the nucleic acid, (ii) a protein coat

TABLE 3/2

EFFECTS OF CHLOROFORM, SODIUM DEOXYCHOLATE AND ACID pH ON ISOLATES C1/66 AND 7F73/1

VIRUS SOURCE	TREATMENT	INFECTIVITY TITRE ($Log_{10}TCD_{50}$ per 0.1ml)
TISSUE CULTURE FLUID i. C1/66	20% chloroform for 30 minutes at room temperature	NIL*
	SALINE CONTROL	4.3
ii. 7F73/1	1:1000 Sodium deoxycholate for 1 hour at room temperature	NIL
	SALINE CONTROL	5.3
iii. 7F73/1	Eagle's '59 for 4 hours at room temperature pH 3.2	NIL
	Eagle's '59 for 4 hours at room temperature Tris pH 7.2 (Control)	6.5

* No infectivity detected at a dilution of 10^{-1} or 10^{-2}, when tested on calf kidney monolayer cultures.

TABLE 4/2

EFFECT OF HEAT ON ISOLATE C1/66 IN THE PRESENCE AND ABSENCE OF 1M $MgCl_2$

VIRUS SOURCE	TREATMENT	INFECTIVITY TITRES ($Log_{10}TCD_{50}$ per 0.1ml)
C1/66	50°C for 60 minutes in the presence of 1M $MgCl_2$	Nil*
	50°C for 60 minutes in the presence of distilled water	2.25
	22°C for 60 minutes in the presence of distilled water	4.75
	22°C for 60 minutes in the presence of 1M $MgCl_2$	4.25

* No infectivity detected at a dilution of 10^{-1} when titrated on calf kidney monolayer cultures.

surrounding the core and protecting the nucleic acid, termed the capsid, composed of a number of hollow structures, the capsomeres and (iii) an outer envelope. In the preparations examined three varieties of particles were observed (Figures 1/2, 2/2 and 3/2). Figure 1/2 shows a spherical particle where the central core is absent the space being filled with phosphotungstic acid. The diameter of this space which reflects the diameter of the core was approximately 750Å. Surrounding the capsid an envelope can be seen, which along its periphery gives the apperance of a double membraned structure. This feature can also be seen in Figure 2/2. The diameter of the particle having an envelope varied from 1350Å to 2300Å. The thickness of the envelope was approximately from 75Å to 100Å. Although not clearly seen, what appears to be projections can be seen along the periphery of the envelope. In contrast to this, Figure 2/2 shows a virus particle where the central core is filled with nucleic acid. In figures 3/2 a third type of virus particle observed is shown. This differs from the previously described particles in that an envelope surrounding the capsid was absent. The capsid is seen to be composed of capsomeres which have the appearance of hollow tubular structures. The exact symmetry of the capsid and the number of capsomeres could not be ascertained with certainty in the preparations examined. The approximate diameter of the capsomeres was in the region of 85Å to 90Å. The capsomeres do not seem to be tightly packed and appear to be arranged in a radial manner (see Figure 1/2).

These particles exhibited a close structural resemblence to

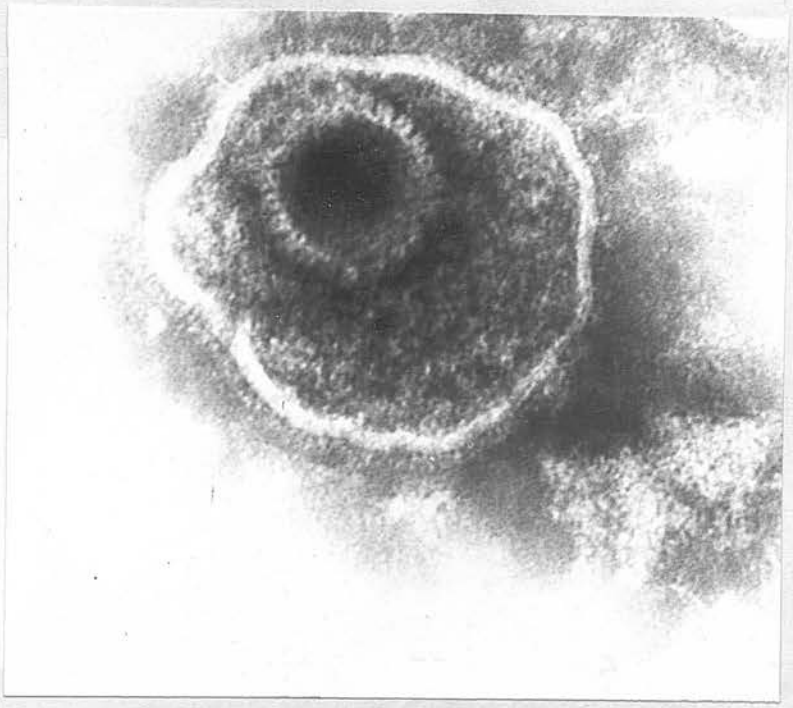

Figure 1/2 Empty and enveloped virus particle. Due to the absence of the central core, this space has been filled with phosphotungstic acid and the particle is described as empty. The envelope is seen to be double membraned.

Isolate C_2/66. Negative contrast staining with phosphotungstic acid x 240,000.

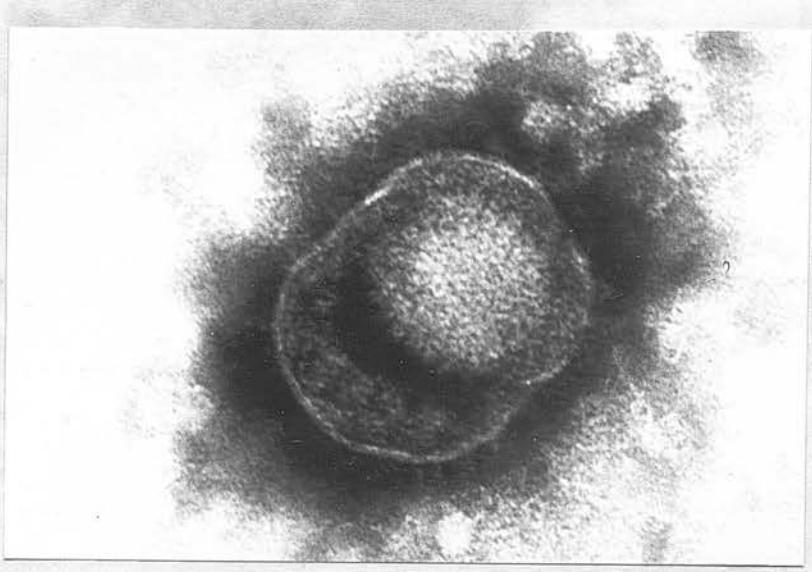

Figure 2/2 Full and enveloped particle. The central core, which is present, is surrounded by the capsid. This particle is described as full, and enveloped (compare with figure 1/2).

Isolate C_2/66. Negative contrast staining with phosphotungstic acid x 240,000.

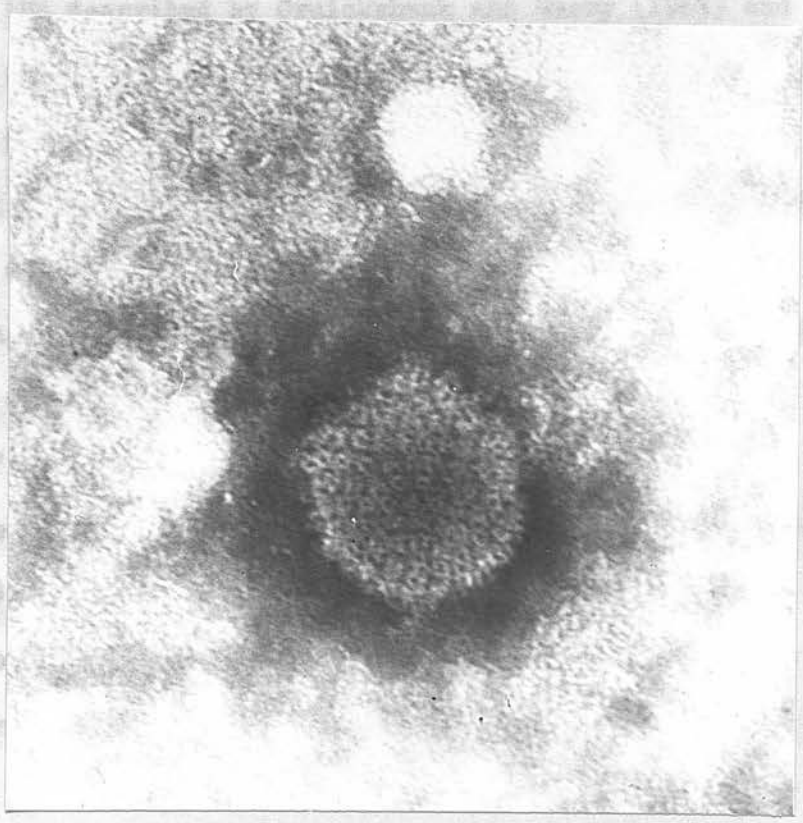

Figure 3/2 Full and naked particle. There is an absence of an envelope surrounding the capsid, whilst the hollow capsomeres can be clearly seen.

Isolate C_2/66. Negative contrast staining with phosphotungstic acid x 240,000.

the virion of herpes simplex described by Wildy et al (1960) and the virion of IBR described by Cruickshank and Berry (1965) and Watrach et al (1966). Wildy et al (1960) and Wildy and Watson (1962) have observed four types of particles in the preparations of herpes simplex virus they have examined. The basis of this classification was on the presence or absence of a central core, the particles being described as "full" or "empty"; and the presence or absence of an outer envelope, the particles being described as "enveloped" or naked. In the preparations of isolate C2/66 examined, three such types of particles, namely empty and enveloped (Figure 1/2), full and enveloped (Figure 2/2) and full and naked (Figure 3/2) were seen. A fourth type, empty and naked particles were not seen.

Cytology

The changes induced in calf kidney cells following infection with approximately 100 TCD_{50} of isolate 7F73/1 were studied by light microscopy. Second generation calf kidney monolayer cultures grown in coverslips were infected with isolate 7F73/1. Inoculated and uninoculated preparations were stained at 24 hourly intervals, after fixation in Bouin's fixative with haematoxylin and eosin as described in the materials and methods section.

Stained coverlip cultures showed that the cytopathic effects occurred as early as twenty-four hours post inoculation. These changes were confined to small foci of cells and occurred within the nucleus. The nuclear changes consisted of margination of the chromatin and disappearance of the nucleoli. The nuclei in these

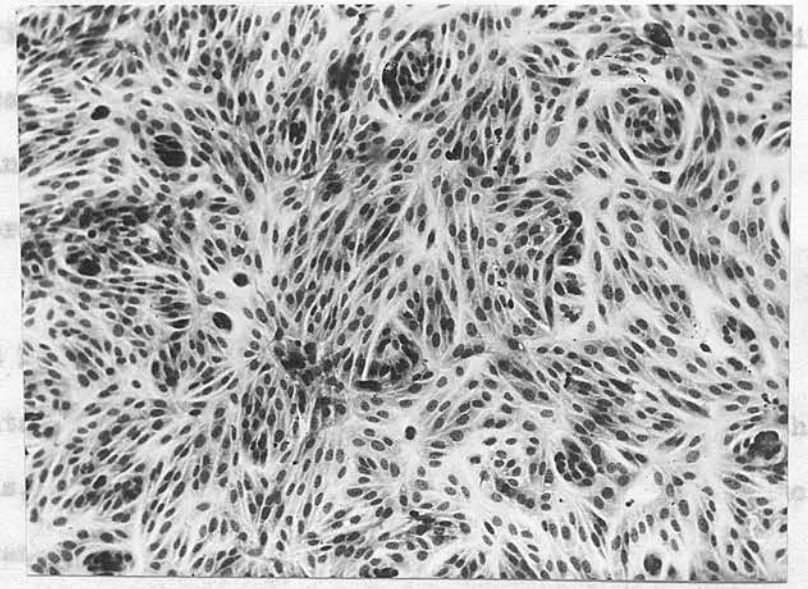

Figure 4/2 Normal 4 day old calf kidney monolayer culture.

Haematoxylin and eosin stained x 110.

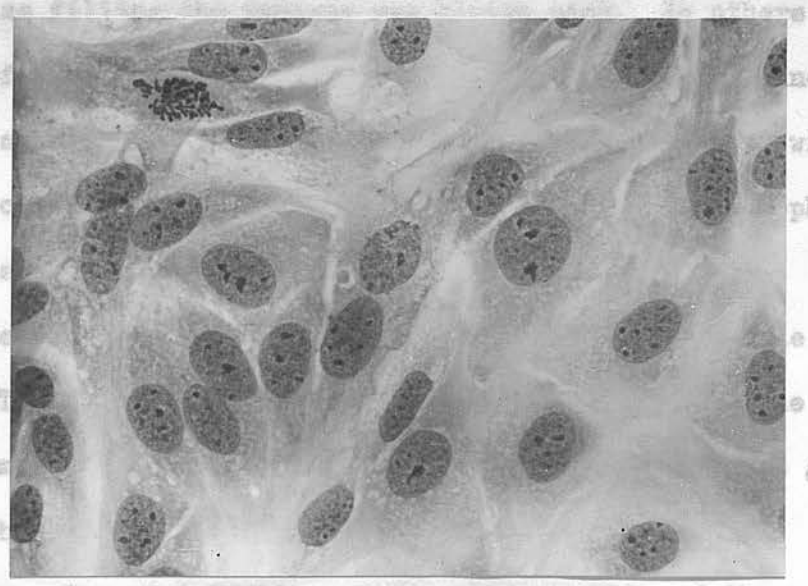

Figure 5/2 Normal 5 day old calf kidney monolayer culture.

Haematoxylin and eosin stained x580.

cells were filled with an homogenous eosinophilic mass. Within some of these cells, a clear unstained space occurred between this central eosinophilic mass and the deeply basophilic staining nuclear membrane, suggestive of a Cowdry type A intranuclear inclusion.

After 48 hours, foci of rounded cells could be seen on an otherwise intact cell sheet (Figure $^{6}/_{2}$). The feature of these rounded cells, was the intensely pyknotic character of the nucleus and the retracted cytoplasm, which stained deeply with eosin. These rounded cells appear in clusters and in some the cytoplasm was drawn out into fine strands. Although the cells in the immediate area around these foci of rounded cells appear to be normal, nuclear changes were evident when examined under the high power. In some cells the marginating chromatin had a beaded appearance which was very basophilic in staining. In these cells the homogenous mass filling the nucleus was bluish pink. In others margination of the chromatin, absence of nucleoli, and an eosinophilic homogenous mass filling the entire nucleus were seen with and without a halo around them. However, the presence of these eosinophilic masses was also associated with the presence of nucleoli and probably represented an earlier stage in the virus growth cycle than the former. These different cytopathic changes seen within the cells of the monolayer culture is probably due to asynchronous growth of virus in the monolayer cultures.

With progression of infection, there was extension of these foci of rounded cells involving the whole sheet. These rounded

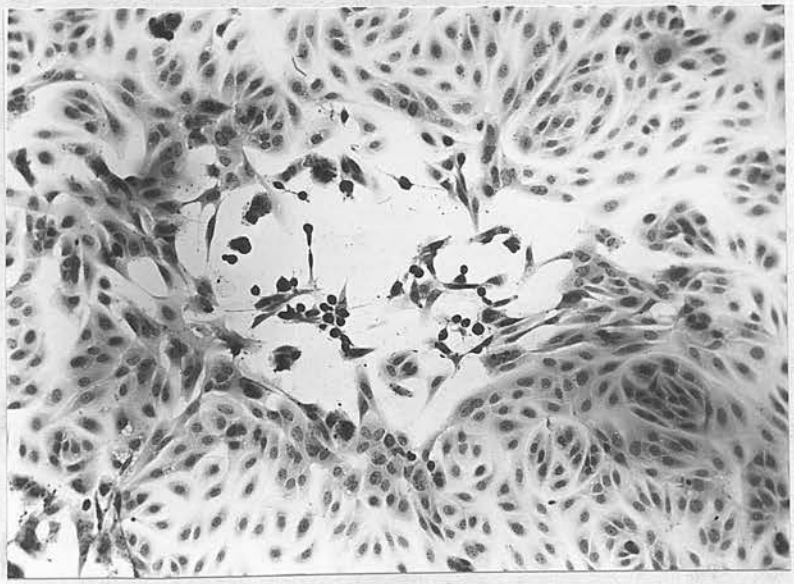

Figure 6/2 4 day old calf kidney monolayer culture, 48 hours after infection with isolate 7F73/1, showing a focal area of necrotic cells.

Compare with Figure 4/2.

Haematoxylin and eosin stained x 110.

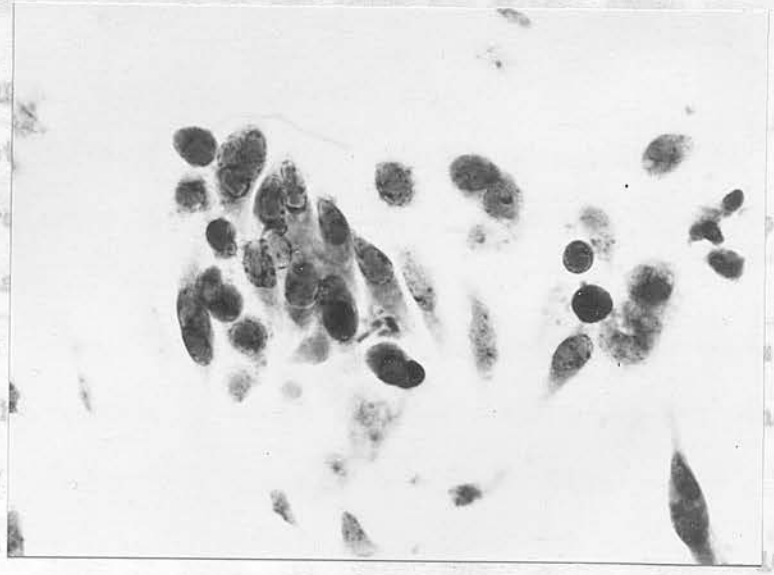

Figure 7/2 5 day old calf kidney monolayer culture 72 hours after infection with isolate 7F73/1, showing Cowdry type A inclusion.

Compare with figure 5/2.

Haematoxylin and eosin stained x 580.

cells sloughed off from the glass surface and 98 hours after infection only a few cells could be seen attached. The majority of the remaining cells were shrunken and pyknotic and connected to each other by fine cytoplasmic strands.

Antigenic Properties of the Isolates

The object of these tests was to investigate a possible serological relationship with other known members of the Herpes group of viruses affecting cattle.

In Tables $^{5}/_{2}$ and $^{6}/_{2}$ the results of neutralization tests are presented. The results in Table $^{5}/_{2}$ were obtained by the constant serum varying virus method and show that hyperimmune serum obtained from Dr. J.H. Darbyshire neutralized 2.1 to 2.2 $\log_{10}$ TCD_{50} of the two isolates, whilst the same serum neutralized 2.8 $\log_{10}$ TCD_{50} of the homologous "Oxford" strain of IBR virus. Hyperimmune rabbit antisera R19 prepared against bovine mammilitis virus (BMV) did not neutralize isolate C2/66. These results indicate that isolates C1/66 and C2/66 are undoubtedly strains of IBR virus.

The antigenic relationship of isolate 7F73/1 to the "Oxford" strain was determined using hyperimmune rabbit serum RS prepared against the "Oxford" strain. Serial two-fold dilutions of anti-serum RS were mixed with serial two-fold dilutions of isolate 7F73/1. After standing overnight at $4^{o}C$, 0.2ml of serum-virus mixtures were inoculated into each of 4 culture tubes. The "Oxford"

TABLE 5/2

NEUTRALIZATION OF ISOLATE C1/66 AND C2/66 BY HYPERIMMUNE RABBIT SERA PREPARED AGAINST "OXFORD" STRAIN OF IBR VIRUS AND BOVINE MAMMILITIS VIRUS

VIRUS STRAIN	LOG NEUTRALIZATION INDEX OF RABBIT SERA PREPARED AGAINST†	
	BMV	"OXFORD"
C1/66	NOT DONE	2.1
C2/66	NIL‡	2.2
"Oxford"	NOT DONE	2.8
Bovine mammilitis virus (BMV)	5.0	NOT DONE

† Antisera diluted to contain 20ND_{50} per 0.1ml.

‡ No neutralization.

strain was also similarly treated. The results of this test are shown in Table 6/2. It was found that dilutions of 1:40 and 1:34 of serum would neutralize 10 and 100 TCD_{50} of the homologous virus. The same serum when diluted 1:24 would neutralize 100 TCD_{50} of the isolate 7F73/1.

Examination of Cattle Sera

Paired serum samples from four animals and single serum samples from cattle in the affected farm were examined for neutralizing antibodies. The object of this was to associate the recovery of virus with the clinical infection present in these animals, at the time of collection of blood samples. The examination of these sera for neutralizing antibody was carried out by the tube neutralization and the plaque neutralization methods.

Tube neutralization method

In this mehtod, two-fold dilutions of serum were mixed with an equal quantity of virus C2/66 (0.5ml) diluted to contain 100 or 10 TCD_{50} per 0.1ml. The mixtures were held at room temperature for 2 hours, after which 0.2ml aliquots of these mixtures were inoculated on to monolayer tube cultures using 3 or 4 tubes for each serum dilution. The tube cultures were incubated in a stationary position at 37°C and final readings made on the fifth day. From the results the ND_{50} of each serum sample was calculated by the Kärber method.

The results of these tests are given in Table 7/2 and 8/2. With the exception of five animals, neutralizing antibody was present

TABLE 6/2

NEUTRALIZATION OF ISOLATE 7F73/1 BY HYPERIMMUNE RABBIT ANTISERUM RS PREPARED AGAINST THE "OXFORD" STRAIN OF IBR VIRUS WHEN TESTED ON CALF KIDNEY MONOLAYER CULTURES

VIRUS STRAIN	VIRUS DOSE USED (TCD_{50} per 0.1ml)*	DILUTION OF RABBIT ANTISERUM PREVENTING CYTOPATHIC EFFECTS IN 50% OF TUBES
"Oxford" IBR virus	100	1:34
	10	1:40
7F73/1	100	1:24
	10	1:40

* TCD_{50} per 0.1ml.

in all the other sera examined. A two to four-fold drop in titre of neutralizing antibody was seen in two animals. The significance of this is not clear, although it is possible that infection within the herd may have been present for some time. The neutralizing titre of serum samples 604/2 and 604/6 were 1:4 or less when tested against 100 TCD_{50} of virus but ranged from 1:5.6 to 1:3.3 when tested against 10 TCD_{50} of virus indicating the presence of antibody. Virus was isolated from steer C (Table 7/2) in the absence of antibody and from steer 7F73 in the presence of antibody (Table 8/2).

Plaque Assay

The infectivity of the isolates for calf kidney cell cultures was determined by the plaque assay procedure. The object of this was to develop this method for the assay of neutralizing antibody from the sera of cattle in the affected farm. The method of infecting monolayer cultures and the overlay procedure were described earlier. The plaque counts observed after 5 days incubation at 37°C in the presence of 4-5% carbon dioxide are plotted against their respective virus dilution in Figure 8/2. A linear relationship between virus concentration and the plaque count was observed within the limits of the dilutions tested. The plaques were clear, round and with irregular edges (Figure 9/2) having a diameter of 2 - 3 mm.

Plaque neutralization test

Plaque neutralization tests were carried out as follows:-

TABLE 7/2

NEUTRALIZING ANTIBODY TITRE OF PAIRED SERUM SAMPLES FROM CATTLE IN THE AFFECTED FARM

ANIMAL NUMBER		NEUTRALIZING ANTIBODY TITRE†	
		Bleed I*	Bleed II**
4471/2 604/11	A	1:9.4	1:4
4445/1 604/2	B	1:13.3	< 1:4
4445/2 604/6	C‡	< 1:4	< 1:4
D		1:2	1:4

* Serum sample obtained during the first visit to the farm.

** Serum sample obtained six weeks later.

† Dilution of serum containing 1 ND_{50} per 0.1ml. when tested against 100 TCD_{50} of virus C2/66. Kärber estimate.

‡ Virus isolated from nasal swab.

4471/2 is the laboratory number of the first serum sample obtained from animal A.

604/11 is the laboratory number of the second serum sample obtained from animal A.

TABLE 8/2

NEUTRALIZING ANTIBODY TITRE OF SINGLE SERUM SAMPLES COLLECTED SIX WEEKS AFTER THE FIRST VISIT FROM CATTLE IN THE AFFECTED FARM

ANIMAL NUMBER	NEUTRALIZING ANTIBODY TITRE
7F75	1:5.0*
7F73†	1:6.7
Steer X	1:5.0
604/3	1:4.7**
604/8	1:13.3
604/1	< 1:4
605/1	< 1:4
604/7	< 1:4
604/10	< 1:4
604/11	1:5.6
604/2	1:3.3
604/6	1:5.0

* Dilution of serum containing 1 ND_{50} per 0.1 ml when tested against 100 TCD_{50} of virus. Kärber estimate.

** Dilution of serum containing 1 ND_{50} per 0.1ml when tested against 10 TCD_{50} of virus. Kärber estimate.

† Virus isolated from nasal swab.

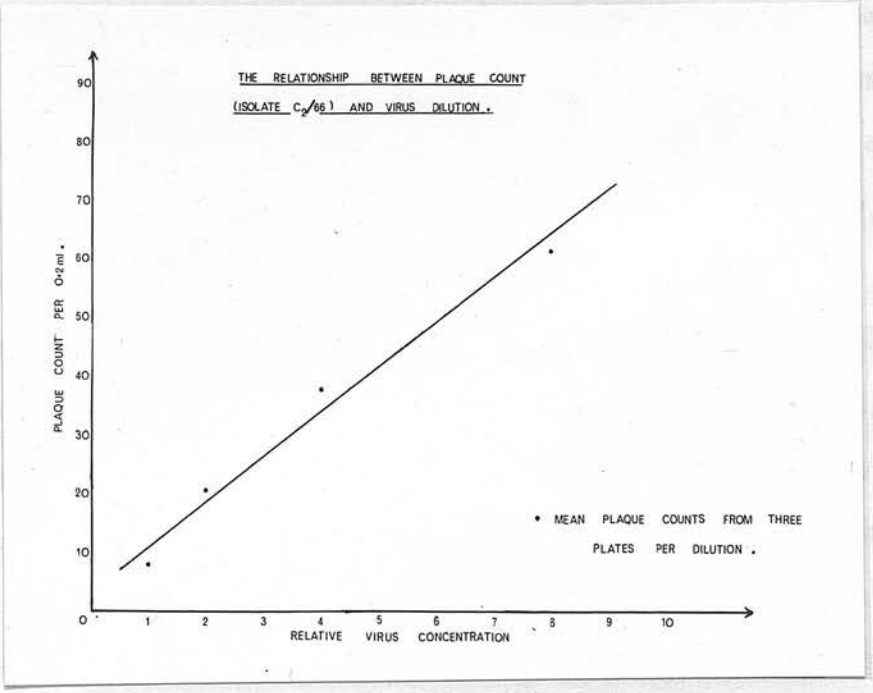

Figure 8/2 Relationship between plaque count and virus concentration.

(Isolate $C_2/66$).

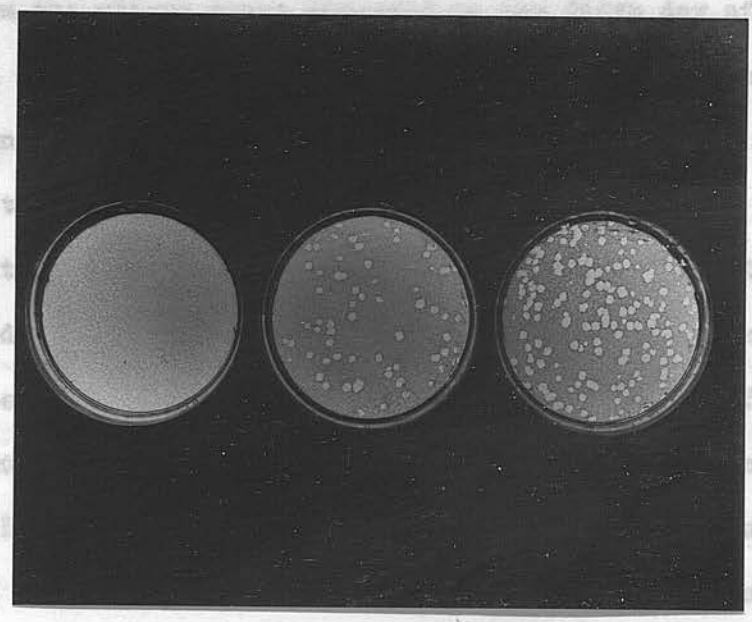

Figure 9/2 Plaques produced by isolate C2/66 on calf kidney monolayer cultures, five days after infection of cell sheets.

(1) To confirm the antigenic relationship of isolates C1/66 and C2/66 to the "Oxford" strain of IBR virus.

The test was done as described in the materials and methods section. From the plaque count obtained on the fifth day after incubation in an atmosphere of 4-5% CO_2 at 37°C, the dilution of serum producing a 50% reduction in the count was calculated by the Kärber method.

The results given in Table 9/2 show that hyperimmune anti-IBR serum obtained from Dr. J.H. Darbyshire completely neutralized the challenge dose of virus C1/66. In Table 10/2 the results of a reciprocal cross plaque neutralization test using hyperimmune rabbit sera R17 and RS prepared against isolate C2/66 and the "Oxford" strain of IBR virus respectively is given. These results confirm that the isolate C1/66 and C2/66 are closely related to the "Oxford" strain of IBR virus.

(2) To detect the present of neutralizing antibody in the cattle sera collected from animals on the affected farm. Dawson and Darbyshire (1964) and Markson and Darbyshire (1966) have indicated that low titres of serum neutralizing antibody in cattle following infection of IBR virus may be due either to low antigenicity of the virus or to the insensitivity of the standard tube neutralization method used for their detection. The results given in Tables 7/2 and 8/2 show the very low levels of neutralizing antibody titres obtained from cattle in the farm, from which IBR virus was isolated. Plaque neutralization tests are accepted as being more sensitive for the detection of antibodies than tube

TABLE 9/2

NEUTRALIZATION OF ISOLATE C1/66 WITH HYPERIMMUNE IBR ANTISERUM† BY THE PLAQUE NEUTRALIZATION TEST ON CALF KIDNEY MONOLAYER CULTURES

	SERUM DILUTION			
	20 ND_{50}	10 ND_{50}	1 ND_{50}	CONTROL
Number of Plaques	0	44	80	93

† The titre (1 ND_{50}) of this antiserum was given as 1:518 for the "Oxford" strain of IBR virus and obtained from Dr. J.H. Darbyshire.

TABLE 10/2

RECIPROCAL CROSS NEUTRALIZATION OF ISOLATE C2/66 AND "OXFORD" STRAIN OF IBR VIRUS BY THE PLAQUE NEUTRALIZATION METHOD

VIRUS STRAIN	HYPERIMMUNE RABBIT SERA NEUTRALIZATION TITRE*	
	R17**	RS***
C2/66	1:30	1:40
IBR "OXFORD"	1:36	1:80

* Kärber estimate of 50% plaque reduction.

** Hyperimmune serum prepared against isolate C2/66.

*** Hyperimmune serum prepared against the "Oxford" strain of IBR virus.

neutralization methods. Accordingly three paired serum samples, A, B and C and three single serum samples 7F73/1, 7F75 and X collected from cattle in the affected farm were also assayed for neutralizing antibody by this method. The results of these tests are given on Table 11/2. Antibody was detected by this method in serum samples 4445/2, 604/6 and 604/2 when tested at a dilution of 1:4. At this same dilution, these samples did not reveal the presence of antibody when tested by the standard tube neutralization method (see Table 7/2). A slowly rising neutralizing antibody titre can be seen in Steer C from which virus was isolated from the nasal passages.

TABLE 11/2

NEUTRALIZING ANTIBODY TITRES OF PAIRED AND SINGLE SERUM SAMPLES COLLECTED FROM CATTLE IN THE AFFECTED FARM, ASSAYED BY THE PLAQUE NEUTRALIZATION TEST

ANIMAL NUMBER		Neutralizing antibody titre† (ND_{50})			
		Experiment I		Experiment II	
		Bleed I‡ (early)	Bleed II‡‡ (late)	Bleed I (early)	Bleed II (late)
4471/2 604/11	A	1:41.1	1:16.5	1:46.6	1:12.9
4445/1 604/2	B	1:49.2	1:8.4	1:32.0	1:5.6
4445/2 604/6	C	1:5.8**	1:7.1	1:3.9	1:9.6
7F75		NCμ	1:10.7	NC	1:10.6
7F73		NC	1:11.8**	NC	1:14.6
Steer X		NC	1:11.4	NC	1:7.9

† Kärber estimate of 50% plaque reduction.

‡ Serum samples obtained during the first visit to the farm.

‡‡ Serum samples obtained six weeks later.

* NC = not collected.

** Virus isolated.

Challenge dose of virus (C2/66) used in the test ≏ 100 - 200 PFU per 0.1ml.

DISCUSSION

DISCUSSION

Viral meningo-encephalitis associated with IBR virus in cattle has not been recorded previously in Britain. The association of this virus with these infections where the mortality was approximately 2.4% illustrates a danger in the intensification of livestock husbandry.

The herd which consisted of about 500 to 700 animals was housed in five pens, all in one shed. The animals originated from diverse sources but the clinical disease was confined only to pens 1 and 2, housing about 100 to 120 animals in each. The outbreak occurred 6 weeks after their arrival from a hill farm and the course of the disease was approximately 2 to 3 weeks. Subsequently a relapse occurred in some convalescing animals of which four were brought into this institute with histories of cerebral involvement and recumbency from 4 weeks to 14 days previously. The clinical disease had two phases - an initial respiratory infection with nasal and occular discharge followed shortly after in some animals by neurological symptoms such as recumbency, with their heads resting on their side, unsteady gait and difficulty in rising. The morbidity was high, 30 - 50% of the animals showing symptoms of an upper respiratory tract infection whilst 17 animals died mainly as a result of cerebral involvement between mid-December 1966 and the end of January, 1967.

Virological investigations of this outbreak resulted in the recovery of 9 virus isolates all producing similar cytopathic effects in calf kidney monolayer cultures. These included isolates

from the brain, frontal sinus exudate and tracheal passages from animals brought in for autopsy during the early phase of the disease to the Veterinary Investigation Centre, Edinburgh, and 4 isolates from the nasal passages of living animals in the farm (Johnston 1967).

Further isolations of virus were made four to six weeks later from two of four animals, isolations being exclusively made from the respiratory passages immediately after slaughter.

Studies on three of these isolates C1/66, C2/66 and 7F73/1 isolated from the nasal passages, brain suspension and the mucosa of the nasal turbinates respectively, showed them to be acid labile, sensitive to lipid solvents and sensitive to heating at 50°C for 1 hour. Electron microscopic observations of concentrated tissue culture fluid revealed particles which were similar in structure to the virion of herpes simplex (Wildy et al 1960) and IBR virus (Cruikshank and Berry 1965, Watrach and Bahnemann 1966). At present the known members of the herpes virus group affecting cattle in the United Kingdom are IBR virus and bovine mammilitis virus. Neutralization tests carried out with hyperimmune rabbit sera prepared against the "Oxford" strain of IBR virus indicated a close antigenic relationship to the former, and the three isolates which were characterized were certainly strains of IBR virus.

IBR meningo-encephalitis in cattle has been reported both from

Australia and America. In the cases reported by Johnston et al (1962) and Gardiner and Nairn (1964) the disease occurred mainly in young calves, 2 weeks to 6 months of age with a high morbidity and mortality which was nearly 50%. No history of an upper respiratory tract infection was present, whilst Gardiner and his colleagues stress that there were no introduction of cattle to this farm for a period of at least 2 years. The American outbreak on the other hand was characterized by a low mortality (11 heifer calves out of 175 young stock) with deaths from neurological symptoms occurring sporadically over a period of 2 years. Again as in the Australian series, no respiratory infection was observed in some of the affected animals.

The similarity of IBR virus both in its basic biophysical and biochemical properties to the virus of herpes simplex and the varied clinical conditions with which it is associated in cattle has led to the belief that the biological behaviour of IBR virus in cattle is similar to herpes simplex in man. Essentially the behaviour of the virus of herpes simplex is to infect young infants and children from 14 months to 3 years of age when a primary infection occurs with or without clinical symptoms. During this period active virus dissemination occurs following the onset of the primary disease and during the period of recovery when no clinical disease is present. It is considered that the virus persists for life in the individual in a latent form. Reactivation of the virus occurs periodically, as a consequence of changes in the constancy of the "milieu intérieur". During this

period, viral activity manifests itself either as a recurrent clinical infection or an inapparent infection with rise of circulating antibody. During this phase too, viral dissemination to a susceptible population may occur (Burnet and Williams 1939, Anderson and Hamilton 1949, Buddingh et al 1953, McNair Scott and Tockumaru 1964).

In cattle, recent evidence suggests a similar biological cycle to be operative. Following experimental intranasal inoculation of young calves up to about 6 months of age, virus excretion occurred up to about 9 to 14 days post inoculation and the disappearance of virus was coincident with rise of antibody. McKercher states that in field cases, virus can be isolated from the nasal passages for 6 to 8 days but rarely after 12 days (McKercher et al 1963). However, he adds in the same paper that following experimental inoculation of calves, virus was isolated on two occasions from nasal secretions 2 to 3 months later. Snowdon (1964) has reported that on inoculation of a 21 month old heifer intravenously, virus was recovered from nasal swabs collected on twooccasions, on the day of infection and 578 days later. On the second occasion, virus was recovered for a period of 6 days following calving by this animal. In addition, Snowdon (1964, 1965) has shown that following intravaginal inoculation of heifers intermittent virus excretion occurred over a period of 147 days. Infectious bovine rhinotracheitis and infectious pustular vulvo-vaginitis (IPV) are different clinical manifestations of the same virus (Gillespie et al 1959, McKercher et al 1959). Initiation of infection of respiratory

passages in field outbreaks may possibly also occur from animals excreting the virus from the vagina as a consequence of close physical contact. McKercher (1959) in studying the dynamics of antibody production in cattle following exposure to IBR virus found that there was considerable variation in some animals. The graphical presentation of his results of the antibody response curve in cattle suggests the occurrence of "booster" responses in some animals indicative of a probable reactivation of a latent infection or a possible exogenous reinfection.

In view of this evidence, the probable initiation of the present outbreak may have been reactivation of a latent IBR virus in one or more animals in pen 1, for there was a sequential spread of the infection from this pen to pen 2. The physiological and emotional stress induced in these animals as a consequence of transportation and changed environmental situation may have been the precipitating factors reactivating the latent virus. The confinement of the infection only to pens 1 and 2 may have been due to the prompt hygiene measures introduced such as improved ventilation. Alternatively the source of infection may have been the animals housed in pens 3 to 5. These animals, though of diverse origin, had been grazing in the farm for a period of 3 weeks prior to their being housed. During this period they may have disseminated virus amongst themselves. Clinically IBR infections are mild under range conditions.

The factors responsible for the severity of the present outbreak and the invasiveness of this virus to the central nervous

system are unknown. McKercher (1959) states that the severe form of infection occurs in conditions where large numbers of cattle are kept in close physical contact, thereby facilitating spread of virus from member-to-member and consequently the virulence of the virus is enhanced. This view, however, does not take into account the response of the individual animal to infection. Markson and Darybshire (1966) inoculated 15 calves two months of age with the "Oxford" strain of IBR virus. Although no clinical encephalitis was produced virus recoveries were made from the central nervous system. Similar observations have also been made by McKercher et al (1963). On the otherhand, Webster and Manktelow (1959) induced clinical encephalitis in two steers following intranasal inoculation of the New Zealand strain of IBR virus.

The association of IBR virus with this outbreak was assessed by assaying serum samples collected from cattle on the farm for neutralizing antibody to isolate C2/66. Antibody titres in these animals were low (1:4 to 1:13) when tested by conventional tube neutralization tests. The paired sera tested showed either an insignificant rise or two to four-fold drop in titre. The presence of low levels or absence of neutralizing antibody in infected herds, in spite of virus isolation have been observed by other workers. Dawson et al (1962) who investigated an outbreak of conjunctivitis and rhinitis in a group of 2 year old cattle in Oxfordshire recovered 12 strains of IBR virus. Although significant rises of neutralizing antibody were present in 4 of 6 convalescent serum samples obtained at 10 day intervals, nevertheless the titres

of these specimens never exceeded 1:60. In a similar outbreak in a small group of cows and calves at pasutre, Darbyshire and Shanks (1963) found very low levels of antibody in the convalescent serum samples, in the order of 1:2 to 1:52. The findings of Snowdon (1964) have been similar. In one herd which he investigated, although virus was isolated, convalescent serum samples obtained from these animals 6 weeks later showed insignificant titres with hardly any rise of antibody except in one animal. Experimental inoculations of IBR virus in cattle have also demonstrated the low, variable and limited response to infection (Snowdon 1964, Markson and Darbyshire 1966). McKercher (1959), however, found a rapid development of antibodies in cattle following intranasal exposure to the virus IBR. These conflicting findings serve further to emphasize the very variable antibody response that occurs in cattle to such infections. On the basis of other virus infections in man and animals the two to four-fold drop in titre seen with 2 paired sera in the present outbreak probably represents a comparatively recent infection.

The assessment of the extent of IBR virus infections is difficult. Virus isolation is the only sure method but is fortuitous even when clinical disease is present. Diagnosis by serology is limited to those animals which respond immunologically. In Britain in assessing the extent of IBR antibodies in cattle, Dawson and Darbyshire (1964) state that 2.1% of cattle had antibodies to this virus. They comment that conventional tube neutralization tests may be insensitive for detection of neutralizing antibody to this

infection. As it appeared unlikely that infection with IBR virus would not produce antibodies a plaque neutralization method was used for the detection and assay of antibodies in nine of these sera. By this method, neutralizing antibody was detected in samples 604/2, 4445/2 and 604/6 when tested at a dilution of 1:4. At this same dilution, no neutralizing antibody was detected by conventional tube neutralization methods. In view of these results it appears that the plaque neutralization method is an improvement on the conventional methods used to detect antibody for IBR virus in cattle. Further work employing this method in sero-epidemiology may indicate a truer picture of the extent of the presence of IBR antibodies in cattle. The technique may be simplified by the use of Leibovitz medium (Leibovitz 1963) supplemented with 4% inactivated horse serum and 15% methyl cellulose, thereby obviating the use of a CO_2 incubator. It was observed that the titre of the virus 7F73/1 when tested by this method was similar to that obtained when the conventional agar overlay method described in the body of this thesis was used (Personal observation).

PART II

- SUMMARY -

A severe respiratory tract infection of cattle characterized by respiratory and nervous symptoms occurred in the Edinburgh area in the period Mid-December 1966 to January 1967. During the early phase of the outbreak, nine viral isolates were recovered from post mortem material at the Veterinary Investigation Centre, Edinburgh. These included isolates from the brain, frontal sinus and trachea. Four further isolates from the nasal passages were made from cattle during this time at the farm.

1. Virus was isolated from two of four convalescing animals (7F42 and 7F73) brought to the institute for neuropathological examination. Isolations of virus were made exclusively from the respiratory passages of these two animals.

2. Three of the isolates, C2/66 a neurogenic strain and two respiratory strains C1/66 and 7F73/1 recovered from animals involved in this outbreak were characterized. They were lipid sensitive, acid labile and showed enhanced inactivation to heat at 50°C for 1 hour in the presence of 1M $MgCl_2$. The structure of the virus particles was similar to the virion of herpes simplex and IBR virus. The three isolates produced a similar cytopathic effect in calf kidney cells consisting of rounding of cells and intranuclear inclusions. Antigenically they were similar to the Oxford strain of IBR virus. It was concluded that the Edinburgh strains were strains of IBR virus.

3. Paired serum samples and single serum samples collected from cattle in the affected farm were assayed for IBR neutralizing antibody, by tube neutralization and plaque neutralization methods.

   a) Neutralizing antibody titres were low < 1:4 to 1:13.3 when tested by the conventional tube neutralization method.

   b) No significant rises of antibody were observed in the paired serum samples tested.

4. Neutralizing antibody in three of the same paired serum samples and 3 single serum samples were examined by the plaque neutralization method. It was found that:

   a) This method was more sensitive for detecting neutralizing antibody to this virus.

   b) A slowly rising antibody was observed in 1 paired serum sample.

ACKNOWLEDGMENTS

I wish to thank Dr. J. Stamp, Director of Moredun Institute, for the opportunity and facilities provided to pursue my studies here in Edinburgh.

I am grateful to Mr. J. Brotherston and Dr. R.H.A. Swain, my supervisors, for thier kindly interest, advice, encouragement and criticism, which they have given me during the course of this work.

Mr. W.S. Johnston, Assistant Veterinary Investigation Officer, Edinburgh, was generous in sharing both the clinical material and the neurogenic strain of IBR virus studied in Part II of this Thesis. Mr. W. Smith kindly examined the para-influenza and IBR viruses under the electron microscope, whilst Mr. D. Thompson examined nasal swabs collected from cattle for bacteria.

I thank the Senate of the University of Edinburgh for awarding me a postgraduate studentship for the period October 1966 to April 1968.

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